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In archaebacteria, there is a doxorubicin efflux pump similar to mammalian P-glycoprotein

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We selected for study an anthracycline-resistant mutant from the archaebacteria *Haloferax volcanii*. This resistance was reversed by a Ca^{2+}-channel antagonist, nifedipine (NDP). This resistance and its reversal by NDP suggest P-glycoprotein (Pgp) to be responsible for maintaining an anticancer drug concentration below the cytotoxic level. Using rhodamine 123 (RH123) as a substrate for Pgp, we then examined whether the resistance to anthracyclines in this bacteria might involve a Pgp-like anthracycline efflux pump. RH123 accumulation by the bacteria was determined with flow cytometry. A steady-state RH123 accumulation by the resistant cells revealed approx. one-fifteenth of that by the wild-type cells, which could be remarkably enhanced by NDP. The other modulators of Pgp, diltiazem and verapamil, also enhanced RH123 accumulation in resistant cells. The uncoupler FCCP completely restored RH123 accumulation in resistant cells to the wild-type cell level. RH123 unidirectional efflux from resistant cells after its preloading revealed much greater than that from wild-type cells, which was remarkably inhibited by FCCP. These confirmed that RH123 low accumulation involves its active efflux mechanism. Taken together, the present study indicated that lower evolutionary archaebacteria might also express a Pgp-like protein very similar to mammalian Pgp.

Introduction

The mammalian *mdr1* gene product P-glycoprotein (Pgp) has been confirmed to function as an active efflux pump of diverse anticancer drugs from multi-drug resistant (MDR) tumor cells [1–5]. The entire coding sequence of mouse or human *mdr1* cDNA was inserted into an expression vector and transfected into drug-sensitive cells, which in turn acquired the MDR phenotype with an impaired accumulation of drug into the cells [6–8]. At present, it is thought that Pgp expression is directly involved in one of the mechanisms for MDR acquisition [8,9]. The highest levels of Pgp expression were consistently found on the apical surfaces and capillaries of normal mammalian tissues [10–12]. Very recently, Pgp was demonstrated to share extensive sequence with numerous bacterial and eukaryotic ATP-dependent transport proteins [13–18], and classified in a superfamily of proteins that transport a varied group of substrates ranging from ions to large proteins and sugar polymers [19–21]. In spite of these findings however, many questions as to (1) what the physiological role and the natural substrates for Pgp are; (2) what the transport mechanism, that is, molecular action is and (3) how Pgp expression is regulated, still remain to be answered. Most importantly, the mammalian Pgp origin, much less that for lower evolutionary organisms, has yet to be found.

In the present study, we attempted to determine whether archaebacteria have a Pgp-like anticancer drug efflux pump to the end of eventually elucidating the Pgp origin with lower evolutionary organisms. We here selected the mutant bacteria with resistance to anthracycline, one of the anticancer drugs that causes Pgp overexpression in tumor cells [1–4]. Using RH123 as a model substrate for Pgp, we then examined the fluorophore accumulations by wild-type and anthracycline-resistant cells. RH123 shares several characteristics with substrates for Pgp expressed in resistant tumor cells [22,23]. Bacteria selected for resistance to

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Abbreviations: CTAB, cetyltrimethylammonium bromide; DAM, daunomycin; DTZ, diltiazem; DXR, doxorubicin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops, 3(*N*-morpholino)propanesulfonic acid; NDP, nifedipine; OX, bis(1,3-dibutylbarbituric acid)trimethine oxonol; Pgp, P-glycoprotein; RH123, rhodamine 123; RH6G, rhodamine 6G; VBL, vinblastine; VCR, vincristine; VRM, verapamil.

anthracycline showed much lower accumulation of RH123, which could be partially restored by NDP, which reverses MDR by inhibiting Pgp [24–26]. RH123 impaired accumulation in the resistant cells was confirmed to involve RH123 active efflux system, which can be inhibited by FCCP. These results taken together suggested that lower evolutionary organisms, such as archaebacteria might have a Pgp-like protein. This find has broad implications and provides new insight into the questions as to where Pgp was derived from and what its natural substrate is.

Materials and Methods

Chemicals. DAM, DTZ, FCCP, NDP and VRM were purchased from Sigma (St. Louis, MO). RH123 was purchased from Molecular Probes (Eugene, OR). RH6G was purchased from Wako (Osaka, Japan). DXR was kindly supplied by Kyowa Hakko Kogyo (Tokyo, Japan). All other reagents were commercial products with analytical grade.

Growth and preparation of cells. Haloferax volcanii was purchased from the National Collections of Industrial & Marine Bacteria (Aberdeen, UK). Cells were grown under standard conditions in the dark [27,28]. We selected the anthracycline-resistant bacteria which can grow normally even in a standard medium containing 1.5 μM anthracycline. Cells used in flow cytometry assays were centrifuged at 9000 $\times g$ for 10 min, washed three times in 4.28 M NaCl, 50 mM Mops buffer at pH 7.0, and resuspended to an appropriate protein concentration. Protein was determined with protein assay kits (Bio-Rad) as a BSA standard.

Fluorophore accumulation by wild-type and resistant cells. Cell suspensions were diluted to 0.1 mg protein/ml with fresh medium containing 5 mM arginine and 5 mM glutamate 5 min prior to measurement [27]. All experiments were carried out at 25°C. A 50-μl aliquot of fluorophore solution was added to 5 ml cell suspension to start RH123 accumulation by cells. The final concentrations of fluorophore were as follows: 0.2 μM OX; 0.15 μM RH123; 0.03 μM RH6G. A 500-μl aliquot of cell suspension was taken at given times, and the RH123 accumulation by cells was measured in EPICS CS, a flow cytometry (Coulter Electronics, Hialeah, FL) equipped with a 500 mW argon laser at 300 mW output power. The laser was tuned to 488 nm excitation for OX and RH123, and 520 nm for RH6G (bandwidth = $\pm$5 nm). Fluorescence emission was detected with bandpass filters of 520 nm for OX and RH123, and 595 nm for RH6G (bandwidth = $\pm$5 nm). Data analysis included single histograms of the relative fluorescence intensity and the conversion of the relative fluorescence intensity to cell sample fluorescence intensity. We further determined a concentration-dependency of steady-state RH123 accumulation in wild-type and resistant cells, varying its concentration form 0.1 μM to 20 μM. RH123 accumulation reached equilibrium within 15 min even with the highest concentration. Therefore, RH123 accumulation was determined after a 15 min incubation, as described above.

Effect of pharmacological agents on RH123 accumulation. RH123 accumulation was started as described above. A 50-μl aliquot of pharmacological agent was added to the cell suspension after a 10-min incubation. The final concentrations of pharmacological agent were as follows: 10 μM for CTAB; 20 μM for other drugs. A 500-μl aliquot of cell suspension was taken at 20 min for assay. None of the drugs interfered with measurement of RH123 fluorescence (data not shown).

Effect of Ca^{2+}-channel antagonist and metabolic inhibitor on rhodamine accumulation. Rhodamine accumulation was started as described above. A 50-μl aliquot of inhibitor (final concentration: FCCP, 10 μM; NDP, 15 μM and 100 μM; DTZ, 100 μM; VRM, 100 μM) was added to the cell suspension after a 10-min

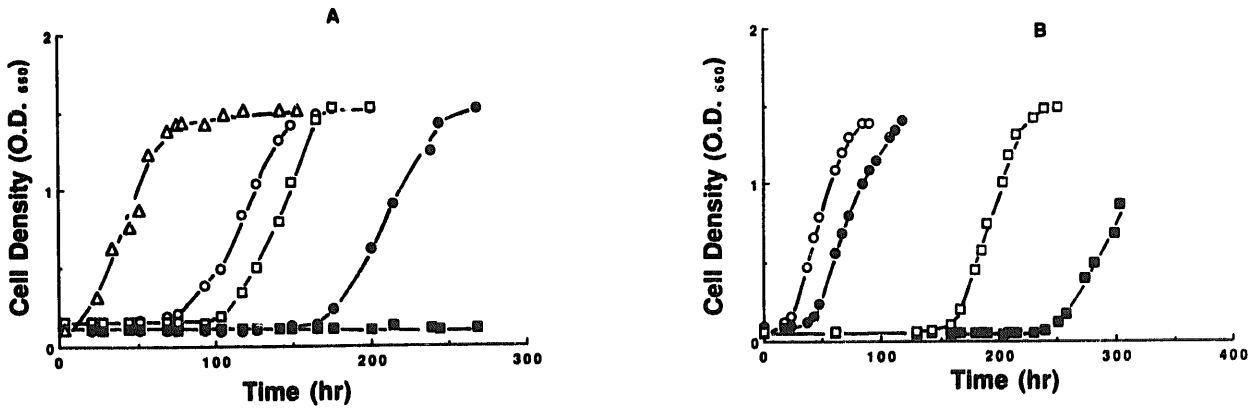

Fig. 1. Effect of doxorubicin on cell growth in wild-type and resistant cells. Panel (A) and (B) represent the cell growth in wild-type and resistant cells, respectively. Cells were grown under standard conditions in the dark [27,28], except that media contained DXR and/or NDP at various concentrations. Cell growth was expressed as optical density at 660 nm. Resistant cells were obtained after multi-step selection with exposure to 1.5 μM DXR. Key: Panel (A): △, control; ○, 1.5 μM DXR; ●, 5 μM DXR; ■, 10 μM; □, 1.5 μM DXR + 10 μM NDP. Panel (B): ○, control; ●, 1.5 μM DXR; □, 1.5 μM DXR + 10 μM NDP; ■, 1.5 μM DXR + 20 μM NDP.

incubation. Fluorescence intensity was measured following a 10-min incubation period. None of the inhibitors interfered with measurement of rhodamine fluorescence (data not shown).

RH123 efflux from wild-type and resistant cells. Cells were incubated with RH123 (8 μM) and/or FCCP (10 μM), as described above. A 1-ml aliquot of cell suspension was taken after a 15 min incubation, and added to 10 ml RH123 free medium at 25°C. RH123 remaining in cells was determined at given times.

Results and Discussion

Effect of anthracyclines on cell growth

DXR inhibited the growth of the wild-type bacteria in a concentration-dependent manner (Fig. 1-A). Lag time in the cell growth increased with anticancer drug concentration. Ca^{2+}-channel antagonist, NDP was observed to potentiate DXR cytotoxicity, whereas NDP solely had no effect on the cell growth. It is important to note that NDP was not directly cytotoxic to cells. On the contrary, inhibition of cell growth in the mutant bacteria selected for resistance to DXR was slightly observed (Fig. 1-B). As far as we know, this represents the first time that the resistance to DXR has been found in a lower evolutionary organism. NDP a well-known potent inhibitor for Pgp was observed to completely reverse the resistant to DXR in this mutant bacteria, resembling one of the features of MDR cells overexpressing Pgp [24–26]. The same bacteria also acquired a similar resistance to the other anthracycline, DAM (data not shown). From these phenomena, we presupposed that the acquisition of resistance to anthracycline might involve a maintenance of lower level anthracyclines by a Pgp-like anthracycline efflux pump overexpressed possibly in resistant cells.

Accumulation of RH123 by wild-type and anthracycline-resistant cells

In order to determine whether a Pgp-like anthracycline efflux pump might be involved in the anthracycline-resistance and its reversal by NDP, accumulation of the Pgp substrate RH123 [22,23] by cells was then examined with flow cytometry. This fluorophore accumulation reached equilibrium within 10 min (Fig. 2). Steady-state RH123 accumulation by resistant cells under kinetically linear condition was approx. one-fifteenth of that by wild-type cells. Strictly speaking, however, the difference in RH123 accumulation between wild-type and resistant cells seems likely to be greater, since RH123 accumulation in wild-type cells revealed much higher than in resistant cells, leading to its fluorescence quenching in wild-type cells. In order to determine the actual difference in RH123 accumulation, we have to measure RH123 accumulated in cells directly.

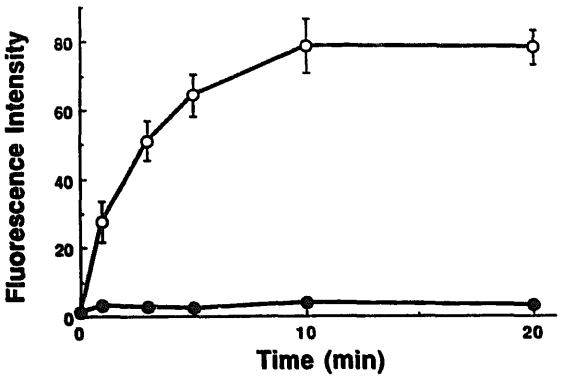

Fig. 2. RH123 accumulation by wild-type and resistant cells. After cells were incubated in a buffer containing 5 mM arginine and glutamate for 5 min at 25°C, a 50-μl aliquot of RH123 was added to the cell suspension (final concentration: 0.15 μM). The amount of RH123 accumulated into cells was determined with flow cytometry (ex: 488 nm, em: 520 nm (bandwidth ±5 nm)). Fluorescence intensity represents RH123 accumulated into cells. Each point represents the mean ± S.E. of six determinations. Key: ○, wild-type cells; ●, resistant cells.

Fig. 3 shows the relationship between the steady-state RH123 accumulation in cells and the concentration. RH123 accumulation in resistant cells was proportional to its concentration in the medium at lower concentrations (less than 4 μM), whereas RH123 accumulation in wild-type cells revealed saturation at more than 2 μM. The saturation of RH123 accumulation in wild-type cells might involve the saturable influx system and/or intracellular binding. Alternatively, the saturation of RH123 accumulation in wild-type cells might be explained by its fluorescence quenching, as aforementioned. The possible explanation for this saturation mechanism could derive from direct measurements of intracellular RH123 amount. To the contrary, RH123 accumulation in resistant cells remarkably increased

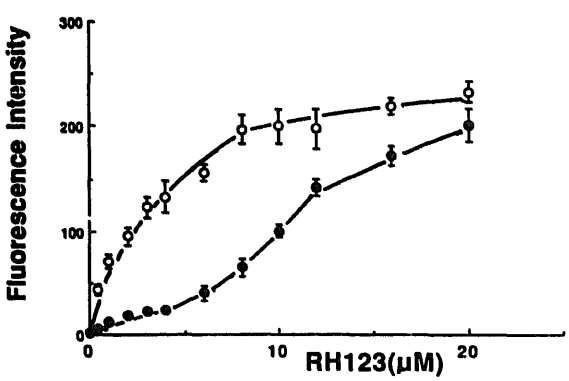

Fig. 3. Concentration-dependence of RH123 accumulation by wild-type and resistant cells. Wild-type and resistant cells were incubated with RH123 at various concentration (0.1–20 μM) as described in Fig. 2. RH123 accumulation was determined after a 15 min incubation. Separate experiments showed that RH123 accumulation reached equilibrium at this time with the highest concentrations. Each point represents the mean ± S.E. of six determinations. Key: ○, wild-type cells; ●, resistant cells.

near 6 μM RH123 and the difference in RH123 accumulation among the two was eliminated more than 20 μM. This remarkable increase in resistant cells seems likely to involve the saturable RH123 efflux system. This concentration-dependence was also observed in steady-state DAM accumulations by wild-type and DAM-resistant Ehrlich ascites tumor cells [29].

Furthermore, we determined the effect of a metabolic inhibitors (FCCP) and Ca^{2+}-channel antagonists (DTZ, NDP, VPM) on RH123 accumulations by wild-type and resistant cells (Fig. 4). 10 μM FCCP completely restored RH123 accumulation in resistant cells to that in wild-type cells. This suggested that RH123-impaired accumulation might involve the active efflux mechanism. We thus determined the unidirectional efflux in wild-type and resistant cells after loading RH123 (Fig. 5). The efflux rate of RH123 in resistant cells was much higher than that in wild-type cells, even if the initial RH123 accumulation in resistant cells was a half of that in wild-type cells. This rapid efflux was remarkably inhibited by FCCP and was reduced to that in wild-type cells, indicating that resistant cells actively extrude RH123. On the other hand, the inhibition of RH123 efflux from wild-type cells by FCCP was slightly observed, although FCCP remarkably enhanced RH123 accumulation (Fig. 4). RH123 concentration in wild-type cells after its preloading revealed much higher than that in resistant cells, possibly resulting in RH123 efflux system saturated. The saturation of RH123 efflux system might be involved in no effect of FCCP on RH123 efflux in wild-type cells. The another possibility is that RH123 fluorescence quenching might apparently counteract the effect of FCCP.

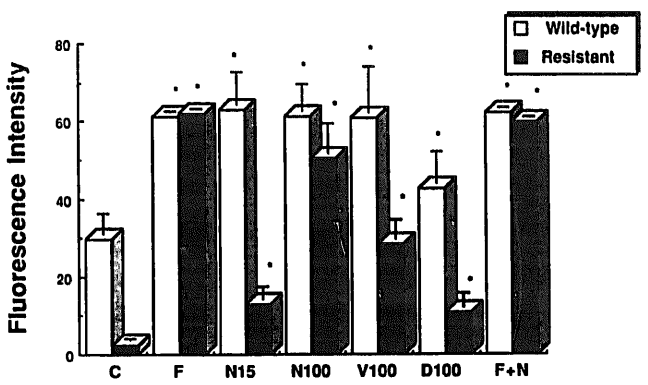

Fig. 4. Effect of metabolic inhibitor and Ca^{2+}-channel antagonist on RH123 accumulation. A 50-μl aliquot of inhibitor solution was added to cell suspension, after a 10-min incubation with 0.15 μM RH123. RH123 accumulation was determined 10 min after inhibitor addition. Each value represents the mean $\pm$ S.E. of seven determinations. Comparisons with control were by paired t-test. * $P < 0.01$. Key: C, control; F, 10 μM FCCP; D100, 100 μM DTZ; V100, 100 μM VRM; N15, 15 μM NDP; N100, 100 μM NDP; F+N, 10 μM FCCP plus 15 μM NDP.

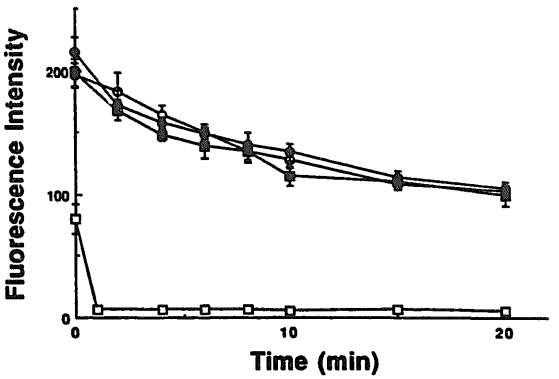

Fig. 5. RH123 efflux from wild-type and resistant cells. Cells were incubated with RH123 (8 μM) and/or FCCP (10 μM) for 15 min as described in Fig. 2. Separate experiments showed that RH123 accumulation reached equilibrium. After RH123 preloading, a 1 ml aliquot of cell suspension was added to 10 ml RH123 free medium at 25°C. RH123 remaining in cells was measured at given times. Each point represents the mean $\pm$ S.E. of five determinations. Key: ○, wild-type cells; □, resistant cells; ●, wild-type cells treated by 10 μM FCCP; ■, resistant cells treated by 10 μM FCCP.

15 μM NDP remarkably enhanced RH123 accumulation in resistant cells. The other modulators of Pgp, DTZ and VRM remarkably enhanced RH123 accumulation in resistant cells at higher concentration. These results coincide with the hallmarks of Pgp in mammalian cells [24–26]. It could be inferred that a Pgp-like protein similar to mammalian Pgp might be expressed in the resistant bacteria. The mechanism for resistance to anthracycline might feasibly involve the maintenance of DXR concentration below cytotoxic levels by this Pgp-like protein. In fact, a preliminary experiment with labeled DXR (data not shown) demonstrated that a much low accumulation of DXR in resistant cells is remarkably enhanced by NDP.

On the other hand, this great difference in RH123 accumulation between the two may be explained by three other possibilities. The first is that RH123 binding to intracellular components may be greatly reduced in resistant cells. RH123 was thought to be bound non-specifically to intracellular components [30]. RH123 accumulation proved equal in wild-type and resistant cells after FCCP exposure, indicating that binding was not altered. Therefore, this mechanism can be ruled out. The second is that the membrane may be depolarized in resistant cells. RH123 has a positive charge and may be highly concentrated by an interior-negative membrane potential (V_m). The V_m value in wild-type cells was determined to be approx. -140 mV in a previous report [31]. If the membrane were depolarized, RH123 accumulation would be impaired. In order to determine whether V_m in resistant cells was changed relative to wild-cells, we then measured V_m utilizing the membrane potential probe OX (Fig. 6). This probe was slowly distributed into cells. OX fluorescence intensity reflecting V_m [32] was

prompted in both cells by membrane depolarization with FCCP, the change in OX fluorescence reflecting that in V_m. The profile of OX fluorescence intensity in resistant cells was superimposed to that in wild-type cells, indicating that the V_m values in resistant cells were determined to be identical to those in wild-type cells. The last is that resistant cells may produce a passive diffusion barrier. This factor would influence the time to reach equilibrium, but at equilibrium the electrochemical potential of RH123 in the cytoplasm would be equal to that in the medium. From electrochemical considerations it is likely that RH123 accumulations by the two cell types would prove equal to each other. Neither can a lower RH123 accumulation by resistant cells at steady state be explained solely by a decreased active inward transport, since a metabolic inhibitor remarkably enhanced a much low RH123 accumulation. This mechanism was also excluded.

Unexpectedly, both FCCP and NDP increased RH123 accumulation by wild-type cells. What mechanism might account for these effects? One may consider that wild-type cells also express the Pgp-like protein: this is feasible considering the fact that high levels of Pgp have been found on the apical surfaces and capillaries in some normal mammalian tissues [10–12], and in drug-sensitive tumor cells [33]. In the present study, NDP was observed to potentiate DXR cytotoxicity in wild-type cells (Fig. 1-A). Although we have not yet identified whether Pgp-like protein might be expressed in wild-type cells, a feasible mechanism for the enhancement of RH123 accumulation in wild-type cells by NDP might involve their expressing a somewhat Pgp-like RH123 efflux pump to maintain its low intracellular concentration level.

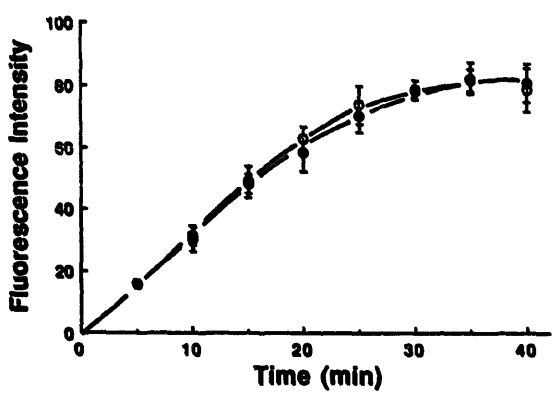

Fig. 6. OX accumulation by wild-type and resistant cells. After cells were incubated in a buffer containing 5 mM arginine and glutamate for 5 min at 25°C, a 50-μl aliquot of OX solution was added to the cell suspension (final concentration: 0.2 μM). The amount of OX accumulated into cells was determined with flow cytometry (ex: 488 nm, em: 520 nm (bandwidth ±5 nm)). Fluorescence intensity represents OX accumulated into cells. Each point represents the mean ± S.E. of four determinations. Key: ○, wild-type cells; ●, resistant cells.

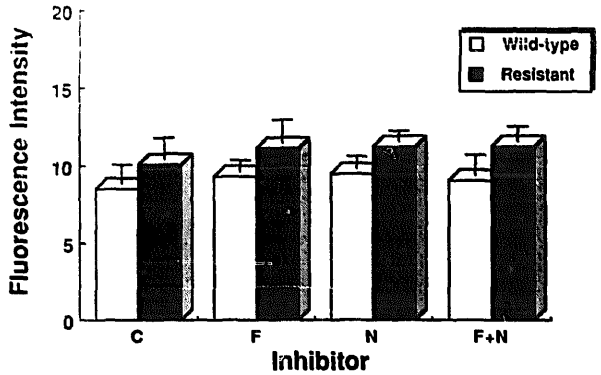

Fig. 7. RH6G accumulation by wild-type and resistant cells. After cells were incubated in a buffer containing 5 mM arginine and glutamate for 5 min at 25°C, a 50-μl aliquot of RH6G solution was added to the cell suspension (final concentration: 0.03 μM). The amount of RH6G accumulated into cells was determined with flow cytometry (ex: 520 nm, em: 595 nm (bandwidth ±5 nm)). RH6G accumulation reached equilibrium in 10 min. We thus measured RH6G accumulation into cells in a 10-min incubation period. RH6G accumulation after inhibitor addition at 10 min was also measured following a 10-min incubation period. Fluorescence intensity represents RH6G accumulated into cells. Each point represents the mean ±S.E. of four determinations. Comparisons were by paired *t*-test, and any comparison did not show significant difference. Key: C, control; F, 10 μM FCCP; N, 15 μM NDP; F+N, 10 μM FCCP plus 15 μM NDP.

Pgp-like drug efflux pump in other bacteria

Very recently, Neyfakh et al. [34] have demonstrated that *Bacillus subtilis* cells selected for their resistance to RH6G have MDR phenotype mimicking that of mammalian MDR cells. MDR in this bacteria has been confirmed to be mediated by the drug efflux pump from cells, which displays a remarkable sequence similarity to tetracycline-efflux pump (Tetp). In order to further determine how the Pgp-like anthracycline efflux pump in the present bacterium is similar to that in *B. subtilis*, we examined RH6G accumulation by the resistant *Haloferax volcanii* in the present study (Fig. 7). The resistant *H. volcanii* showed neither impaired accumulation for RH6G. Furthermore, both FCCP and Ca^{2+}-channel antagonist had no effect on RH6G accumulation. This resulted in our consideration that the Pgp-like drug efflux pump in the resistant cells might not extrude RH6G from cells, nevertheless Pgp in mammalian MDR cells recognizes and transports RH6G [23,35]. On the other hand, a minimal difference in RH6G accumulation between the two might be explained by another possibility that RH6G with its higher partition coefficient relative to RH123 would be transported more rapidly through the cell membrane, and therefore be more able to overcome the efficiency of Pgp-like drug efflux pump system. Using rhodamine dyes, Lampidis et al. [35] demonstrated that the difference in the uptake by sensitive and resistant tumor cells between RH123 and RH6G correlated with the difference in the lipophilicity, being an important fac-

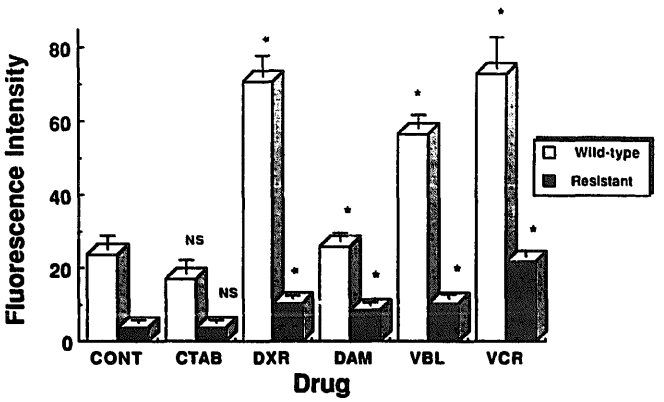

Fig. 8. Effect of other anticancer agents on RH123 accumulation. A 50-μl aliquot of pharmacological agent was added to cell suspension, after 10 min incubation with 0.15 μM RH123. RH123 accumulation was determined 10 min after pharmacological agent addition. Each value represents the mean ± SE of four determinations. Comparisons with control were by paired t-test: *, $P < 0.01$; NS, not significantly different. Key: CONT, control; CTAB, 10 μM CTAB; DXR, 20 μM DXR; DAM, 20 μM DAM; VBL, 20 μM VBL; VCR, 20 μM VCR.

tor in determining their ability to override Pgp in MDR cells. However, the difference in RH6G accumulation between the sensitive and MDR cells still revealed great (6-fold difference), although the difference in RH6G accumulation was smaller relative to that in RH123 accumulation. Consequently, the higher lipophilicity of RH6G may not fully account for the minimal difference in RH6G accumulation between wild-type and resistant cells. It is interesting to note that the Pgp-like drug efflux pump recognizes and transports RH123, but not RH6G. The feasible explanation for this recognition could drive from determination of the binding sites. A fundamental, but as yet unanswered question in Pgp biology, still remains how a single protein can recognize and transport a broad group of diverse compounds.

It was further confirmed that RH123 efflux by the Pgp-like protein was not inhibited by the other compound CTAB, to which the *B. subtilis* acquired resistance [34]. On the other hand, RH123 accumulation in resistant cells was remarkably enhanced by anthracycline (DAM and DXR) and vinca alkaloid (VBL and VCR)(Fig. 8), which are confirmed to be substrates for mammalian Pgp [1,3,4]. This different spectrum of drugs transported suggests that the Pgp-like anthracycline efflux pump in *H. volcanii* might be different from the Tetp-like pump in *B. subtilis*. However, how this Pgp-like anthracycline efflux pump is similar to mammalian Pgp has yet to be elucidated.

In this report we showed evidence that in archaebacteria, there might exist an energy-dependent drug efflux pump with features mimicking mammalian Pgp, which resulted in our hypothesizing that the DXR resistant bacteria arise as a result of amplification of the gene encoding the Pgp-like drug efflux pump. In order to confirm whether Pgp-like drug efflux pump is involved, we must clone this hypothetical gene, and clarify how this pump is homologous to Pgp in a primary sequence and whether this gene confer MDR phenomenon. We must also perform another biochemical approach to identify Pgp-like drug efflux pump utilizing photoaffinity probes [36,37] and monoclonal antibodies for mammalian Pgp [5,33].

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