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Arginine 482 to glycine mutation in ABCG2/BCRP increases etoposide transport and resistance to the drug in HEK-293 cells

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Abstract. Resistance to etoposide has been associated with the overexpression of P-glycoprotein and MRP1 in human tumor cells. However, the role of BCRP in resistance to etoposide has not been clearly established, especially the significance of arginine 482 mutations in drug transport (cellular uptake and efflux). Different levels of resistance to etoposide have been recently observed in cells expressing BCRP in terms of cytotoxicity. The aim of this work was to study the effects of these mutations on the functional involvement of BCRP in etoposide transport. HEK293 cells were transfected with an empty vector (HEK/V), the vector bearing the wild-type BCRP (HEK/R482), the mutant arginine-482-glycine (HEK/R482G) or the mutant arginine-482-threonine (HEK/R482T). MTT assay was used to study the cytotoxic effect of etoposide and [^{3}H]-etoposide was used to determine cellular drug uptake and efflux. Data show that HEK/R482G cells displayed the highest levels of resistance to etoposide. Cellular [^{3}H]-etoposide uptake was lower in HEK/R482, HEK/R482G and HEK/R482T cells compared to HEK/V cells. In addition, cellular [^{3}H]-etoposide uptake in HEK/R482G was the lowest. Drug efflux measurements showed that fumitremorgin C was able to increase the residual cellular [^{3}H]-etoposide uptake in BCRP-transfected cells and especially in HEK/R482G ones. Our data show that the R482G mutation in BCRP is able to increase efflux of etoposide and that mutation analysis at codon 482 may be of clinical importance in cancers treated with etoposide.

Introduction

One of the mechanisms of resistance to anticancer chemotherapy has been linked to the overexpression of ABC membrane transporters leading to the decrease of the intracellular drug concentration. These proteins include P-glycoprotein/*MDR1* (ABCB1) (1), multidrug resistance-associated protein/*MRP* (ABCC) (2) and the ABC half transporter breast cancer resistance protein/*BCRP* (ABCG2) (3,4).

Resistance to etoposide has been mainly associated with the overexpression of P-glycoprotein and MRP1 (1,2). Different levels of resistance to etoposide have been recently observed in cells expressing BCRP (5,6). Murine cells selected for resistance to doxorubicin that overexpress the mouse Bcrp1 gene were also resistant to etoposide (7). Moreover, the reduced cellular accumulation and resistance to etoposide in cells lacking P-glycoprotein (P-gp) and Mrp1 proteins have been related to the overexpression and/or amplification of the *Bcrp1* gene (8).

Human BCRP gene was initially identified in resistant breast carcinoma cells selected for resistance to doxorubicin and verapamil. In these cells the BCRP gene has been shown to be mutated in the exon 5 leading to the substitution of the arginine 482 for a threonine (R482T). The same amino acid is also mutated in another cell line but substituted for a glycine (R482G) (9,10). The affinity of BCRP to several substrates has also been found altered by the mutation arginine 482 (9,11).

Nevertheless, although the role of BCRP in etoposide resistance is well established in murine cells, its functional involvement in human in terms of drug transport (cellular uptake and efflux), as well as the significance of arginine 482 mutations has not been investigated. We studied the resistance to etoposide and its cellular transport in HEK293 cells transfected by the human wild-type BCRP or its R482G and R482T mutants. We show that cells expressing the R482G mutant display higher resistance level to etoposide than the wild-type BCRP or the mutant R482T genes.

Materials and methods

Drugs and chemicals. Etoposide was supplied by Merck (Lyon, France). [^{3}H]-etoposide (25 μCi/mol) was obtained from Moravek Biochemicals (Brea, CA). Mitoxantrone, novobiocin and MTT were obtained from Sigma Chemicals (St. Quentin Fallavier, France). Fumitremorgin C (FTC)

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was kindly provided by R.W. Robey and Professor S. Bates (National Cancer Institute, NIH, Bethesda, MD, USA).

Cell lines. The human embryonic kidney HEK293 cells transfected with empty vector (HEK/V) or BCRP (HEK/R482 and HEK/R482G and HEK/R482T) were kindly provided by R.W. Robey and S. Bates (12). Cells were grown as monolayer in MEM (Invitrogen, Paris, France) supplemented with 10% fetal calf serum and 100 μg/ml penicillin, and 100 μg/ml streptomycin. The human ovarian carcinoma subline 2008/MRP1 which is stably transfected with *MRP1* cDNAs and their parental cells 2008 were kindly provided by Professor P. Borst (13). The 2008 cell lines were cultured in the RPMI-1640 (Invitrogen) supplemented with 10% fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin.

mRNA analysis. RNA was extracted from $5x10^6$ cells using the RNAeasy mini kit and DNAse I (Qiagen, Paris, France) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using random hexamers, MMLV reverse transcriptase and reaction buffer provided in the Superscript First-Strand Synthesis System kit (Invitrogen). Real-time quantification was performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) using 5 μl of diluted cDNA, 350 nM of forward and reverse primers and SYBR-Green PCR Master Mix in a 25-μl reaction volume under following conditions: 95˚C for 10 min, followed by 40 cycles of 94˚C for 45 sec, 60˚C for 45 sec, 72˚C for 45 sec and finally 72˚C for 10 min. Primers for *BCRP* (forward 5'-CAGGTCTGTTTCAATCTCACA-3' and reverse 5'-TCCATATGGTGGAATGCTGAAG-3') and *MRP1* (forward 5'-GAAGGCCATCGGACTCTTCA-3' and reverse CAGCGCGGACACATGGT) were designed using the Primer Express software (Applied Biosystems). Primers for TBP (forward 5'-GCACAGGAGCCAAGAGTGAA-3' reverse 5'-TCACAGCTCCCCACCATGTT-3') were used according to (14). Quantification of TBP as an endogenous control gene was performed to standardize the amount of cDNA added to each reaction. Data are presented as relative expression of *BCRP* or *MRP1* using the comparative threshold cycle (Ct) method. ΔCt is the difference of Ct values of target gene and the houskeeping gene TBP. Relative expression of target genes in *BCRP* or *MRP1* transfected cells as compared to HEK/V or 2008 cells was expressed as $\Delta\Delta$Ct = ΔCt (*BCRP*- or *MRP1*-transfected cells) - ΔCt (HEK/V or 2008). The N-fold expression in target genes of *BCRP* or *MRP1* was expressed as $N = 2^{-\Delta\Delta Ct}$ (15).

Flow cytometry analysis. The cells were fixed and permeabilized using IntraPrep as recommended by the manufacturer (Immunotech, Marseille, France). Cells were incubated 45 min at 4˚C with the monoclonal antibodies BXP34 at 5 μg/ml (Alexis Biochemicals, San Diego, CA) or MRPm6 at 2.5 μg/ml (Alexis Biochemicals, San Diego, CA) for BCRP and MRP1 labelling respectively. For control, cells were incubated with the mouse IgG1 isotype (Dako A/S, Trappes, France). Protein detection was detected with the F(ab')2 fragment of rabbit anti-mouse IgG-FITC diluted to 1/50 (Immunotech). Cells were analysed using a flow cytometer Becton-Dickinson (Mountain View, CA, USA). FITC was excited at 488 nm with an argon ion laser. Fluorescence emission was collected after passage through a 530-nm band pass filter. Data protein expression was determined as ratio of mean fluorescence of monoclonal antibodies MRPm6/IgG1 or BXP-34/IgG1 control.

MTT cytotoxicity assay. Cells were plated in flat-bottom 96-well plates at a density of 2000 cells per well and allowed to attach for 24 h at 37˚C in 5% CO_2. Drugs at various concentrations with or without novobiocin (10 μM) or FTC (1 μM) were added to the cells before incubation for 72 h. A solution of MTT (20 μl, 2.5 mg/ml) was added to each well for 3 h at 37˚C. Medium was then removed and 200 μl of dimethylsulfoxide was added to each well. Optical densities were measured at 540 nm using a series 750 microplate reader (Cambridge Technology, Watertown, MA, USA). The IC_{50} was determined as the drug concentration which resulted in a 50% growth inhibition. Resistance factor (RF) was calculated by dividing the IC_{50} obtained for the resistant cell line by the IC_{50} obtained for the parental cell line.

Etoposide accumulation. Etoposide accumulation was performed by 24 h pre-plating of $5x10^5$ cells/well in 6-well plates. Wells were then refreshed with 1 ml of complete medium containing 2 μM etoposide spiked with 0.2 μCi/ml [^{3}H]-etoposide (Moravek, Brea, CA), with or without 10 μM FTC or 100 μM novobiocin. After incubation for 2 h at 37˚C, plates were then placed on ice, the medium was removed, and cells were washed once with ice-cold PBS and then solubilized with 0.2 ml of Solvable (Packard, Meridian, CT). Etoposide content was determined by scintillation counting. All drug cellular accumulation measurement were performed in triplicate wells.

Drug efflux. For drug efflux analysis, cells were preloaded with 2 μM [^{3}H]-etoposide for 2 h. The cells were washed with PBS after incorporation of the drugs. Then the cells were incubated in fresh medium containing or not 10 μM FTC for 1 h. After incubation, [^{3}H]-etoposide remaining in the cells was measured as described above.

Statistical analysis. Values are means ± SD. Means were considered to be significantly different using an appropriate unpaired t-test.

Results

BCRP and MRP1 mRNA analysis in transfected cells. Real-time quantification PCR assays have been performed determine the relative expression level of *BCRP* and *MRP1* mRNA. A high expression level of *BCRP* mRNA was observed in HEK293 cells transfected with the BCRP genes, as compared to HEK/V cell line (Table I). The relative expression level of *BCRP* mRNA in HEK/R482, HEK/R482G and HEK/R482T cell lines was 347-, 247- and 315-fold and is higher than in HEK/V cells, respectively. The relative expression level of *MRP1* mRNA was 35-fold higher in 2008/MRP1 cell line than in parental 2008 cell line.

BCRP protein expression. Flow cytometry was used to determine the BCRP expression in HEK293 cells (Fig. 1 and Table II). BCRP-transfected cells presented an increased BCRP protein level, as compared to HEK/V cells. As previously

Table I. Relative mRNA expression levels of *BCRP* and *MRP1* as estimated by real-time RT-PCR in HEK293 and 2008 transfected cells.[a]

	HEK/V	HEK/R482	HEK/R482G	HEK/R482T	2008	2008/MRP1
Ct TBP	21.20	20.59	20.43	20.07	25.13	25.31
Ct BCRP	28.41	19.36	19.69	18.98	-	-
Ct MRP1	-	-	-	-	27.20	22.23
ΔCt	7.21	-1.23	-0.74	-1.09	2.07	-3.08
ΔΔCt	-	-8.44	-7.95	-8.30	-	-5.15
$2^{-\Delta\Delta Ct}$	-	347.29	247.28	315.17	-	35.51

[a]The fold change of *BCRP* or *MRP1* expression in the *BCRP*- and *MRP1*-transfected cell lines was determined as described in Materials and methods. Results represent the mean of three independent RNA extractions.

Table II. Flow cytometry analysis of MDR proteins in HEK/293 and 2008 cells. Protein expression was determined as ratio of mean fluorescence of monoclonal antibodies MRPm6/IgG1 or BXP-34/IgG1 control.

	HEK/V	HEK/R482	HEK/R482G	HEK/R482T	2008	2008/MRP1
BCRP	1.56	20	25	21	-	-
MRP1	-	-	-	-	1.02	4.44

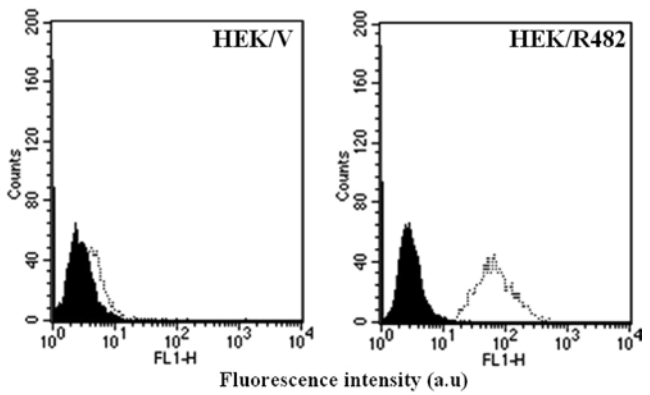

Figure 1. Analysis of BCRP protein expression in HEK/V and HEK/R482 cells by flow cytometry. Each histogram shows the overlay of the specific BXP34 antibody (white) with the isotype IgG1 control (black).

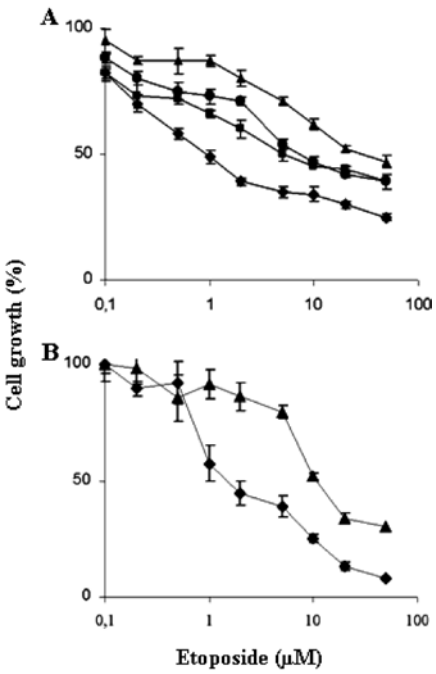

Figure 2. (A) Cytotoxic effect of etoposide in HEK293 (♦), HEK/R482 (■), HEK/R482T (●) and HEK/R482G (▲) cell lines. (B) Cytotoxic effect of etoposide in 2008 (♦) and 2008/MRP1 (▲) cells. Cell growth was determined using MTT assay. Standard deviation has been calculated with five independent experiments.

reported, BCRP protein levels are similar between wild-type and mutants (12). According to data reported by Hooijberg *et al* (13), a 4.3-fold increased expression of MRP1 was observed in 2008/MRP1 cells as compared to 2008 cells (Table II).

Cytotoxic effects of mitoxantrone and etoposide. The cytotoxic effect of etoposide has been determined in BCRP-transfected cells and in control HEK/V cells. As control, experiments have also been performed using the well-known BCRP substrate, mitoxantrone (16) and 2008/MRP1 cell line as a positive control for etoposide resistance (17) (Fig. 2 and Table III).

Results indicate that the expression of wt BCRP (HEK/R482) confers 5-fold resistance to etoposide, as compared to parental cells (HEK/V). Interestingly, the resistance factor was significantly increased to 30-fold in the glycine mutant (HEK/R482G). In contrast, resistance to etoposide in HEK/R482T cell line remains in the same range of HEK/R482 cell line. For mitoxantrone, the resistance observed was not significantly different between HEK/R482 cell line and the

Table III. IC_{50} of mitoxantrone and etoposide in HEK293 and 2008 cells in the presence or not of novobiocin or FTC.[a]

		Cell line									
		HEK/V	HEK/R482		HEK/R482G		HEK/R482T		2008	2008/MRP1	
Drug	Inhibitor	IC_{50}	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	IC_{50}	RF
Mitoxantrone	-	0.15	1.17	11.33	3.10	20.67	3.00	20.00			
	Novobiocin	0.08	0.08	1.00	0.50	6.25	0.90	11.25			
	FTC	0.12	0.11	0.92	0.21	1.75	0.32	2.67			
Etoposide	-	1.00	5.00	5.00	30.00	30.00	8.00	8.00	0.70	6.00	8.57
	Novobiocin	1.00	2.00	2.00	4.50	4.50	8.20	8.20			
	FTC	1.00	1.30	1.30	8.20	8.20	3.20	3.20			

[a]IC_{50} values are the means of three independent experiments, each performed in triplicate. Resistance factor (RF) is the ratio of the IC_{50} observed in MRP1- or BCRP-transfected cells and the IC_{50} observed in vector transfected cells.

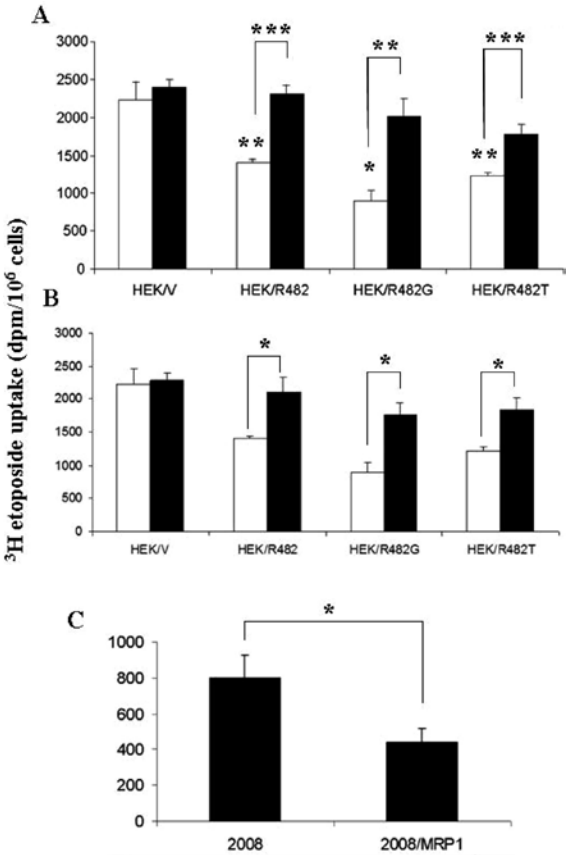

Figure 3. [^{3}H]-etoposide uptake after a 2-h treatment of HEK293 cells in the presence (black columns) or absence (white columns) of 100 μM novobiocin (A) or 10 μM FTC (B). (C) [^{3}H]-etoposide uptake in 2008 and 2008/MRP1 cells. Cells were incubated with [^{3}H]-etoposide (2 μM) for 2 h. Standard deviation has been calculated with three independent experiments. DPM: disintegration per minute. *p<0.01; **p<0.02; ***p<0.05.

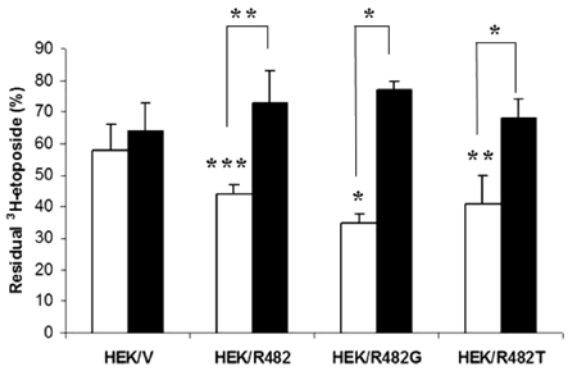

Figure 4. Intracellular residual [^{3}H]-etoposide in HEK293 cell lines. Cells were treated with 2 μM [^{3}H]-etoposide for 2 h, then washed and incubated in fresh medium for 1 h in the presence (black column) or not (white column) of 10 μM FTC. Cells were washed before measurement of cellular residual [^{3}H]-etoposide. Data are the means of three independent experiments. *p<0.01; **p<0.02; ***p<0.05.

two mutants and was ranging between 11- to 20-fold (Fig. 3 and Table III).

The resistance to mitoxantrone and etoposide in HEK/R482 cells was modulated by the BCRP inhibitors novobiocin (18,19) and FTC (20) (Table III). However, novobiocin was able to modulate resistance to etoposide to a lesser extent than FTC. By contrast, FTC was able to reverse resistance to etoposide moderately in HEK/R482G cell line as compared to novobiocin. Novobiocin was not able to modulate resistance to etoposide in HEK/R482T cell line. This suggests that mutant BCRP proteins presented altered properties for pharmacologic modulation (Table III).

Intracellular accumulation of etoposide is reduced in BCRP-transfected cells. The difference between the levels of resistance to etoposide shown in Table III suggested that mutant BCRP may induce an alteration of the cellular accumulation of etoposide. To address this issue, intracellular accumulations of [^{3}H]-etoposide were measured in vector and BCRP-transfected HEK293 cells after 2 h of treatment (Fig. 3A and B).

The [^{3}H]-etoposide uptake represented 63 (p<0.02), 40 (p<0.01) and 54% (p<0.02), respectively, in HEK/R482, HEK/R482G and HEK/R482T, of the value measured in HEK/V cells (Fig. 3A and B). Moreover, the [^{3}H]-etoposide uptake is significantly reduced in HEK/R482G cells as compared to HEK/R482 cells (p<0.05). The [^{3}H]-etoposide uptake is also reduced in HEK/R482T cells as compared to HEK/R482 cells, although

this does not reach stastical significance. As a positive control, [^{3}H]-etoposide uptake in 2008/*MRP1* cells was estimated and compared to 2008 cells. The uptake of [^{3}H]-etoposide in 2008/*MRP1* cells is reduced by 2-fold ($p<0.01$), as compared to 2008 cells (Fig. 3C).

In order to confirm these data, we treated the cells simultaneously with etoposide and 100 μM novobiocin. In all BCRP-transfected cell lines the intracellular concentration of [^{3}H]-etoposide has been found increased (Fig. 3A). [^{3}H]-etoposide uptake reached 92 ($p<0.05$), 77 ($p<0.02$) and 81% ($p<0.05$) in HEK/R482, HEK/R482G and HEK/R482T cells, respectively, when compared to the values observed in the absence of novobiocin. Moreover, a similar effect was observed when BCRP-transfected cells were treated with 10 μM FTC (Fig. 3B). This suggests that the resistance to etoposide is actually due to its transport by BCRP.

Etoposide efflux. In order to confirm the transport of etoposide by BCRP, we examined the efflux of etoposide in BCRP-transfected HEK293 cells. Cells were treated with 2 μM [^{3}H]-etoposide for 2 h, and washed out, in the presence or not of FTC, for an additional hour. All the intracellular [^{3}H]-etoposide concentrations were normalized with the intracellular concentrations measured before the washes. In the absence of FTC, after 1 h of washing, the intracellular [^{3}H]-etoposide concentration was of 58% of the initial concentration in HEK/V cells. However, in transfected cells it represented 44 ($p<0.05$), 35 ($p<0.01$) and 41% ($p<0.02$) of the initial concentration in HEK/R482, HEK/R482G and HEK/R482T cells, respectively (Fig. 4). The intracellular [^{3}H]-etoposide concentration is significantly lower in HEK/R482G cells as compared to HEK/R482 cells ($p<0.05$). However, this does not reach stastical significance when HEK/R482T cells were compared to HEK/R482 cells. In the presence of FTC, cellular residual [^{3}H]-etoposide was not significantly increased in HEK/V cells (Fig. 4). However, in HEK/R482, HEK/R482G and HEK/R482T cells treated with FTC, the cellular residual [^{3}H]-etoposide was increased to 73 ($p<0.02$), 77 ($p<0.01$) and 68% ($p<0.01$) of the initial concentration, and was significantly higher than that observed in the absence of FTC (Fig. 4).

Discussion

The human BCRP has been shown to confer resistance to mitoxantrone, anthracyclines and camptothecins (3,4). In drug-selected cell lines, two different mutations leading to a transition from arginine 482 to threonine (R482T) and glycine (R482G) respectively, have been observed (9,10). R482T mutation confers high-level resistance to anthracyclines (21), and cells with R482G or R482T mutation are able to efflux more efficiently rhodamine 123 (9). Moreover, R482G mutation seems to confer relatively less resistance to camptothecins. All these data suggest that amino acid 482 has a crucial role in BCRP function and that mutation of this amino acid significantly changes substrate specificity, thus altering the drug resistance phenotype.

BCRP has been recently involved in resistance to etoposide. Moreover, when P-glycoprotein (P-gp)- and Mrp1-deficient mouse cells are selected for resistance to etoposide, amplification and overexpression of Bcrp1 emerged as the dominant resistance mechanism (8). In order to study the role of this protein in resistance to etoposide in human, we used the embryonic HEK293 cells transfected with the wild-type BCRP (HEK/R482) or its two mutants R482G (HEK/R482G) and R482T (HEK/R482T) (5). As compared with HEK/V cells, all of transfected cell lines displayed resistance to mitoxantrone and transported efficiently the BCRP substrate Hoechst 33342 (Table III and data not shown). Determination of rhodamine 123 uptake confirmed that HEK/R482G and HEK/R482T cells transported efficiently this probe when compared with HEK/R482 cells (data not shown).

In our study, we have shown that wild-type BCRP, R482T and especially R482G mutant can confer significant resistance to etoposide, the HEK/R482G cells showing an IC_{50} value six-fold higher than in HEK/R482 cells (Table III). In this case, the resistance may be correlated with a decrease in the etoposide intracellular concentration (Fig. 3). Nevertheless, HEK/R482 and HEK/R482T cells were only 5- and 8-fold resistant, respectively, to etoposide (Table III). This suggest that R482G mutation confer to the BCRP protein a better affinity for etoposide than the mutation R482T, or a better efficiency in drug efflux, as suggested by our results (Fig. 3).

The BCRP inhibitors FTC and novobiocin was able to significantly modulate resistance to etoposide and with a higher efficiency in the two mutants (Table III). In the presence of FTC, cellular uptake of etoposide was significantly increased and particularly in HEK/R482 and HEK/R482G cells. Etoposide efflux measurements showed that inhibition of BCRP with FTC leads to a significant increase in intracellular concentration of etoposide in BCRP-transfected cell lines, as compared to untreated cells (Fig. 4). Allen and co-workers have shown that Bcrp1-mediated etoposide resistance was reversed by two structurally different BCRP/Bcrp1 inhibitors, GF120918 and Ko143 (8). The same authors suggested that mutation at R482 was not necessary for Bcrp1-mediated resistance to etoposide because the Bcrp1 expressed in the etoposide-resistant cells was wild-type at this position (22). Our data show clearly that R482G mutation is able to increase resistance to etoposide. Since several recent studies have demonstrated the role of BCRP expression in drug resistance in cancer (4) and given previous evidence that even low levels of drug transporters may have a substantial effect on the basal resistance to substrate drugs (23), BCRP may be taken into account as a potential contributor to resistance to etoposide. Finally, the mutation analysis at codon 482 of BCRP may be of great importance in clinical cancers treated with etoposide. Recently, only two studies have analysed the BCRP mRNA sequence on patient samples (24,25).

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