# Anti-cancer drugs and glutathione stimulate vanadate-induced trapping of nucleotide in multidrug resistance-associated protein (MRP)

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**Abstract Multidrug resistance-associated protein (MRP), a member of the ABC superfamily transporters, functions as an ATP-dependent efflux pump that extrudes cytotoxic drugs from the cells. Although glutathione has been considered to play an important role in the function of MRP, there is no convincing evidence that glutathione directly interacts with MRP. Here we demonstrate that vanadate-induced trapping of 8-azido-ATP in MRP was stimulated in the presence of glutathione, oxidized glutathione and the anti-cancer drugs VP-16 and vincristine. MRP in membrane from a human *MRP* cDNA transformant was specifically photolabeled with 8-azido-[$\alpha$-$^{32}$P]ATP by the vanadate-trapping technique. Vanadate and $Mg^{2+}$ were required for trapping of nucleotides, and vanadate trapping of nucleotides was inhibited by excess ADP as well as ATP. These results suggest that a stable inhibitory complex MRP·MgADP·Vi, an analog of the MRP·MgADP·Pi transition state complex, is formed in the presence of vanadate. Glutathione as well as anti-cancer drugs would directly interact with MRP, and stimulate the formation of the transition state of the ATPase reaction of MRP.**

*Key words:* ABC transporter; Multidrug resistance-associated protein; Multidrug resistance; ATP hydrolysis; Glutathione

## 1. Introduction

Multidrug resistance (MDR) of tumor cells is a major obstacle in cancer chemotherapy. This phenomenon is frequently associated with the expression of P-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP), both members of the ABC superfamily of transporters [1]. Pgp and MRP function as ATP-dependent efflux pumps that extrude cytotoxic drugs from the cells before the drugs reach their intracellular targets, thus conferring resistance to many structurally dissimilar anti-cancer drugs, such as the *Vinca* alkaloids, colchicine, actinomycin D, epipodophyllotoxins, taxol, and anthracyclines [2–6]. However, the mechanism of transport for MRP could be different from that for Pgp, because the depletion of intracellular glutathione by buthionine sulfoximine (BSO) results in a complete reversal of resistance to anti-cancer drugs of some cell lines expressing MRP [7,8], but BSO has no effect on Pgp-mediated MDR.

It has been reported that MRP transports leukotriene $C_4$, a glutathione conjugate, and that MRP mediates ATP-dependent transport of vincristine in the presence of glutathione [9–12]. These results suggest that glutathione is important in the function of MRP. However, the mechanism by which glutathione helps in vitro ATP-dependent vincristine transport and in vivo efflux of anti-cancer drugs from cells by MRP is unclear. There is no convincing evidence that glutathione directly interacts with MRP.

MRP-mediated transport is ATP-dependent, and non-hydrolyzable ATP analogs cannot support MRP-mediated transport [9–12]. The mechanism of ATP hydrolysis by Pgp has been studied [13–15] and it was recently reported that the apparent affinity for nucleotides of Pgp was greatly increased in the presence of vanadate [16,17], probably because a stable and reversible inhibitory complex was generated by trapping nucleotide at the catalytic site. Although MRP and Pgp share only 15% amino acid identity, the 'ATP binding cassette' regions of these proteins are highly homologous [18]. We therefore expected that the mechanism of ATP hydrolysis by MRP could be analogous to that by Pgp, and that vanadate trapping with 8-azido-ATP followed by UV irradiation might cause specific labeling of MRP as in the case of Pgp.

## 2. Materials and methods

### 2.1. Materials

The monoclonal antibody C219 was purchased from Centocor, and QCRL-1 [19] was provided by Dr. S. Cole, Queen's University, Kingston, Ont., Canada. Human MRP expression vector pJ3Ω-MRP [4] was provided by Dr. G.J.R. Zaman, Netherlands Cancer Institute. Vinblastine, colchicine, actinomycin D, taxol, and doxorubicin were purchased from Wako Pure Chemical Industries. VP-16 was from Sigma. 8-Azido-[$\alpha$-$^{32}$P]ATP was purchased from ICN Biomedicals.

### 2.2. Transfection and drug resistance assay

Human cultured KB-3-1 cells were propagated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum under 5% $CO_2$ at 37°C. KB-3-1 was cotransfected with pJ3Ω-MRP and pSV2neo with LipofectAMINE (Gibco) according to the manufacturer's directions. Cells were first selected in the presence of 0.8 mg/ml gentamicin (G418) for 10 days. The mass populations of gentamicin-resistant colonies were further selected in stepwise increasing concentrations (5, 20, 40 ng/ml) of doxorubicin. KB-3-1 cells transfected with pSV2neo formed no colonies in media containing 5 ng/ml of doxorubicin. The $IC_{50}$ (the drug concentration that inhibits cell growth by 50%) was measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from dose-response curves for increasing concentrations of vinblastine, colchicine, actinomycin D, taxol, doxorubicin, and VP-16.

### 2.3. Vanadate trapping of 8-azido-ATP and subsequent photoaffinity labeling of MRP

Membrane proteins (20 μg) were incubated with 200 μM vanadate, 3 mM $MgSO_4$, 2 mM ouabine, 0.1 mM EGTA, 20 μM 8-azido-[$\alpha$-$^{32}$P]ATP, and 40 mM Tris-HCl (pH 7.5), in a total volume of 20 μl for 10 min at 37°C. (Variations of these conditions are described in the figure legends.) The reactions were stopped by addition of 400 μl of ice-cold Tris-EGTA buffer (0.1 mM EGTA, 40 mM Tris-HCl (pH

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*Abbreviations:* MDR, multidrug resistance; Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; BSO, buthionine sulfoximine; FBS, fetal bovine serum


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7.5)), and free ATP, $Mg^{2+}$, and vanadate were removed after centrifugation (15000×*g*, 10 min, 4°C). The pellet was washed in the same buffer and was resuspended in 8 μl of Tris-EGTA buffer, placed on ice, and irradiated for 5 min (λ = 254 nm, 5.5 mW/cm$^2$). Samples were electrophoresed on SDS-polyacrylamide gel, then autoradiographed. The amount of trapped 8-azido-[α-$^{32}$P]ATP in MRP was measured by counting the radioactivity of the band of MRP excised from a SDS gel. Experiments were done in duplicate or triplicate.

## 3. Results

A stable transformant, KB/MRP, was made by introducing an expression vector, pJ3Ω-MRP, containing the human *MRP* cDNA into a drug-sensitive human carcinoma cell line, KB-3-1. MRP was detected as a 190-kDa protein with the MRP-specific monoclonal antibody QCRL-1 in KB/MRP membrane, but not in KB/MDR1 membrane (Fig. 1A). Pgp was not detected in KB/MRP membrane (Fig. 1B). Pgp or MRP was not detected in the parental KB-3-1 membrane. The spectrum of multidrug resistance of KB/MRP cells was similar to that of KB/MDR1 (Fig. 1C): they were resistant to doxorubicin, VP-16, vinblastine, colchicine, actinomycin D, and taxol. But patterns of drug resistance were quite characteristic: KB/MRP cells were preferentially resistant to VP-16 and KB/MDR1 cells to taxol as reported previously [3,4].

Pgp has been reported to have a drug-stimulated ATPase activity [13], and hence drug transport and ATP hydrolysis are considered to be coupled directly. In the presence of vanadate, Pgp forms a stable and reversible inhibitory complex with MgADP·Vi after ATP hydrolysis [16,17]. Because MRP-mediated transport requires ATP and non-hydrolyzable ATP analogs cannot support it [9–12], MRP-mediated transport is also considered to be coupled to ATP hydrolysis. The vanadate trapping technique was so effective for investigating catalytic sites of Pgp that we first examined if vanadate induces trapping of nucleotides in MRP as it does in Pgp.

When membrane proteins from KB/MRP cells were incubated with vanadate, $Mg^{2+}$, and 8-azido-[α-$^{32}$P]ATP, followed by UV irradiation, a 190-kDa protein was specifically photoaffinity-labeled (Fig. 2, lane A4). No specific photoaffinity-labeled protein was observed in membrane proteins from KB-3-1 (lane A1), and the mobility in SDS-PAGE of the photoaffinity-labeled protein in KB/MRP membrane was identical to that of MRP in the Western blot (data not shown), indicating that MRP in KB/MRP membrane was photolabeled with 8-azido-[α-$^{32}$P]ATP in the presence of vanadate. Both vanadate and $Mg^{2+}$ were required for the photoaffinity labeling (lanes A2 and A3). Vanadate trapping was inhibited by an excess of ATP (lane A5) or ADP (lane A6). These results suggest that the apparent affinity for ATP of MRP was quite low and was greatly increased in the presence of vanadate.

We examined the effect of substrates for MRP on vanadate trapping. Vanadate trapping of nucleotides was increased about 3-fold by VP-16 (Fig. 2, lanes B4 and D4) and vincristine (lane C3), 1.5-fold by doxorubicin (lanes B6 and D6), and 1.2–1.3-fold by verapamil (lanes B2 and D2), vinblastine (lanes B3 and D3), and taxol (lanes B5 and D5). These results corresponded to the resistance spectrum of MRP-overexpressing cells including KB/MRP in this report: preferential resistance to VP-16 and vincristine, moderate resistance to doxorubicin, and low resistance to taxol and vinblastine. These results suggested that VP-16 and vincristine interacted with MRP directly in the absence of glutathione, although transport of these drugs by MRP was reported to be dependent on intracellular glutathione [7,8].

Then we examined the effect of glutathione on vanadate trapping of nucleotides in MRP, and found that 20 μM glutathione (Fig. 2, lanes B7, C2, and D7) and oxidized glutathione (lanes B8 and D8) increased vanadate trapping of nucleotides about 3-fold. These results suggest that glutathione interacts with MRP directly. Vanadate trapping of nucleotides was further stimulated by 1 mM and 3 mM glutathione (lanes C4 and C6). The effect of 3 mM glutathione was weaker than that of 1 mM glutathione, suggesting that the physiological concentration of glutathione may have a maximal stimulatory effect on the ATPase activity of MRP. Interestingly, the effects of 1 mM (lane C5) and 3 mM glutathione (lane C7) on vanadate trapping were rather depressed in the presence of 20 μM vincristine.

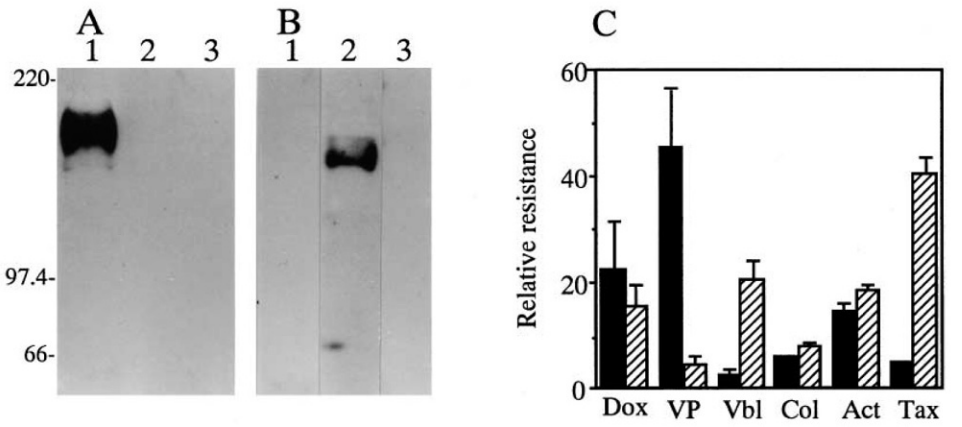



Fig. 1. Immunoblot analysis of MRP and Pgp in membrane protein fractions of KB/MRP, KB/MDR1, and untransfected KB-3-1 cells with the anti-MRP monoclonal antibody QCRL-1 (A) or with the anti-Pgp monoclonal antibody C219 (B). Lane 1, KB/MRP; lane 2, KB/MDR1; lane 3, KB-3-1. Membrane proteins (20 μg) were separated in a 7% SDS-polyacrylamide gel. Molecular size standards are indicated in kDa on the left. C: Relative resistance of KB/MRP (black bars) and KB/MDR1 cells (hatched bars). Relative resistance was calculated by dividing the $IC_{50}$ values (concentration required for inhibiting growth rate by 50%) against each drug by those of KB-3-1 cells. Each bar represents the average of three experiments.

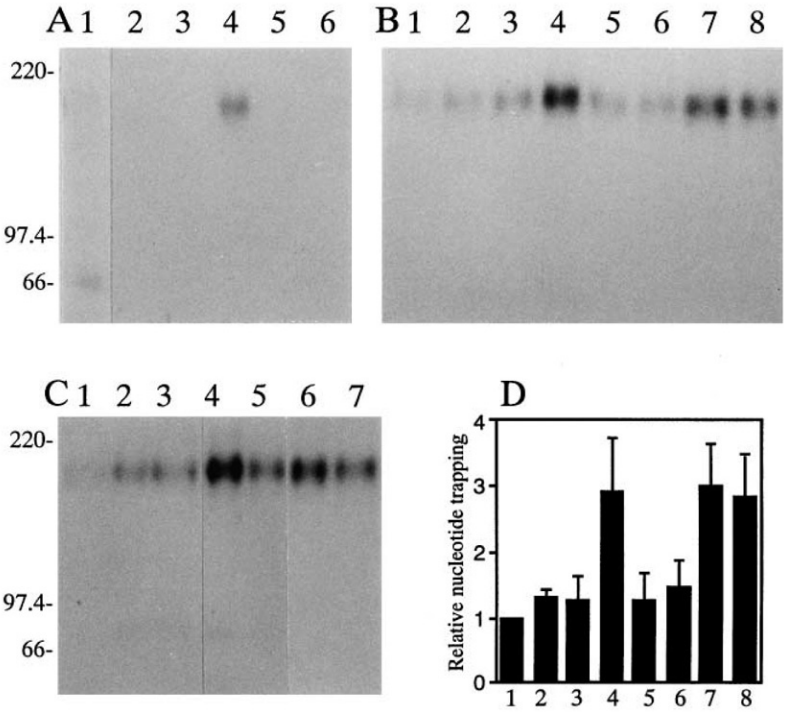
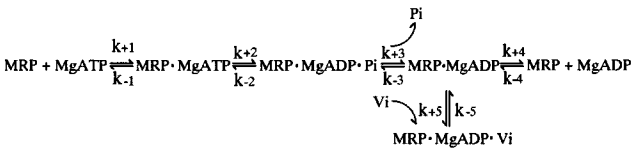



Fig. 2. Stimulation of vanadate-induced trapping of nucleotide in MRP by anti-cancer drugs and glutathione. A: Photoaffinity labeling of MRP in plasma membrane with 8-azido-ATP in the presence of $Mg^{2+}$ and vanadate. Plasma membrane proteins (20 μg) from KB-3-1 (lane 1) or from KB/MRP (lanes 2–6) were reacted with 20 μM 8-azido-[α-$^{32}$P]ATP, 200 μM vanadate, and 3 mM $MgSO_4$, and analyzed as described in Section 2. Incubations were done in the absence of vanadate (lane 2) or $MgSO_4$ (lane 3), or in the presence of 2.5 mM ATP (lane 5), or ADP (lane 6). Experiments were done three times independently. B: Stimulation of vanadate-induced trapping by anti-cancer drugs and glutathione. Plasma membrane proteins (20 μg) from KB/MRP were reacted with 20 μM 8-azido-[α-$^{32}$P]ATP, 200 μM vanadate, and 3 mM $MgSO_4$ in the presence of 20 μM verapamil (lane 2), vinblastine (lane 3), VP-16 (lane 4), taxol (lane 5), doxorubicin (lane 6), glutathione (lane 7), or oxidized glutathione (lane 8). Experiments were done three times independently. C: Plasma membrane proteins (20 μg) from KB/MRP were reacted with 20 μM 8-azido-[α-$^{32}$P]ATP, 200 μM vanadate, and 3 mM $MgSO_4$ in the presence of 20 μM glutathione (lane 2), or vincristine (lane 3), 1 mM glutathione (lane 4), 1 mM glutathione and 20 μM vincristine (lane 5), 3 mM glutathione (lane 6), 3 mM glutathione and 20 μM vincristine (lane 7). Experiments were done twice independently. D: Relative stimulation of vanadate trapping of nucleotides in MRP. The amount of trapped 8-azido-[α-$^{32}$P]ATP in MRP was measured by counting the radioactivity of the band of MRP excised from a SDS gel shown in B. Relative stimulation was calculated by dividing the amount of trapped nucleotide by that of lane B1. Each bar represents the average of three independent experiments.

## 4. Discussion

We showed that MRP in KB/MRP membrane could be specifically photolabeled with 8-azido-[α-$^{32}$P]ATP by the vanadate trapping technique. Vanadate and $Mg^{2+}$ were required for trapping of nucleotides, and vanadate trapping of nucleotides was inhibited by excess ADP as well as ATP. Vanadate-induced trapping of nucleotides at the catalytic site was extensively studied with myosin [20,21] and Pgp [16,17], and in both cases vanadate was accepted as an analog of inorganic phosphate and a stable and reversible inhibitory complex was generated by trapping MgADP·Vi at the catalytic site. These results suggest that MRP, like Pgp, has no high-affinity binding site for MgATP, but forms a metastable state (E·MgADP·Pi, where E is MRP or Pgp) after hydrolysis, and the phosphate ion is released before MgADP. A minimal reaction pathway for vanadate-induced trapping of nucleotides in MRP could be speculated to be like Scheme 1. The first catalytic turnover in MRP produces an intermediate containing ADP, and then vanadate binds to this intermediate to form a stable inhibitory complex, MRP·MgADP·Vi. MgADP could also be trapped at the catalytic site by pathway $k_{-4}$, and this would be why excess ADP inhibited vanadate-induced trapping of nucleotide. This inhibitory complex would be an analog of the MRP·MgADP·Pi transition state complex.




Scheme 1.

Cell lines overexpressing MRP are resistant to VP-16, vincristine, and doxorubicin, and these drugs were reported to be substrates for MRP to transport [3,4]. On the other hand, cell lines overexpressing MRP showed only low resistance to vinblastine and taxol. Verapamil has only a weak effect on drug resistance of KB/MRP cells (data not shown). Vanadate trapping of nucleotides was increased about 3-fold by VP-16 and vincristine, and weakly or marginally by doxorubicin, vinblastine, taxol, and verapamil. These results indicate that preferential substrates for MRP to transport stimulate vanadate-induced trapping of nucleotide in MRP, i.e. the formation of the transition state of the ATPase reaction of MRP.

Glutathione has been considered to be important in the function of MRP, but there was no convincing evidence that GSH directly interacts with MRP. In this work, we

demonstrated that glutathione strongly stimulated vanadate-induced trapping of nucleotides in MRP, suggesting that glutathione as well as anti-cancer drugs would directly interact with MRP. It is assumed that MRP contains a bipartite binding site for hydrophobic moieties of substrates and glutathione that would allow binding of covalent or non-covalent drug-glutathione complexes, or the sequential binding of glutathione and drug [12]. The depressing effects of 20 μM vincristine on vanadate trapping induced by 1 mM and 3 mM glutathione (Fig. 2) may suggest that binding sites for anti-cancer drugs and glutathione are not independent but overlap or closely interact with each other.

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