

# Effect of the Multidrug Resistance Protein on the Transport of the Antiandrogen Flutamide<sup>1</sup>

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## ABSTRACT

Prostate cancer is the most common noncutaneous malignancy of American men. Although it can be initially treated with androgen deprivation therapy, tumors that relapse become resistant to future hormonal manipulation. We previously found that the multidrug resistance protein (MRP), MRP1, is overexpressed in advanced stage and grade human prostate cancer and is negatively regulated by p53. In this study, we sought to determine whether the cellular accumulation of the antiandrogen flutamide, a drug commonly used in the treatment of prostate cancer, is affected by MRP1 expression. There were significant differences between the wild-type and MRP1-overexpressing cells in efflux and accumulation of flutamide and hydroxyflutamide, its active metabolite. In contrast, transport of dihydrotestosterone was not affected by MRP1. Treating the cells with leukotriene D<sub>4</sub>, a known MRP1 substrate, or VX-710, an MRP1 modulator, restored flutamide and hydroxyflutamide accumulation. Finally, intracellular glutathione depletion with buthionine sulfoximine or energy depletion using 2-deoxy-D-glucose/sodium azide restored flutamide accumulation to that of parental cells while incubating the cells at 4°C abolished MRP1-mediated transport. In summary, these studies indicate that flutamide and hydroxyflutamide but not dihydrotestosterone are transported by MRP1 and that these findings may contribute to our understanding of resistance to hormone refractory prostate cancer.

## INTRODUCTION

Prostate cancer is the most common noncutaneous malignancy in American men (1). When diagnosed at an early stage, *i.e.*, before cancer cells escape the capsule of the prostate, treatment with surgery or radiation can produce a 5-year survival of 95% (1). When prostate cancer recurs or is diagnosed at an advanced stage, standard treatment includes pharmacological or surgical castration (2, 3).

Most prostate cancers require androgens for growth and development. Flutamide is a nonsteroidal antiandrogen that acts as a competitive inhibitor of dihydrotestosterone for the androgen receptor (2) and is often used as a part of initial treatment. Flutamide and its active metabolite hydroxyflutamide block expression of genes with promoters that contain androgen response elements and also prevent androgen-dependent stabilization of the androgen receptor (4). Although >50% of patients initially respond to androgen deprivation, most patients relapse within a median of 12–18 months, at which time, they are resistant to additional hormonal therapy (5). Retreatment with antiandrogen therapy or chemotherapy of patients with hormone-refractory disease has not been shown to significantly improve the overall survival of 2–3 years (1).

Several factors may contribute to resistance after relapse from androgen deprivation therapy. These include increased affinity of the receptor for testosterone because of mutation of the androgen receptor

(6, 7), increased androgen receptor-mediated transcription through activation of cyclic AMP-dependent protein kinase A (8), and decreased apoptosis attributable to mutations in p53 (9).

We recently found that progression from benign prostate epithelium to high-grade prostate cancer correlated with the expression of drug resistance proteins (10). Specifically, our results indicated that overexpression of MRP<sup>3</sup>1 correlated with expression of mutant p53 (10) and that the expression of MRP1 was repressed by the wild-type protein (11).

MRP1 is a  $M_r$  190,000 membrane protein belonging to the ABC family of transporters. MRP1 pumps negatively charged substrates from the cytosol to the extracellular environment (12–14). Cotransport with glutathione (*e.g.*, doxorubicin and vincristine; Refs. 15, 16) or conjugation with glutathione, glucuronides, or sulfates (*e.g.*, leukotrienes) can enhance transport of MRP1 substrates (16, 17).

The structural features of drugs that are transported by ABC transporters have been extensively studied (18, 19). We noted that the antiandrogen flutamide shares several of these characteristics, including a weak negative charge and an electron-withdrawing trifluoro group attached to a hydrophobic ring (Refs. 18, 19; Fig. 1). Therefore, the purpose of this study was to determine if MRP1 expression affected the transport of flutamide in human cancer cells.

## MATERIALS AND METHODS

**Drugs.** Leukotriene D<sub>4</sub>, buthionine sulfoximine, sodium azide, and 2-deoxy-D-glucose were obtained from Sigma Chemicals (St. Louis, MO). [<sup>3</sup>H]Flutamide (10–13 Ci/mmol) and [<sup>3</sup>H]hydroxyflutamide (9 Ci/mmol) were obtained from Moravsek Biochemicals (Brea, CA). 5 $\alpha$ -Dihydro[1 $\alpha$ , 2 $\alpha$ (n)-<sup>3</sup>H] testosterone was obtained from Amersham Pharmacia (Bedminster, NJ). The MRP1 primary antibody, QCRL-1, and P-glycoprotein antibody, C219, were obtained from Signet Laboratories (Dedham, MA). The goat antimouse secondary antibody was obtained from Dako (Carpenteria, CA). The MRP6 antibody was obtained through Santa Cruz Biotechnology (Santa Cruz, CA). The MRP1 modulator VX-710 was a gift from Vertex Pharmaceuticals (Cambridge, MA).

**Cell Lines and Culture Conditions.** The parental KB3-1 and the MRP1-expressing cell line KB4D-10 (a gift from Vertex Pharmaceuticals) were grown in DMEM with 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. KB4D-10 cells were transfected with an MRP1 expression vector containing a full-length cDNA and grown in the same conditions as its parental cell line, except that the media contained 10 ng/ml doxorubicin. PC-3, the MRP1-overexpressing cell line PC-3-ADR (a gift from Dr. William Nelson, Johns Hopkins, Baltimore, MD), and the P-glycoprotein overexpressing cell line MCF-7-ADR were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. PC-3-ADR cells were selected for MRP1 by adding increasing amounts of doxorubicin and selecting viable colonies; these cells were maintained in 100 nM doxorubicin. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>/95% air. Cell lines were free of *Mycoplasma* and fungi and were discarded after 3 months; new cell lines were obtained from frozen stocks.

**Western Analysis.** Cell lysates were prepared, and protein concentrations determined as previously described (11) with slight modifications. Briefly,

<sup>3</sup> The abbreviations used are: MRP, multidrug resistance protein; BSO, buthionine sulfoximine; PBS, Phosphate Buffered Saline; ABC, ATP binding cassette.

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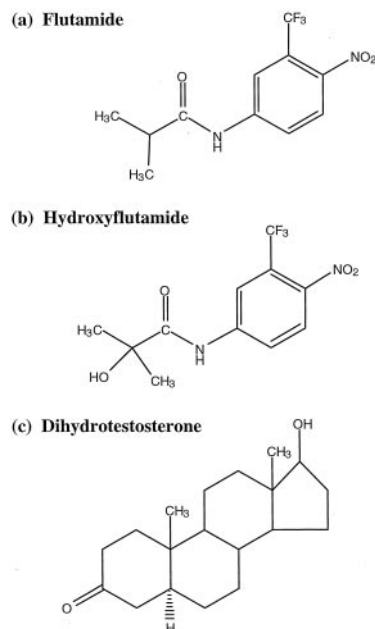


Fig. 1. Structure of flutamide (a), its active metabolite hydroxyflutamide (b), and dihydrotestosterone (c).

cells were lysed with radioimmunoprecipitation assay buffer [10 mM sodium phosphate (pH 7.2), 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 2 mM EDTA, supplemented with fresh 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 50  $\mu\text{g}/\text{ml}$  leupeptin], and the supernatant removed after centrifugation at  $16,000 \times g$  for 30 min. The supernatant was sonicated using a Virsonic 100 sonicator at 3 W. Fifty  $\mu\text{g}$  of protein were separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in PBS with 0.05% Tween 20, washed, and incubated with the MRP1 antibody QCRL-1 overnight. Binding of the primary antibody was detected using a goat antimouse horseradish peroxidase-conjugated secondary antibody. Binding was detected using the enhanced chemiluminescence detection system (Amersham Pharmacia) as described in the manufacture's protocol.

**Drug Accumulation, Uptake, and Efflux.** Drug accumulation and uptake were determined by seeding cells ( $5.0 \times 10^4$ – $1.0 \times 10^5$  cells/ml/well) onto 24-well plates. After 48 h, cells were washed with serum-free media containing 25 mM HEPES and incubated with 60 nM [ $^3\text{H}$ ]flutamide, 60 nM [ $^3\text{H}$ ]hydroxyflutamide, or 20 nM  $5\alpha$ -dihydro[1 $\alpha$ , 2 $\alpha$ (n)- $^3\text{H}$ ] testosterone for varying periods of time. Additional experiments were performed by incubating the cells at  $4^\circ\text{C}$  for 1 h or using glucose-free media supplemented with 15 mM sodium azide and 50 mM 2-deoxy-D-glucose for 30 min before drug incubation. The reactions were stopped by adding ice-cold PBS, and the cells were lysed immediately with 1% SDS. For measuring drug efflux, cells ( $5.0 \times 10^4$ – $1.0 \times 10^5$  cells/ml/well) were seeded onto 24-well plates, grown for 48 h, and washed with serum-free media. The cells were then incubated with [ $^3\text{H}$ ]flutamide or [ $^3\text{H}$ ]hydroxyflutamide for 30 min and washed twice with ice-cold PBS. Two hundred and fifty  $\mu\text{l}$  of serum-free media were added to each well and incubated for varying periods of time. The appearance of drug in fresh media in separate wells was measured over time by liquid scintillation counting.

## RESULTS

**MRP1 Expression in Cell Lines.** To determine the effect of MRP1 overexpression on flutamide accumulation, we used cell lines that overexpress MRP1 either by transfection or selection. Fig. 2 confirms the overexpression of MRP1 as compared with the parental controls and the lack of detectable P-glycoprotein in the drug-resistant cell lines.

**Effect of MRP1 on Transport of Flutamide.** To assess whether overexpression of MRP1 changes flutamide accumulation, we com-

pared the accumulation of [ $^3\text{H}$ ]flutamide, [ $^3\text{H}$ ]hydroxyflutamide, and [ $^3\text{H}$ ]dihydrotestosterone in MRP1-overexpressing cells to that of parental cell lines. Fig. 3 demonstrates that KB4D-10 and PC-3-ADR cells accumulated significantly less [ $^3\text{H}$ ]flutamide (Fig. 3A) and [ $^3\text{H}$ ]hydroxyflutamide (Fig. 3B) than the parental controls. This difference was observed after 15 s of incubation; steady-state accumulation was achieved after 1 min (Fig. 3 and Table 1). In contrast, the accumulation of [ $^3\text{H}$ ]dihydrotestosterone was not affected by MRP1 expression (Fig. 3C and Table 1).

To verify that the decrease in flutamide accumulation in MRP1 cells was energy dependent, we preincubated cells with sodium azide and 2-deoxy-D-glucose for 30 min. Under these conditions, steady-state accumulation of flutamide and hydroxyflutamide was restored to that of the parental cells (Fig. 4). In addition, incubation of the cells at  $4^\circ\text{C}$  abolished the MRP1-mediated flutamide transport (Fig. 5). In both instances, these conditions had no effect on accumulation of flutamide and hydroxyflutamide in sensitive cells (data not shown).

We next asked whether the difference observed in steady-state drug accumulation in MRP1 cells was attributable to changes in drug efflux. Fig. 6 shows that the efflux of [ $^3\text{H}$ ]flutamide (Fig. 6A) and [ $^3\text{H}$ ]hydroxyflutamide (Fig. 6B) into drug-free media was significantly increased in cell lines overexpressing MRP1.

Leukotriene D4 enters cells passively (20), is transported by MRP1, and can competitively block the transport of MRP1 substrates (21). To test whether leukotriene D4 could compete for [ $^3\text{H}$ ]flutamide and [ $^3\text{H}$ ]hydroxyflutamide transport, cell lines were incubated with the antiandrogens after a 3-h preincubation with leukotriene D4. Fig. 7 demonstrates that leukotriene D4 restored the accumulation of flutamide and hydroxyflutamide in cell lines overexpressing MRP1 but had no effect on the accumulation of the antiandrogens in parental cells.

Glutathione enhances transport of MRP substrates by either direct conjugation or cotransport (13, 22). To test the effects of glutathione on flutamide transport, we depleted cellular glutathione with BSO and measured the effect on flutamide accumulation. As shown in Fig. 8, BSO increased the accumulation of flutamide in MRP1-overexpressing cell lines but had no effect on parental cells.

To determine if VX-710, an inhibitor of MRP1 (23, 24), affected the transport of the clinically active agent hydroxyflutamide, we studied its effect on hydroxyflutamide accumulation. Fig. 9 demonstrates that VX-710 increased the accumulation of hydroxyflutamide in a dose-dependent manner. There was no effect of the drug on the transport of hydroxyflutamide in wild-type cells (data not shown).

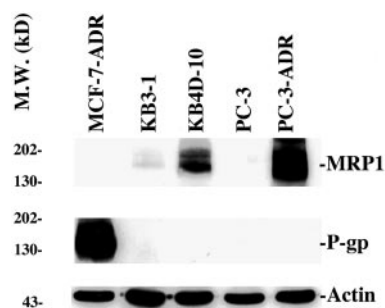


Fig. 2. Expression of MRP1 in KB4D-10 and PC-3-ADR cancer cell lines. Fifty  $\mu\text{g}$  of cell lysate from each cell line were resolved on an 8% SDS-PAGE and probed with antibodies to MRP1 (QCRL-1; Signet Laboratories),  $\beta$ -actin (Sigma Chemicals) or P-glycoprotein (Signet Laboratories) as described in "Materials and Methods." The results are a representative of three separate experiments.

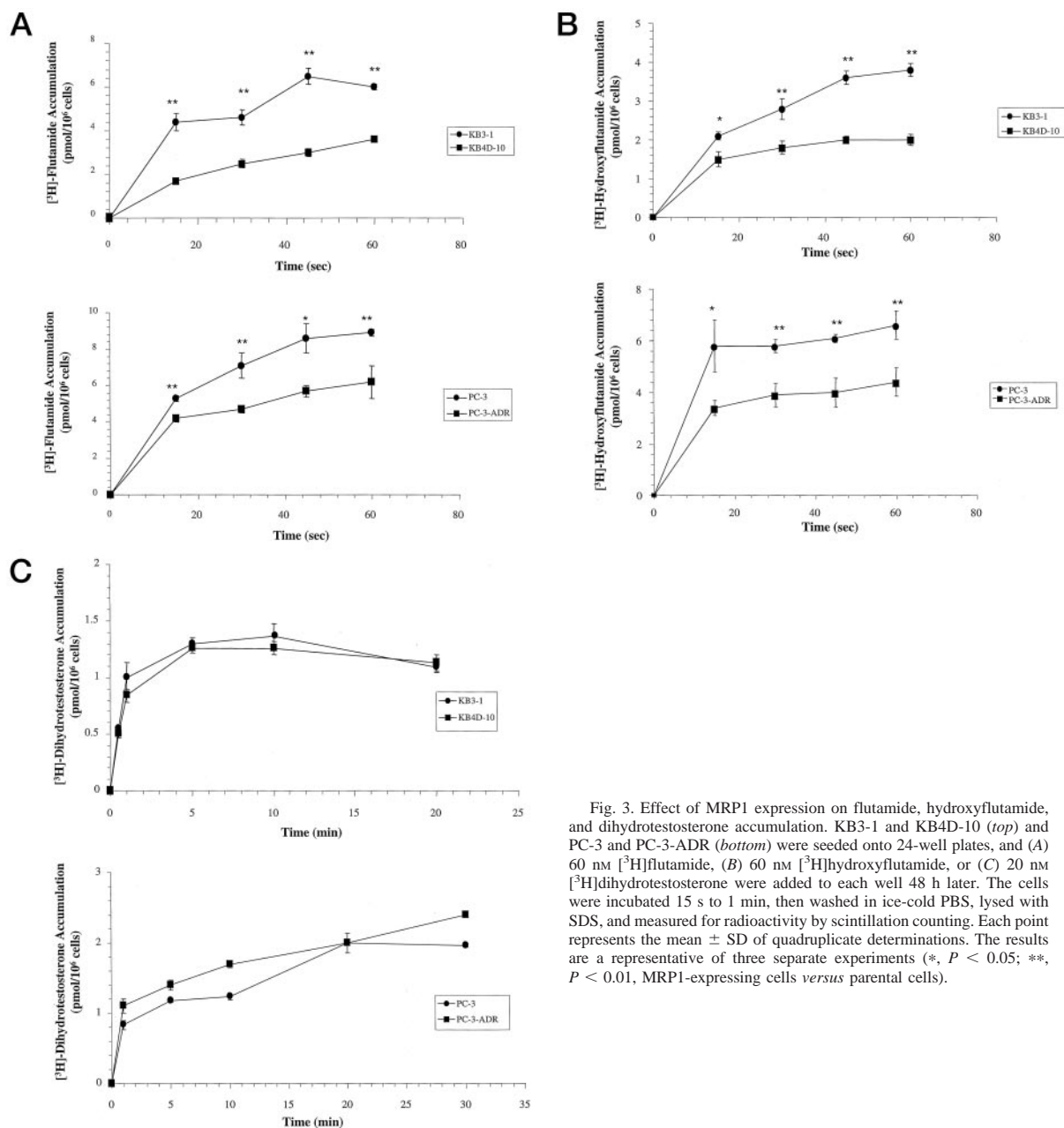


Fig. 3. Effect of MRP1 expression on flutamide, hydroxyflutamide, and dihydrotestosterone accumulation. KB3-1 and KB4D-10 (*top*) and PC-3 and PC-3-ADR (*bottom*) were seeded onto 24-well plates, and (A) 60 nM [ $^3$ H]flutamide, (B) 60 nM [ $^3$ H]hydroxyflutamide, or (C) 20 nM [ $^3$ H]dihydrotestosterone were added to each well 48 h later. The cells were incubated 15 s to 1 min, then washed in ice-cold PBS, lysed with SDS, and measured for radioactivity by scintillation counting. Each point represents the mean  $\pm$  SD of quadruplicate determinations. The results are a representative of three separate experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , MRP1-expressing cells *versus* parental cells).

## DISCUSSION

Resistance to antiandrogen therapy is a hallmark of advanced prostate cancer (1). We had several reasons to suspect that resistance to the antiandrogen flutamide might be mediated by MRP1. First, we had previously shown that MRP1 expression increased with advanced disease (10). Second, the structure of flutamide resembled other compounds transported by MRP1 and P-glycoprotein (18). In this study, we provide evidence for the first time that the antiandrogens flutamide and hydroxyflutamide are transported by MRP1.

Steady-state accumulation of flutamide and hydroxyflutamide is decreased (Fig. 3), and efflux is increased (Fig. 6) in cells that overexpress MRP1. To rule out that the rapid alterations in transport represented membrane binding, cells were pretreated with sodium azide and 2-deoxy-D-glucose or incubated at 4°C. Energy depletion or incubation at 4°C abolished the differences in drug accumulation between sensitive and resistant cells, consistent with the energy dependence of MRP-mediated transport. Steady state was achieved

rapidly; the accumulation of flutamide and hydroxyflutamide was the same at 30 min as at 1 min (Table 1). To verify that the effect was not transient, we incubated the cells up to 6 h and saw a similar observation (data not shown).

Table 1 Steady-state accumulation of flutamide, hydroxyflutamide, and dihydrotestosterone in sensitive and MRP1 cell lines<sup>a</sup>

Cell line	Flutamide	Hydroxyflutamide	Dihydrotestosterone
		(pmol/10 <sup>6</sup> cells)	
PC-3	7.4 $\pm$ 0.3 <sup>b</sup>	4.6 $\pm$ 0.6 <sup>b</sup>	1.1 $\pm$ 0.1
PC-3-ADR	5.9 $\pm$ 0.8	3.1 $\pm$ 0.6	1.2 $\pm$ 0.2
KB3-1	6.9 $\pm$ 0.9 <sup>c</sup>	5.2 $\pm$ 0.6 <sup>b</sup>	1.4 $\pm$ 0.6
KB4D-10	4.5 $\pm$ 0.7	3.9 $\pm$ 0.5	1.7 $\pm$ 0.3

<sup>a</sup>  $5.0 \times 10^4$ – $1.0 \times 10^5$  cells/ml/well were seeded onto 24-well plates and allowed to grow for 48 h. The cells were washed with serum-free media then [ $^3$ H]flutamide, [ $^3$ H]hydroxyflutamide, or [ $^3$ H]dihydrotestosterone were added and incubated for 30 min. Drug accumulation was measured as described in "Materials and Methods." Each value represents the mean  $\pm$  SD of quadruplicate determinations (<sup>b</sup>,  $P < 0.05$ , <sup>c</sup>,  $P < 0.01$  comparing the accumulation of drugs in MRP1-overexpressing cell lines to that of the parental controls).

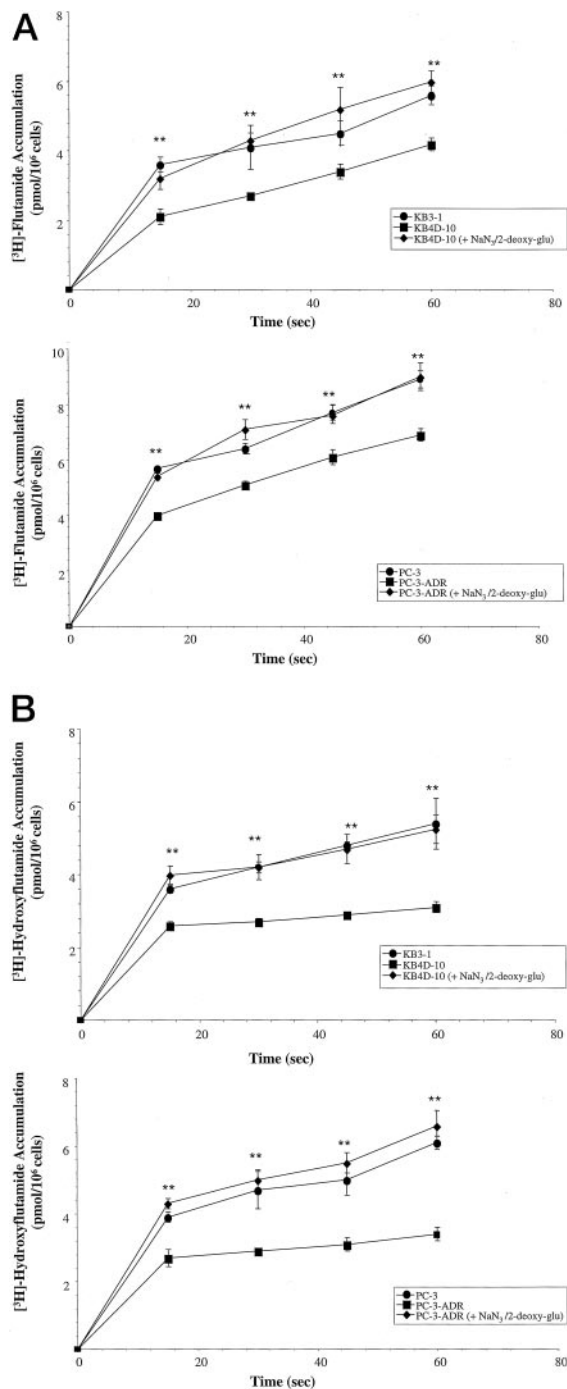


Fig. 4. Effect of sodium azide and 2-deoxy-D-glucose on flutamide and hydroxyflutamide accumulation. KB3-1 and KB4D-10 (top) and PC-3 and PC-3-ADR (bottom) were seeded onto 24-well plates. Forty-eight h later, the cells were incubated for 30 min in glucose-free media supplemented with 15 mM sodium azide and 50 mM 2-deoxy-D-glucose. The accumulation of (A) [ $^3\text{H}$ ]flutamide or (B) [ $^3\text{H}$ ]hydroxyflutamide was assayed as described in "Materials and Methods." Each point represents the mean  $\pm$  SD of quadruplicate determinations. The results are a representative of three separate experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , MRP1 cells treated *versus* untreated).

Several lines of evidence indicate that MRP1 rather than other MRP family members or other ABC transporters mediate the transport of flutamide. First, P-glycoprotein was not detected in the cell lines (Fig. 2); second, flutamide transport was inhibited by leukotriene D4 (Fig. 7) and BSO (Fig. 8), inhibitors of MRP-family transporters (MRP1, MRP2, MRP3, and MRP6; Refs. 13, 17, 25); third, we excluded MRP2 and MRP3 as flutamide transporters because they are ex-

pressed in both the parental lines and resistant lines (26), yet BSO (Fig. 8), leukotriene D4 (Fig. 7), and VX-710 (Refs. 23, 24; Fig. 9) did not affect flutamide transport in parental cells. These data narrowed the possibility to MRP1 and MRP6. Western analysis revealed no differences in MRP6 expression in sensitive and resistant cell lines (data not shown). Finally, the KB4D-10 cells, which were transfected with a MRP1 expression vector, reproduced the effects on flutamide transport that was seen with the selected cell lines. Currently, there are no fully selective MRP1 substrates to use as a positive control.

Transport of both flutamide and its active metabolite hydroxyflutamide (27) are affected by MRP1 to a similar extent (Fig. 3). Therefore, hydroxylation by the cytochrome p450 enzyme CYP1B1 does

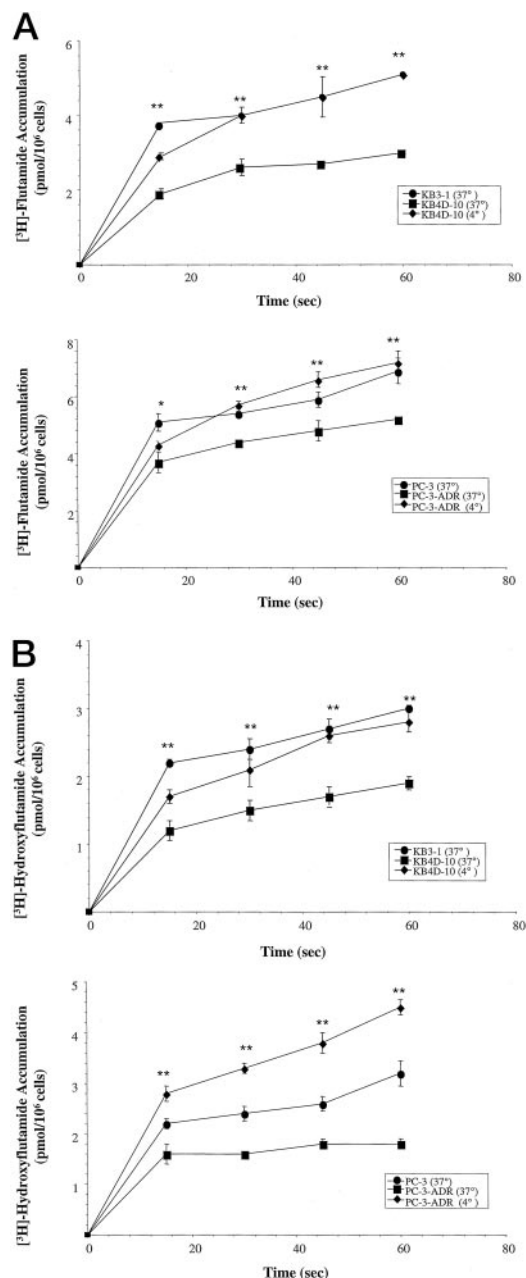


Fig. 5. Effect of temperature on flutamide and hydroxyflutamide accumulation. KB3-1 and KB4D-10 (top) and PC-3 and PC-3-ADR (bottom) were seeded onto 24-well plates. Forty-eight h later, the cells were incubated for 1 h at 4°C. The accumulation of (A) [ $^3\text{H}$ ]flutamide or (B) [ $^3\text{H}$ ]hydroxyflutamide was assayed as described in "Materials and Methods." Each point represents the mean  $\pm$  SD of quadruplicate determinations. The results are a representative of three separate experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , MRP1 cells treated *versus* untreated).



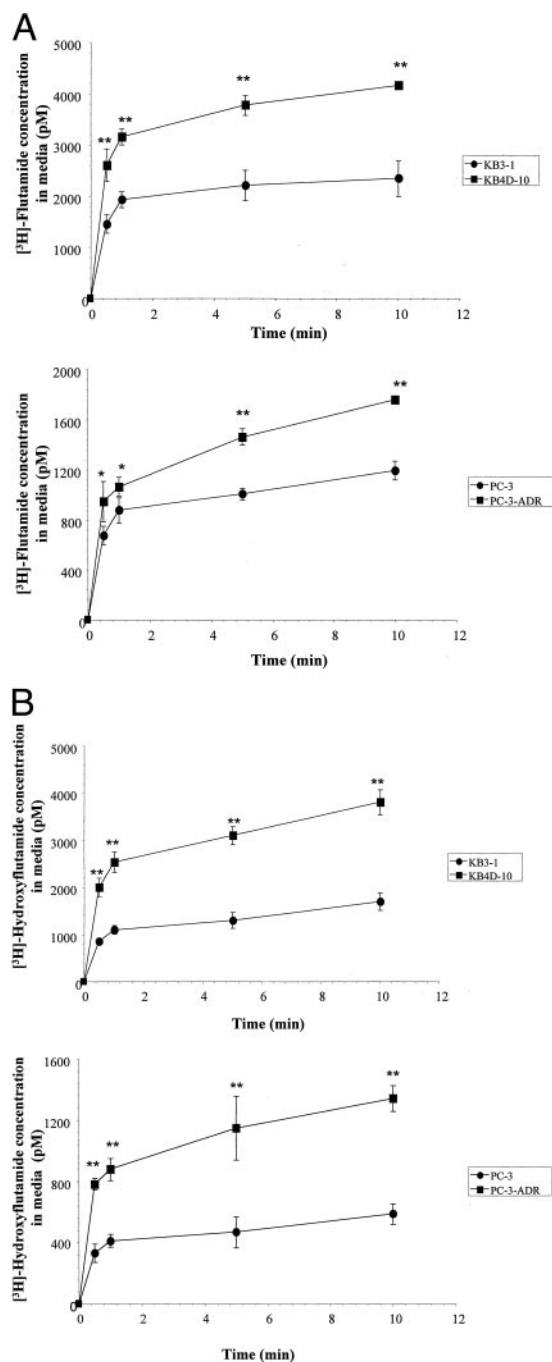


Fig. 6. Effect of MRP1 expression on efflux of flutamide and hydroxyflutamide. KB3-1 and KB4D-10 (*top*) and PC-3 and PC-3-ADR (*bottom*) cells were seeded onto 24-well plates, and 60 nM (A) [<sup>3</sup>H]flutamide or (B) [<sup>3</sup>H]hydroxyflutamide were added 48 h later. The cells were allowed to accumulate drug for 30 min, then washed twice with ice-cold PBS, and resuspended in serum-free media. The appearance of [<sup>3</sup>H]flutamide and [<sup>3</sup>H]hydroxyflutamide in fresh media at various time points was measured by scintillation counting. Each point represents the mean  $\pm$  SD of quadruplicate determinations. The results are a representative of three separate experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , MRP1-expressing cells *versus* parental cells).

not influence transport of the active metabolite (28, 29). It was not possible to determine the effect of MRP1 overexpression on sensitivity of these cell lines to antiandrogen therapy because the cells are androgen independent, and insufficient quantities of hydroxyflutamide are available for cell viability studies.

Flutamide shares structural similarities with other MRP1 substrates. These characteristics include a negative charge on an aromatic ring containing a strong electron withdrawing group (-CF<sub>3</sub>) (Fig. 1; Refs.

12, 13, 18). Other compounds that are transported by MRP1 that contain these structural features include the short-chain lipid 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoyl]-sphingosine glucosylceramide (30) and 2,4 dinitrophenyl-S-glutathione (16, 18).

The emergence of hormone-refractory prostate cancer is a major factor in the demise of patients with this disease. Our current studies build on previous results to create a scenario by which the function of

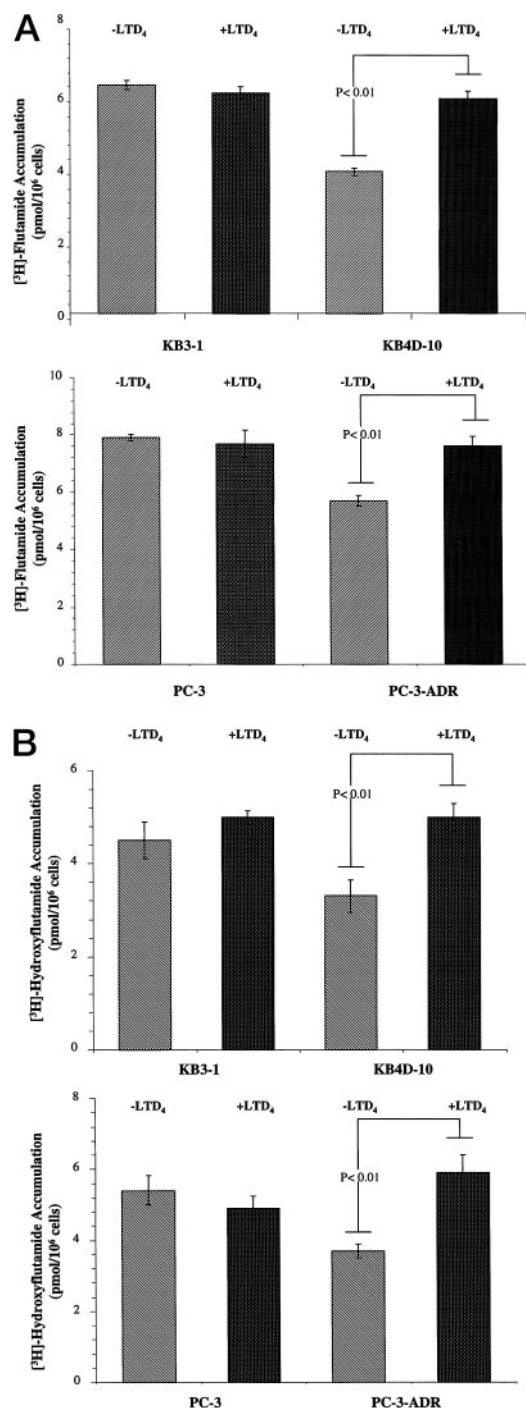


Fig. 7. Effect of leukotriene D4 on accumulation of flutamide and hydroxyflutamide. KB3-1 and KB4D-10 (*top*) and PC-3 and PC-3-ADR (*bottom*) were seeded onto 24-well plates, and the accumulation of (A) [<sup>3</sup>H]flutamide or (B) [<sup>3</sup>H]-hydroxyflutamide was determined after a 3-h preincubation with 2  $\mu$ M leukotriene D4 as described in Fig. 2 and "Materials and Methods." Each point represents the mean  $\pm$  SD of quadruplicate determinations. The results are a representative of three separate experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , MRP1 cells treated *versus* untreated).

p53 may contribute to androgen independence. When studying the expression of drug resistance proteins in *de novo* human prostate cancer, we were struck by the coexpression of mutant p53 and MRP1 in surgical samples (10). We demonstrated that MRP1 expression is repressed by wild-type p53 and that expression is increased when p53 is inactivated by mutation (11). We found that dihydrotestosterone is not transported by MRP1 (Fig. 3C, Table 1), and these data suggest that in the presence of MRP1, dihydrotestosterone can still accumulate in cells and interact with androgen receptors, whereas the interaction

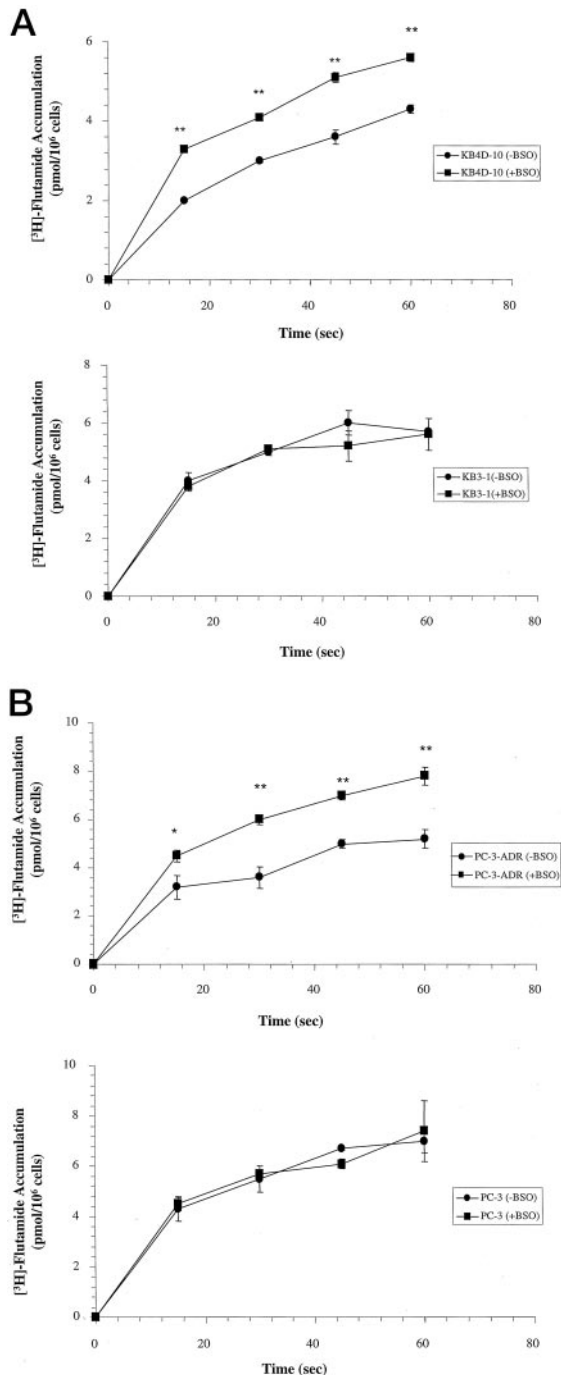


Fig. 8. Effect of glutathione depletion on the accumulation of flutamide. A, KB3-1 (bottom) and KB4D-10 (top) and (B) PC-3 (bottom) and PC-3-ADR (top) cells were seeded onto 24-well plates, and 50  $\mu\text{M}$  BSO added 24 h later. Drug accumulation was assayed as described in "Materials and Methods." Each point represents the mean  $\pm$  SD of quadruplicate determinations. The results are a representative of three separate experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , MRP1 cells treated *versus* untreated).

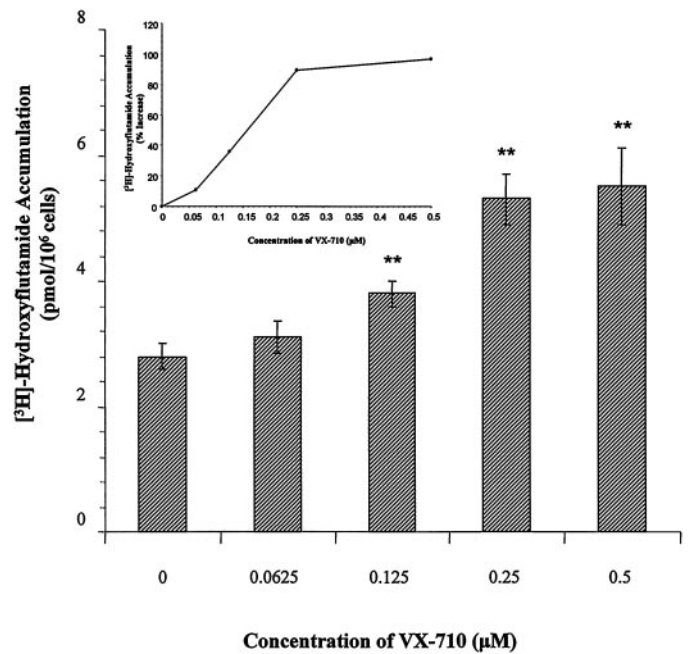


Fig. 9. Effect of VX-710 on the accumulation of hydroxyflutamide. PC-3-ADR cells were plated onto 24-well plates and allowed to grow for 48 h before VX-710 was added for 3 h. Drug accumulation was assayed as described in Fig. 4 and "Materials and Methods." The insert signifies the percentage increase over control (no addition of VX-710). Each point represents the mean  $\pm$  SD of quadruplicate determinations. The results are a representative of three separate experiments (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  MRP1 cells treated *versus* untreated).

of the antiandrogen with this target will be decreased. Our data suggest that in addition to the known effects of p53 on apoptosis (9), loss of p53 function may lead to resistance to antiandrogen drugs through MRP1-mediated drug transport.

In summary, we have shown that flutamide is transported by MRP1 based on drug accumulation and efflux studies and the ability of leukotriene D4, BSO, and VX-710 to restore steady-state concentrations. At the same time, cell lines that overexpress MRP1 retain baseline accumulation of dihydrotestosterone. These results suggest an unexpected role of MRP1 in the process of resistance to antiandrogen therapy of prostate cancer that could potentially be addressed through the use of MRP1 modulators.

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