

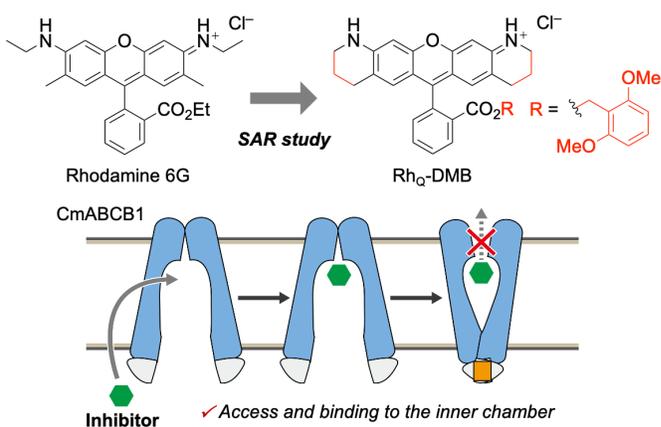
Structure–ATPase activity relationship of rhodamine derivatives as potent inhibitors of P-glycoprotein CmABC B1

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ABSTRACT: Understanding the transport and inhibition mechanisms of substrates by P-glycoprotein (P-gp) is one of important approaches in addressing multidrug resistance (MDR). In this study, we evaluated a variety of rhodamine derivatives as potential P-gp inhibitors targeting CmABC B1, a P-gp homologue, focusing on their ATPase activity. Notably, a Q-rhodamine derivative with an *o,o'*-dimethoxybenzyl ester moiety (Rh_Q-DMB) demonstrated superior affinity and inhibitory activity, which was further confirmed by a drug susceptibility assay in yeast strains expressing CmABC B1. Results from a tryptophan fluorescence quenching experiment using a CmABC B1 mutant suggested that Rh_Q-DMB effectively enters and binds to the inner chamber of CmABC B1. These findings underscore the promising potential of Rh_Q-DMB as a tool for future studies aimed at elucidating the substrate-bound state of CmABC B1.

KEYWORDS: Multidrug resistance, ATP-Binding cassette transporter, Rhodamine, Inhibitor, Structure–activity relationship

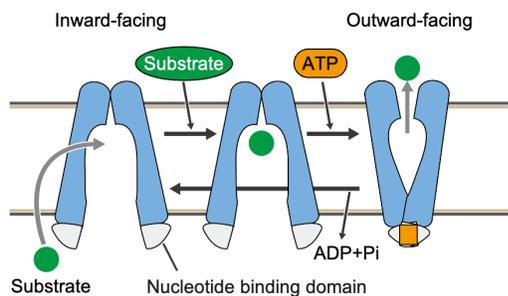


Multidrug resistance (MDR) poses a significant challenge in cancer treatment, rendering cancer cells resistant to a broad spectrum of drugs with varying structures and mechanisms of action.^{1,2} According to recent studies on the MDR of cancer cells,^{3–5} the overexpression of ATP-binding cassette (ABC) transporters plays a pivotal role in facilitating MDR activity in cancer cells. The ABC transporter superfamily in humans is comprised of 48 members, of which three are primarily implicated in MDR.⁶ P-glycoprotein (P-gp), also known as MDR1 or ABCB1, is the earliest example identified to be associated with MDR.^{7,8} P-gp is capable of recognizing and transporting a wide range of substances, including toxic xenobiotics, endogenous metabolites, and chemotherapeutic

drugs such as paclitaxel and doxorubicin.^{9,10} Thus, P-gp reduces the intracellular accumulation of these drugs, eventually causing drug resistance.^{11,12}

For years, P-gp has been identified as a potential therapeutic target to overcome MDR. The strategy of combining P-gp modulators with anti-cancer drugs has been viewed as a promising avenue to counteract P-gp-mediated MDR.¹³ However, despite extensive research for development of an efficient P-gp modulators over the past several decades,^{14–17} there is still much room for improvement. One of the major obstacles is that the mechanisms governing substrate transport and inhibition, including (a) the structure of P-gp when bound to the substrate or post-substrate

(a) Mode of action of (Cm)ABC B1



(b) This work

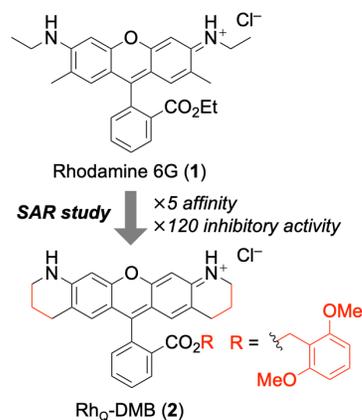
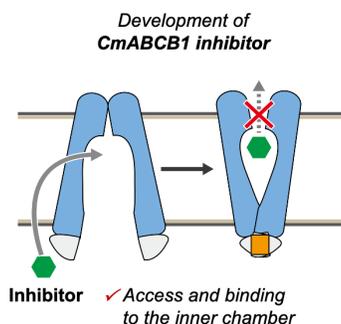
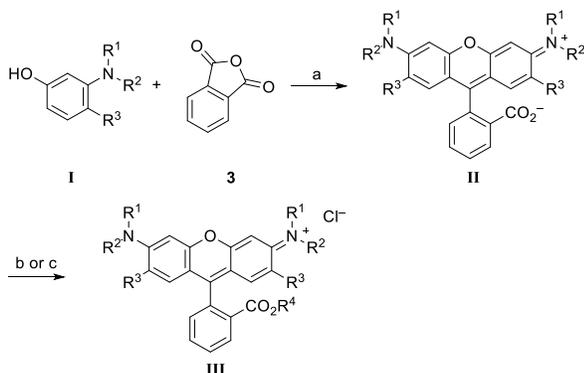


Figure 1. (a) Mode of action of ABC B1; (b) Development of a CmABC B1 inhibitor.

efflux and (b) the substrate binding site and mode of transportation, remain elusive. On a positive note, advances in structural biology have begun to shed light on the three-dimensional structures of P-gps.^{17–22} Even the structure of human P-gp bound to a substrate has been revealed by a cryo-EM technique.^{17,23–25}

In the transport cycle, the ABCB1 transporter initially assumes an inward-facing conformation, exposing the substrate-binding cavity (Figure 1a). This conformation facilitates the binding of the substrate to the transmembrane domain. Following this, ATP binds to the nucleotide-binding domain, triggering a transition to an outward-facing conformation. In this state, the substrate-binding cavity collapses, resulting in the transport of the substrate out of the cell. However, comprehensive structural data including each inward- and outward-facing state have only been sporadically reported, and often in conjunction with the use of different mutants. To deepen our understanding of the transport process, it is crucial to examine the structure of these state in wild-type P-gp or at least in the same mutant form. In line with our ongoing research into the molecular mechanisms of substrate transport by P-gp, we have elucidated the high-resolution inward- and outward-facing structures of *Cyanidioschyzon marolae* P-gp (CmABCB1), a eukaryotic homolog of ABCB1 with a similar amino acid sequence, structure, and substrate specificity to human P-gp, using X-ray crystallography.^{19,21} However, it is crucial to note that the substrate-bound state of CmABCB1 remains a puzzling aspect of our research. Addressing this gap is critical for a comprehensive understanding of the molecular dynamics of this transporter. To explore the substrate-bound state of CmABCB1, we focused on the development of inhibitors that can enter the inner chamber of CmABCB1 but are not readily effluxed (Figure 1b). Among the potential substrates of CmABCB1, we selected rhodamines, well-known transport substrates for various ABC transporters including human P-gp, for a structure-activity relationship study, which was informed by our two key precedents: (a) rhodamine 6G (**1**) has exhibited stimulation and inhibition activities toward CmABCB1,¹⁹ and (b) tryptophan quenching analysis revealed that several commercially available rhodamines can access the inner chamber of CmABCB1.²⁶ In addition to these findings, our plan was also drawn from the pioneering work of Raub and Dettly, who developed P-gp inhibitors based on the sulfur-rhodamine derivatives.¹⁴ In this study, we synthesized a variety of rhodamine derivatives and evaluated their affinity and inhibitory activity against CmABCB1, leading to the discovery of the novel Q-rhodamine (Rh_Q; Q for tetrahydroquinoline) derivative **2**, Rh_Q-DMB, as a potent P-gp inhibitor, a tool for future studies aimed at elucidating the substrate-bound state of CmABCB1.

Based on an established synthetic method, 19 rhodamine derivatives were synthesized as shown in Scheme 1.²⁷ Aminophenols **I** and phthalic



Scheme 1. Synthesis of rhodamine derivatives

Reagents and conditions: (a) *o*-dichlorobenzene, 175 °C; (b) alcohol, EDC·HCl, DMAP, CH₂Cl₂, rt; (c) alkyl iodide, Cs₂CO₃, DMF, rt

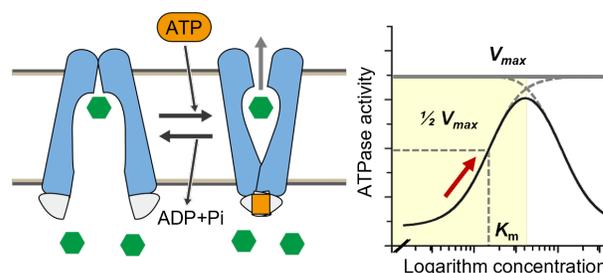
anhydride (**3**) were heated in *o*-dichlorobenzene at 175 °C to give the corresponding carboxylate **II** with a rhodamine skeleton in one step. The subsequent esterification with various alcohols was conducted under standard conditions using a condensation reagent (EDC·HCl, DMAP) or alkyl iodide under basic conditions (Cs₂CO₃). Purification by silica gel column chromatography (pre-washed with MeOH, eluent: CHCl₃/MeOH) afforded the target compound **III**. Comprehensive synthetic procedures for each rhodamine derivative are detailed in the Supporting Information.

The ATPase activities of the synthesized rhodamine derivatives were evaluated to establish a SAR. In general, ABC transporters hydrolyze ATP to ADP and phosphate while effluxing substrates out of the cell,²⁸ a property also exhibited by CmABCB1.¹⁹ Our previous research has illuminated that CmABCB1, when in micelles, exhibits high thermal stability, making it amenable to purification followed by quantitative kinetic analyses of ATPase activity.¹⁹

CmABCB1 exhibits low basal ATPase activity in the absence of a transport substrate but shows higher ATPase activity when substrates are present, a phenomenon termed substrate-stimulated ATPase activity in human P-gp (Figure 2).^{19,29,30} During titration with P-gp substrates, ATPase activity initially increases (Figure 2a), reaching a maximum before declining at higher substrate concentrations (Figure 2b) to form a characteristic bell-shaped profile analyzed using modified Michaelis-Menten kinetics.^{31–34} The decrease in ATPase activity is attributed to substrate inhibition, a common feature of transporters and enzymes. Along these lines, we evaluated the affinity (K_m) and inhibitory activity (K_i) of the substrates against CmABCB1. The parameters are derived from the equation described in the Supporting Information. Substrates with a low K_m value induce the structural change of the transporter by entering its inner chamber, whereas ones with a low K_i value inhibit substrate transport and are not readily effluxed. Thus, the ideal candidate of the inhibitor consistent with our aim would possess low values of both K_m and K_i .

Upon screening rhodamine derivatives, Rh_Q-DMB (**2**) emerged as a potent inhibitor, exhibiting an affinity 5 times greater and an inhibitory

(a) Stimulation at low substrate concentration



(b) Inhibition at high substrate concentration

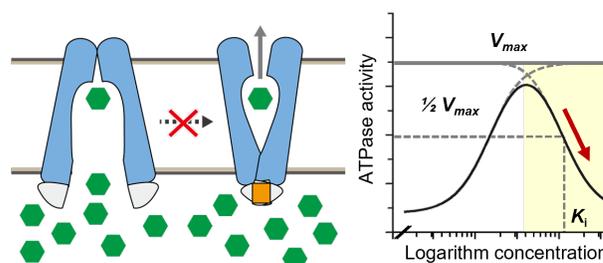


Figure 2 (a) The behavior of CmABCB1 at low substrate concentration showing substrate-stimulated ATPase activity. (b) The behavior of CmABCB1 at high substrate concentration showing substrate inhibition.

activity 120 times higher than the reference compound (Figure 3), i.e., rhodamine 6G (**1**), as detailed below.

Table 1 shows the results of the affinity and inhibitory activity of the rhodamine derivatives **1** and **4–8** with different substituents, mainly in the tricyclic xantheno core. The parent compound, rhodamine 6G (**1**), exhibited moderate affinity ($K_m = 2.9 \mu\text{M}$) and inhibitory activity ($K_i = 690 \mu\text{M}$). By comparison, the corresponding butyl ester **4** showed a slight improvement in inhibitory activity and serves as a benchmark for

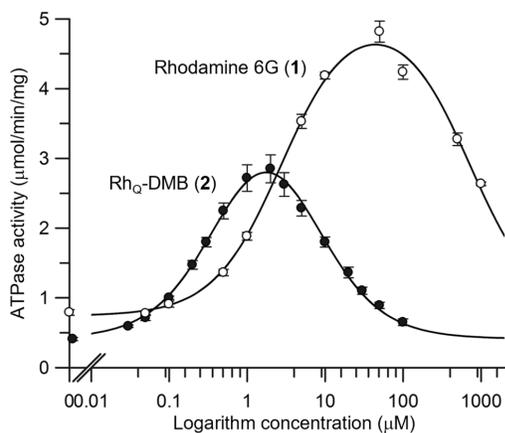


Figure 3 Substrate-concentration dependence of CmABC1 ATPase activity. The ATPase activity was measured as a function of rhodamine 6G (**1**) or RhQ-DMB (**2**) concentration with 5 mM ATP at 37 °C in a buffer solution of 50 mM Tris-HCl (pH 7.5 at 37 °C), 150 mM NaCl, and 0.05% (w/v) β -DDM for 30 min. Data are means \pm SD ($n = 3$). The solid line is a fit of the equation to the data as described in the Supporting Information (Eqs. 1, S4).

subsequent studies. *N*-Butyl derivative **5** showed improved inhibitory activity ($K_i = 160 \mu\text{M}$), although its affinity remained unchanged ($K_m = 3.2 \mu\text{M}$). The introduction of a pyrrolidine ring (**6**) led to improvements in both affinity ($K_m = 1.1 \mu\text{M}$) and inhibitory activity ($K_i = 110 \mu\text{M}$).

Further improvements were observed with Q-rhodamine **7**, a pentacyclic rhodamine derivative with two fused piperidine rings, which exhibited a significantly improved inhibitory activity ($K_i = 14 \mu\text{M}$), while maintaining an affinity ($K_m = 1.7 \mu\text{M}$) comparable to that of **6**. By comparison, X-rhodamine **8** displayed only moderate inhibitory activity with a K_i value of 43 μM .

Having identified the optimal structure for the xantheno moiety, we proceeded to investigate variations in the ester moiety as detailed in Table 2. Given that the inner chamber of CmABC1 is predominantly hydrophobic and rich in aromatic residues,¹⁹ we hypothesized that the incorporation of an aromatic ring could hopefully enhance the affinity and inhibitory activity through an aromatic interaction. This idea aligns with findings that aromatic moieties increase binding affinity toward other homologues of P-glycoprotein.^{35–37} Pleasingly, the introduction of both a benzyl group (**9**) and a naphthyl group (**10**) led to improvements in both affinity and inhibitory activity. Rhodamine derivative **11** with a phenethyl group, i.e., an aromatic substituent that is one carbon longer than a benzyl group, showed reduced affinity ($K_m = 1.4 \mu\text{M}$). Next, the substituents of the benzyl group were further investigated (**2, 12–21**). It was found that the introduction of one substituent at the *meta*-position, whether an electron-donating or electron-withdrawing group, was effective with respect to affinity in all case: A benzyl group with methyl (**12**), dimethylamino (**13**), methoxy (**14**), trifluoromethyl (**15**), and nitro (**16**) groups led to improved affinity. In terms of inhibitory activity, derivatives **15** and **16** with an electron-withdrawing group showed a moderate reduction ($K_i = 18 \mu\text{M}$ for **15** and $K_i = 12 \mu\text{M}$ for **16**).

Table 1. Effect of the xantheno moiety on affinity and inhibitory activity toward CmABC1.^a

Compound	Structure	K_m (μM)	K_i (μM)	Compound	Structure	K_m (μM)	K_i (μM)
1		2.9 ± 0.14	690 ± 40	6		1.1 ± 0.095	110 ± 20
4		4.6 ± 0.18	560 ± 180	7		1.7 ± 0.29	14 ± 1.0
5		3.2 ± 1.1	160 ± 95	8		1.5 ± 0.25	43 ± 13

^a K_m and K_i values were obtained from three independent repeats except **6**. Data of **6** were obtained from four independent repeats.

Table 2. Effects of the ester moiety on affinity and inhibitory activity toward CmABC1.^a

Compound	R ⁴	K _m (μM)	K _i (μM)	Compound	R ⁴	K _m (μM)	K _i (μM)
7		1.7 ± 0.3	14 ± 1	16		0.55 ± 0.03	12 ± 2
9		0.81 ± 0.12	8.9 ± 0.7	17		3.9 ± 2.0	1.2 ± 0.5
10		0.77 ± 0.23	9.6 ± 2.0	18		2.3 ± 1.0	6.3 ± 1.8
11		1.4 ± 0.3	8.1 ± 1.6	19		0.50 ± 0.10	8.2 ± 0.8
12		0.56 ± 0.10	8.4 ± 1.3	20		0.73 ± 0.16	9.1 ± 2.0
13		0.52 ± 0.09	10 ± 3	21		0.31 ± 0.11	31 ± 5
14		0.58 ± 0.10	8.2 ± 1.7	2		0.55 ± 0.13	5.8 ± 1.3
15		0.44 ± 0.11	18 ± 4	(Rh _Q -DMB)			

^aK_m and K_i values were obtained from three independent repeats.

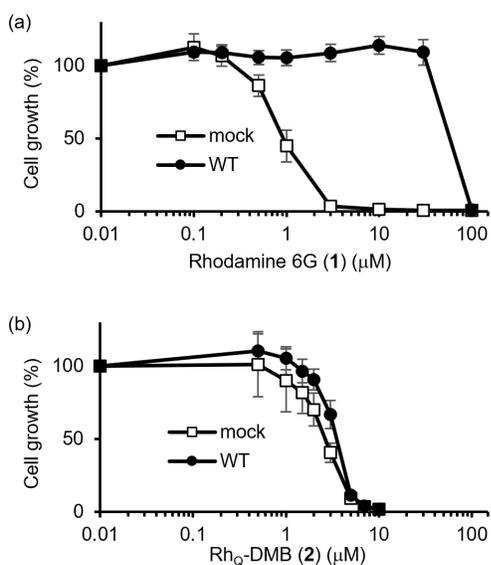


Figure 4. Drug susceptibility assay in *S. cerevisiae* AD1-8u⁻ cells in YPD medium for 30 °C for 15–17 h. AD1-8u⁻ cells expressing WT CmABC1 (●) were grown in various concentrations of drugs. For

each drug assayed, mock-transfected AD1-8u⁻ cells (□) were also grown. Data are means ± SD (n = 3).

We subsequently evaluated the impact of varying the positions and numbers of substituents (**2**, **17–21**), selecting the methoxy and trifluoromethyl groups as electron-donating and electron-withdrawing groups, respectively. Derivatives with one trifluoromethyl group in the *ortho* position (**17**) or two in the *meta* position (**18**) showed a clear reduction in affinity. The data for compound **17** present an unusual scenario where the value of K_m exceeds K_i. This might indicate that substrate inhibition occurs notably earlier than the point at which the hyperbolic response curve approaches saturation, a phenomenon that is illustrated in Figure 2. By comparison, the methoxy group, depending on the number and position, had a positive effect on both affinity and inhibitory activity. Among several derivatives with *o*- (**19**), *p*- (**20**), *m,m'*- (**21**), and *o,o'*-dimethoxy group(s) (**2**), *o,o'*-dimethoxybenzyl derivative **2** (Rh_Q-DMB) showed well-balanced affinity and inhibitory activity with a 5-fold improvement in affinity (K_m = 0.55 μM) and a 120-fold improvement in inhibitory activity (K_i = 5.8 μM) compared to the parent rhodamine 6G (**1**).

To gain insights into the mechanism of action of Rh_Q-DMB (**2**), we conducted drug susceptibility assays with *Saccharomyces cerevisiae* AD1-8u⁻, a drug-sensitive yeast which lacks seven major transporters of the ABC

family associated with multi-drug efflux (Figure 4).³⁸ Rhodamines are well-known growth inhibitors in yeast; therefore, strains lacking CmABC1 accumulate rhodamines even at low concentrations and thus show low resistance. In contrast, strains containing CmABC1 show enhanced resistance due to the efflux of the rhodamines. Indeed, while rhodamine 6G (**1**) did not inhibit cell growth in the CmABC1-positive strain up to 30 μM (Figure 4a, WT), CmABC1-negative strains demonstrated reduced growth at significantly lower concentrations (1 μM) (Figure 4a, mock). This supports the idea that **1** is actively effluxed by CmABC1. With these results in mind, we conducted further assays using Rh_Q-DMB (**2**) (Figure 4b). If **2** indeed functions as an inhibitor of CmABC1, we would expect it to hinder cell growth once inside the cell, regardless of whether the transporter is present. Pleasingly, both CmABC1-positive and -negative strains exhibited similar growth inhibition at concentrations above ca. 1 μM . This suggested that **2** was less likely to be effluxed by CmABC1 and is in alignment with its high ATPase inhibitory activity.

Our above findings confirmed that Rh_Q-DMB (**2**) effectively inhibits CmABC1. However, while ATPase stimulation does point to potential substrate entry into the inner chamber, it does not definitively confirm inhibition is occurring because of interactions within that space. Instead, the substrate might inhibit CmABC1 by interacting with either intracellular or extracellular domains, without actually entering the chamber. To better understand this situation, we have recently developed a method of tryptophan (Trp) fluorescence quenching to observe substrate binding within the CmABC1 chamber.^{26,39,40} The method employs the 4WY/M391W mutant, a CmABC1 mutant that four of six

Compound **2** was added at increasing concentrations to a 13 $\mu\text{g}/\text{mL}$ solution of purified mutant CmABC1 (4WY/M391W) in buffer at 25 °C. Fluorescence emission at 340 nm was recorded at 25 °C following excitation at 290 nm.

intrinsic Trp residues are removed and one extrinsic tryptophan residue is introduced at the top of the inner chamber, i.e., the probable substrate-binding site, enabling to verify substrate entry into the chamber via site-specific fluorescence quenching from the Trp391 residue.²⁶ Note that the mutant has essentially the same structure and activity as the WT. We measured fluorescence quenching of the extrinsic Trp391 residue of the 4WY/M391W mutant in micelles after mixing with Rh_Q-DMB (**2**) (Figure 5a). The data showed concentration-dependent quenching of the tryptophan fluorescence in 4WY/M391W by **2** without a shift in the emission maxima.²⁶ The plot in Figure 4b illustrates the quenching ratio of the tryptophan emission at 340 nm as a function of the concentration of **2**. The tryptophan quenching ratio reached over 30% at saturation levels of **2** (Figure 5b), suggesting that **2** enters the inner chamber in a manner consistent with other major substrates such as rhodamine 6G (**1**), nifedipine, and tetraphenylphosphonium as we reported previously.²⁶

In this study, a variety of rhodamine derivatives were synthesized and evaluated for their affinity and inhibitory activity toward CmABC1 contained in micelles to discover a novel inhibitor. The SAR study suggested that Rh_Q-DMB (**2**), a Q-rhodamine derivative bearing an *o,o'*-dimethoxybenzyl ester moiety, exhibited the most potent affinity and inhibitory activity ($K_m = 0.55 \mu\text{M}$, $K_i = 5.8 \mu\text{M}$) against CmABC1 as measured by an ATPase activity assay. The results of the drug susceptibility assay using yeast strains expressing CmABC1 indicated that **2** was barely effluxed by CmABC1. The tryptophan fluorescence quenching experiment showed a concentration-dependent quenching of tryptophan fluorescence, indicating that **2** enters the inner chamber. These results indicate that **2** has the desired characteristics of being able to enter the inner chamber, but is hardly effluxed by CmABC1. Future efforts will focus on elucidating the substrate-bound state of CmABC1 through structural biology techniques, such as X-ray crystallography and cryo-electron microscopy, to provide a more comprehensive understanding of the functional dynamics. Simultaneously, further studies are being conducted, encompassing ADMET analysis and in vivo evaluations, to advance the development of ABCB1 inhibitors as a strategy to MDR.

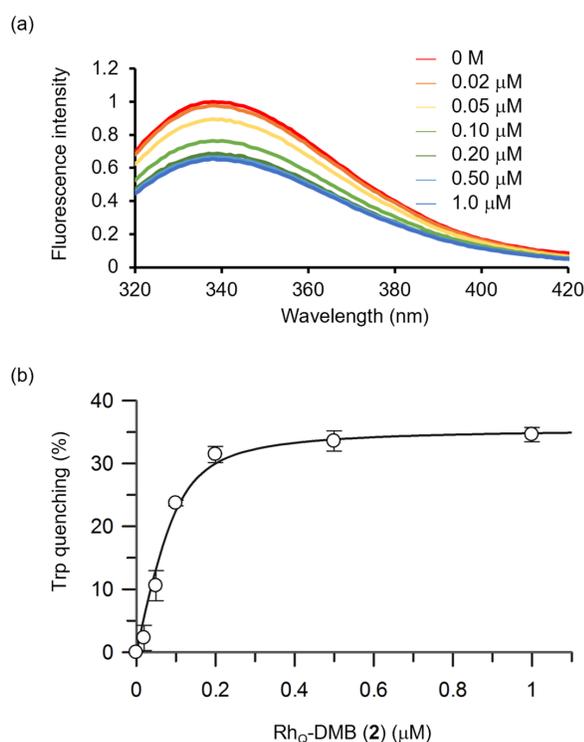


Figure 5. (a) Fluorescence emission spectra for the purified CmABC1 mutant (4WY/M391W) (13 $\mu\text{g}/\text{mL}$) in a buffer solution of 20 mM Tris-HCl (pH 7.5 at 25 °C), 150 mM NaCl, and 0.05% (w/v) β -DDM containing Rh_Q-DMB (**2**) (0–1.0 μM). Fluorescence emission was recorded at 25 °C following excitation at 290 nm. (b) Quenching of the extrinsic tryptophan fluorescence of P-gp by sequential addition of **2**.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <http://pubs.acs.org>.

Cell lines and cell culture, preparation and spectra data, the results of ATPase assay, HPLC chromatogram of representative compounds, and the ¹H NMR and ¹³C NMR spectra of new compounds (PDF)

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Author Contributions

K.T. and H. K. conceived the idea and supervised the project. S.M. and R.T. synthesized the compounds and performed the ATPase assay. S.M. performed the drug-susceptibility assay and tryptophan quenching assay. R.M., K.M. performed the purification of wild-type CmABC1. S.M. and H.T. wrote the manuscript and K.T. and H.K. revised the manuscript. All authors approved the final version of the manuscript to be published.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ADP, adenosine diphosphate; ATP, adenosine triphosphate; cryo-EM, cryogenic electron microscopy; β -DDM, *N*-dodecyl- β -maltoside; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; EDC-HCl, 3-ethylcarbodiimide hydrochloride; ESI, electrospray ionization; EtOAc, ethyl acetate; EtOH, ethanol; HIS, histidine; IC₅₀ half maximal inhibitory concentration; MeOH, methanol; MGY, minimum glycerol; PCR, polymerase chain reaction; Pi, inorganic phosphate; SAR, structure activity relationship; SD, standard deviation; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran; TLC, thin layer chromatography; Tris, tris(hydroxymethyl)aminomethane; YPD, yeast extract-peptone-dextrose.

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