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Research Paper

Cellular Phototoxicity Evoked Through the Inhibition of Human ABC Transporter ABCG2 by Cyclin-dependent Kinase Inhibitors *In vitro*

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Purpose. The physiological importance of the human ATP-binding cassette (ABC) transporter ABCG2 has been recognized with regard to porphyrin-mediated photosensitivity. Functional impairment owing to inhibition of ABCG2 by drugs or its genetic polymorphisms may lead to the disruption of porphyrin homeostasis, which in turn causes cellular toxicity.
Materials and Methods. We evaluated the impact on photosensitivity of the inhibition by cyclin-dependent kinase (CDK) inhibitors of ABCG2 function. For this purpose, we established new methods for photosensitivity assays by using Flp-In-293 cells and plasma membrane vesicles prepared from Sf9 insect cells. With the new methods, we subsequently tested CDK inhibitors, i.e., purvalanol A, WHI-P180, bohemine, roscovitine, and olomoucine.
Results. Among CDK inhibitors tested, purvalanol A was found to be the most potent inhibitor (IC_{50}= 3.5 μM) for ABCG2-mediated hematoporphyrin transport. At a concentration of 2.5 μM, it evoked the photosensitivity of ABCG2-expressing Flp-In-293 cells treated with pheophorbide a. WHI-P180 moderately inhibited ABCG2 function, exhibiting weak phototoxicity. In contrast, the phototoxicity of bohemine, roscovitine, and olomoucine were minimal in our assay system.
Conclusions. It is suggested that the planar structure is an important factor for interactions with the active site of ABCG2. The present study provides a new approach to studying drug-induced phototoxicity *in vitro*.

KEY WORDS: ABCG2 (BCRP/MXR); cyclin-dependent kinase inhibitor; pheophorbide a; phototoxicity; porphyrin.

INTRODUCTION

There is a strong requirement that screening for photochemical reactivity should be included in the preclinical stage or the development phase for new drugs. The phototoxicity risk associated with drugs may depend on both environmental and genetic factors. Recently, the physiological importance of the human ATP-binding cassette (ABC) transporter ABCG2 has been recognized with regard to porphyrin-mediated photosensitivity (1,2). Functional impairment owing to inhibition of ABCG2 by drugs or its genetic polymorphisms may lead to the disruption of porphyrin homeostasis (3,4).

Human ABCG2 was originally identified as an ATP-dependent drug efflux pump that mediates drug resistance of cancer cells and affects the pharmacological behavior of a variety of drugs (5–7). Overexpression of ABCG2 reportedly confers cancer cells resistance to anticancer drugs, such as topotecan, irinotecan (CPT-11), and mitoxantrone (8–11). In addition, ABCG2 protein is expressed endogenously in placental trophoblast cells, the epithelium of the small intestine and liver canalicular membrane, as well as in ducts and lobules of the breast. Apical localization in the epithelium of the small intestine and colon indicates a possible role for human ABCG2 in regulating the uptake of p.o. administered drugs as well as xenobiotics (12). On the other hand, mice lacking Abcg2 displayed a previously unknown type of protoporphyria, where intestinal absorption of a chlorophyll metabolite, pheophorbide a, was remarkably enhanced, and erythrocyte levels of protoporphyrin IX were increased about tenfold (2). ABCG2 appears to be responsible for the cellular homeostasis of porphyrins and their related compounds (13,

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ABBREVIATIONS: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; CDK, cyclin-dependent kinase; D-MEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; EGTA, ethyleneglycol-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetracetic acid; EKI-785, N-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butynamide; FCS, fetal calf serum; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; HOMO, highest occupied molecular orbital; IC_{50}, concentration leading to a 50%-inhibition; K_m, Michaelis–Menten constant; MXR, mitoxantrone resistance; MO, molecular orbital; QSAR, quantitative structure-activity relationship; Tris, tris (hydroxymethyl)aminomethane.

14). In this regard, we have provided evidence that ABCG2 transported hematoporphyrin and pheophorbide a in an ATP-dependent manner (3). Furthermore, we have most recently reported that functional impairment owing to inhibition of ABCG2 by drugs, such as imatinib, or its genetic polymorphisms may lead to photosensitivity through the disruption of porphyrin homeostasis (3,4). Indeed, side-effects related to photosensitization have been reported in patients treated with imatinib mesylate (15,16).

Özvegy-Laczka *et al.* (17) first demonstrated by *in vitro* assay that gefitinib, imatinib, and N-[4-[(3-bromophenyl) amino]-6-quinazolinyl]-2-butynamide (EKI-785) interact with ABCG2 at submicromolar concentrations, whereas other multidrug transporters, i.e., ABCB1 and ABCC1, showed much lower reactivity toward these drugs. Our quantitative structure-activity relationship (QSAR) analysis revealed that one amine bonded to one carbon of a heterocyclic ring is an important component for the interaction with the ABCG2 protein (18). In addition, fused heterocyclic ring(s) and two substituents on a carbocyclic ring of the fused heterocyclic ring(s) are also important chemical moieties for the interaction with ABCG2 (18). Interestingly, many protein kinase inhibitors carry such structural components within their molecules.

The human genome encodes more than 500 protein kinases and this protein kinase family has been the subject of intensive research for the development of novel anticancer drugs (19). Gefitinib and imatinib are new anticancer drugs that have been developed as inhibitors for EGFR tyrosine kinase and BCR/ABL kinase, respectively. Recently, another class family of protein kinases has been attracting particular attention. Namely, the cyclin-dependent kinases (CDKs), which regulate critical processes of cell cycle progression and gene transcription essential for cancer cell survival (20). In cancer, CDKs are deregulated in different ways, such as overexpression of cyclin E (21) and loss of $p16^{INK4A}$, a CDK inhibitor (22). Thus, chemical small molecules that specifically regulate or inhibit CDKs are of great interest in drug discovery and development for cancer chemotherapy.

In the present study, we aimed to examine the impact of the inhibition by CDK inhibitors of ABCG2 function on photosensitivity. By using Flp-In-293 cells and plasma membrane vesicles prepared from Sf9 insect cells, we established new methods for photosensitivity assays. Furthermore, we identified structural components that are important for CDK inhibitors to interact with ABCG2. In this paper, we provide evidence that inhibition of ABCG2 by certain CDK inhibitors can enhance the risk of photosensitivity, suggesting a new scheme for the assessment of drug-induced phototoxicity risk.

MATERIALS AND METHODS

Chemicals and Biological Reagents

The following compounds and therapeutic drugs were purchased from the commercial sources indicated in parentheses: ATP, hematoporphyrin, purvalanol A, bohemine, roscovitine, and olomoucine (Sigma-Aldrich, St. Louis, MO, USA); WHI-P180 (Calbiochem-Novabiochem GmbH, Darmstadt, Germany); pheophorbide a (Frontier Scientific, Inc., UT, USA); L-glutamine (Wako Pure Chemical Industries, Ltd., Osaka, Japan); creatine kinase, creatine phosphate, ethyleneglycol-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetracetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), Tris (hydroxymethyl)aminomethane (Tris), 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), and high-glucose Dulbecco's modified Eagle's medium (D-MEM) (Nacalai Tesque, Inc., Kyoto, Japan); fetal calf serum (FCS) (Dainippon Pharmaceuticals, Osaka, Japan); antibiotic-antimycotic cocktail solution and hygromycin B (Invitrogen, Carlsbad, CA, USA). All other chemicals used were of analytical grade.

Expression of ABCG2 in Sf9 Cells

Competent DH10Bac E. coli cells were transformed by the ABCG2-pFastBac1 plasmids, as described previously (3,4). Then, the ABCG2 cDNA was transposed into a bacmid, which is a baculovirus shuttle vector carrying the baculovirus genome, in DH10Bac cells with the aid of a helper plasmid. The recombinant bacmid was isolated and purified.

Insect Spodoptera frugiperda Sf9 cells were grown in NIM-EX Insect serum-free medium (NOSAN Corporation, Yokohama, Japan) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen Co., Carlsbad, CA, USA) with gentle shaking at 27°C. Sf9 cells were then transfected with the ABCG2-recombinant bacmid in the presence of Cellfectin® reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's protocol. Ninety-six hours after the transfection, the culture medium containing the recombinant baculovirus was harvested by centrifugation. To amplify recombinant baculovirus, Sf9 cells were further infected with the harvested virus and maintained at 27°C for 72 h. After the incubation, the culture medium was harvested by centrifugation. This process was repeated two times.

Sf9 cells (1×10^6 cells/ml) were infected with the amplified recombinant baculoviruses and cultured in NIM-EX Insect serum-free medium at 27°C with gentle shaking. Three days after the infection, cells were harvested by centrifugation. Cells were subsequently washed with PBS, collected by centrifugation, and stored at −80°C until used.

Preparation of the Plasma Membrane Vesicles from Sf9 Cells

Plasma membrane vesicles were prepared from ABCG2-expressing Sf9 cells as described previously (3,4). The frozen cell pellet was thawed quickly, diluted 40-fold with a hypotonic buffer (0.5 mM Tris/HEPES, pH 7.4, 0.1 mM EGTA), and then homogenized with a Potter–Elvehjem homogenizer. After centrifugation at 2,000×*g* for 10 min, the supernatant was further centrifuged at 100,000×*g* for 30 min. The resulting pellet was suspended in 0.25 M sucrose containing 10 mM Tris/HEPES, pH 7.4. The crude membrane fraction was layered over 40% (*w*/*v*) sucrose solution and centrifuged at 100,000×*g* for 30 min. The turbid layer at the interface was collected, suspended in 0.25 M sucrose containing 10 mM Tris/HEPES, pH 7.4, and centrifuged at 100,000×*g* for 30 min. The membrane fraction was collected and resuspended in a small volume (150 to 250 μl) of 0.25 M sucrose containing 10 mM Tris/HEPES, pH 7.4. After the protein concentration was measured with the BCA Protein

Assay Kit (Nacalai Tesque, Inc., Kyoto, Japan), the membrane solution was stored at −80°C until used.

High-Speed Detection of ABCG2-Mediated Hematoporphyrin Transport

The frozen stocked membrane solution was thawed quickly at 4°C. Plasma membrane vesicles (50 μg of protein) were incubated with 20 μM hematoporphyrin in the presence or absence of 1 mM ATP in 30 μl of the standard incubation medium (0.25 M sucrose and 10 mM Tris/HEPES, pH 7.4, 10 mM creatine phosphate, 100 μg/ml of creatine kinase, and 10 mM $MgCl_2$) at 37°C for 10 min. After a specified incubation period, the reaction mixture was mixed with 80 μl of ice-cold stop solution (10 mM EDTA, 0.25 M sucrose and 10 mM Tris/HEPES, pH 7.4), and then 50 μl of the resulting solution was loaded onto a 96-well separation plate (100 μl of bed volume) packed with Sephadex G-25 equilibrated with 0.25 M sucrose and 10 mM Tris/HEPES, pH 7.4 (Fig. 2A). The plate was immediately centrifuged in a swing-type rotor at 1,600×*g* for 5 min, whereby the eluate was collected into a 96-well micro plate. The eluate in each well was mixed with 250 μl of 10 mM NaOH solution to dissolve plasma membrane vesicles. Hematoporphyrin in the resulting solution was quantitatively analyzed by measuring its fluorescence in a Fluoroskan Ascent FL (Thermo Labsystems, Helsinki, Finland; excitation at 405 nm; emission at 612 nm).

Expression of ABCG2 in Flp-In-293 Cells

Flp-In™-293 cells (Invitrogen, Carlsbad, CA, USA) were maintained in high-glucose Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% (*v*/*v*) heat-inactivated FCS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. The number of viable cells was determined from counts made in a hemocytometer with Trypan Blue dye exclusion.

Flp-In-293 cells were transfected with the ABCG2-pcDNA5/FRT vector, the Flp recombinase expression plasmid pOG44, and LipofectAmine™-2000 (Invitrogen, Carlsbad, CA, USA) as described previously (23,24). Single colonies resistant to hygromycin B (Invitrogen, Carlsbad, CA, USA) were picked and subcultured. Selection of positive colonies was performed by immunoblotting, as described previously (25). Mock cells were prepared by transfecting Flp-In-293 cells with pcDNA5/FRT and pOG44 vectors in the same manner as described above.

Cellular Photosensitivity Assay with 96-well Plates

The effect of pheophorbide a on the photosensitivity of mock or ABCG2-expressing Flp-In-293 cells was measured as described previously (3,4). Briefly, cells were seeded in 96-well plates (15,000 cell/well) and cultured at 37°C for 24 h (Fig. 2B). Pheophorbide a was added to the culture medium at various concentrations and then incubation was continued in the dark for 4 h. Subsequently, the culture medium was replaced with fresh medium. Cells were then exposed to light for 90 min in the cell culture chamber, where 96-well plates were placed on a light viewer (Hakuba Model 7000PRO). Cells were again incubated in the dark for 24 h, and cell viability was measured by the MTT assay as described previously (4). Thereafter, 100 μl of 10% (*w*/*v*) SDS in PBS was added to the culture medium, and the mixture was incubated at 37°C overnight. The absorbance of formazan, a metabolite of MTT, in the resulting solution was photometrically measured at a test wavelength of 570 nm and a reference wavelength of 630 nm in a Multiskan JX system (Dainippon Pharmaceuticals Co., Osaka, Japan). IC_{50} values were calculated from dose-response curves (i.e., cell survival *vs*. drug concentration).

MO Calculations of CDK Inhibitors

To clarify the three-dimensional (3D) structures of CDK inhibitors, we performed ab initio molecular orbital (MO) calculations based on the restricted Hartree–Fock (RHF) level of theory. The basis set used were MIDI-4, and MO calculations were performed on a work station cluster (NEC Express5800–120Rc-1 geon 2.4GHz × 2) of 32 nodes with the program package AMOSS, which had been developed by NEC (Tokyo, Japan).

RESULTS

Selection of CDK Inhibitors

Our QSAR analysis (18) has revealed that structural components represented by the descriptors of H12, D01, D02, and OH positively contributed to the inhibition of ABCG2. Table I summarizes the meanings of those descriptors and the corresponding chemical fragmentation codes (CFC). H12 had the largest positive coefficient, suggesting that one amine bonded to one carbon of a heterocyclic ring is an important component for the interaction with the ABCG2 protein. In addition, fused heterocyclic ring(s) and two substituents on a carbocyclic ring of the fused heterocyclic ring(s) are also important chemical moieties for the interaction with ABCG2. As shown in Fig. 1, the chemical fragmentation codes of

Table I. Descriptors and Chemical Fragmentation Codes (CFC) Involved in CDK Inhibitors

Descriptor	CFC	Description
H12		Amine bonded to heterocyclic carbon
	H121	One
	H122	Two
D01		Substituents on a heterocyclic ring of a fused-ring heterocyclic system
	D013	Two atoms of a fused heterocyclic ring bear substituents
D02		Substituents on a carbocyclic ring of a fused-ring heterocyclic system
	D023	Two carbon atoms of a fused carbocyclic ring bear substituents
OH		Hydroxy groups
	H401	One –OH group
	H441	One –OH group bonded to aromatic carbon
M531	M531	One carbocyclic system with at least one aromatic ring

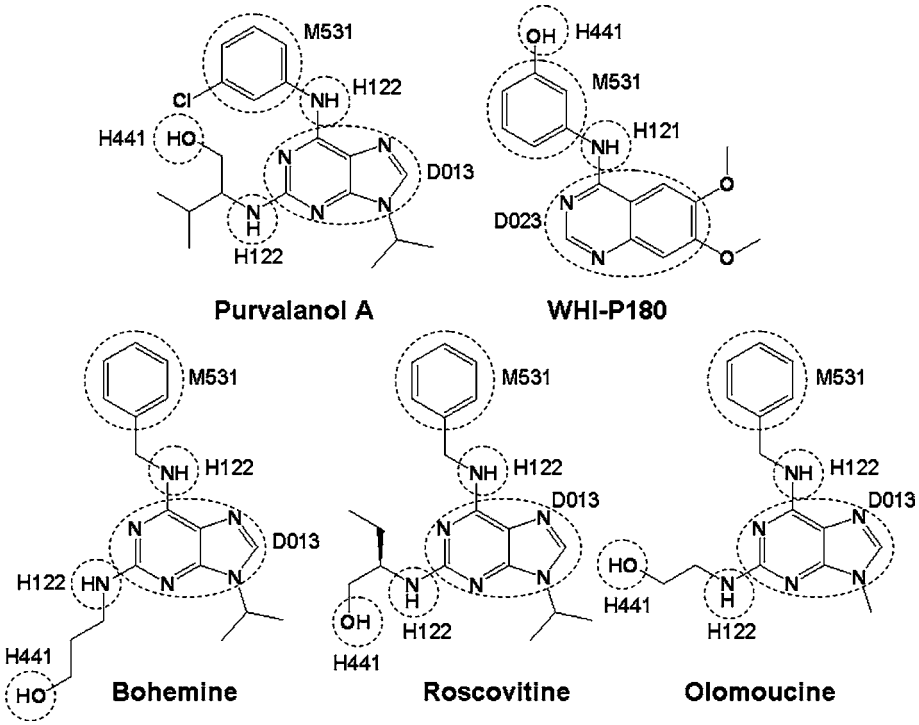

Fig. 1. Chemical structures of CDK inhibitors: purvalanol A, WHI-P180, roscovitine, bohemine, and olomoucine. Chemical fragmentation codes involved in these CDK inhibitors were deduced by using the Markush TOPFRAG program (http://thomsonderwent.com/products/patentresarch/markushtopfrag/) (Derwent Information Ltd., London, UK) as described previously (18).

purvalanol A, WHI-P180, bohemine, roscovitine, and olomoucine involve H121, H122, D013, D023, and H441 as positive contributors. Based on the QSAR analysis, we hypothesized that those CDK inhibitors would interact with the ABCG2 protein.

Inhibition of ABCG2-Mediated Porphyrin Transport by CDK Inhibitors

To examine our hypothesis, we detected the inhibition of ABCG2-mediated porphyrin transport by purvalanol A, WHI-P180, bohemine, roscovitine, and olomoucine. For this purpose, we have developed a high-speed *in vitro* screening system by using 96-well gel filtration plates and plasma membrane vesicles prepared from Sf9 cells expressing human ABCG2 (Fig. 2A). With this new method, the throughput of inhibition screening has been enhanced more than ten times, as compared with the original method described previously (3).

Figure 3A shows the time courses for hematoporphyrin transport into the membrane vesicles in the presence or absence of ATP. The activity of ATP-dependent hematoporphyrin transport was very low in plasma membrane vesicles prepared from mock-infected Sf9 cells (data not shown). The K_m value for hematoporphyrin was estimated to be 6.6 μM (Fig. 3B). As demonstrated in Fig. 4, purvalanol A, WHI-P180, bohemine, and roscovitine inhibited ABCG2-mediated hematoporphyrin transport, whereas olomoucine showed little inhibition in the concentration range of 0–30 μM. With the high-speed screening system, IC_{50} values were calculated to be 3, 10, 21, and 21 μM, respectively. Among the CDK inhibitors tested, purvalanol A was the strongest inhibitor for ABCG2.

Photosensitization of ABCG2-Expressing Flp-In-293 Cells by CDK Inhibitors

Based on the ABCG2-inhibition results, we attempted to evaluate the effect of purvalanol A, WHI-P180, bohemine, roscovitine, and olomoucine on the photosensitivity of ABCG2-expressing Flp-In-293 cells. Since those CDK inhibitors could inhibit the proliferation of Flp-In-293 cells, however we first determined an optimal concentration range for each compound. Namely, we incubated both ABCG2-expressing and mock Flp-In-293 cells with each CDK inhibitor at different concentrations (0–50 μM) under the standard conditions for 72 h, and thereafter determined the cell growth. The respective maximal tolerance concentrations for purvalanol A, WHI-P180, bohemine, roscovitine, and olomoucine were estimated to be 3, 5, 10, 10, and 30 μM.

To evaluate the effects of those CDK inhibitors on the photosensitivity of ABCG2-expressing Flp-In-293 cells, we incubated ABCG2-expressing Flp-In-293 cells with varying concentrations of pheophorbide a in the presence of 2.5 μM purvalanol A, 5 μM WHI-P180, 10 μM roscovitine, or 10 μM bohemine for 4 h. After a 90-min exposure to visible light and the following incubation without CDK inhibitors and pheophorbide a for 20 h, the cell viability was determined according to the protocol shown in Fig. 2B.

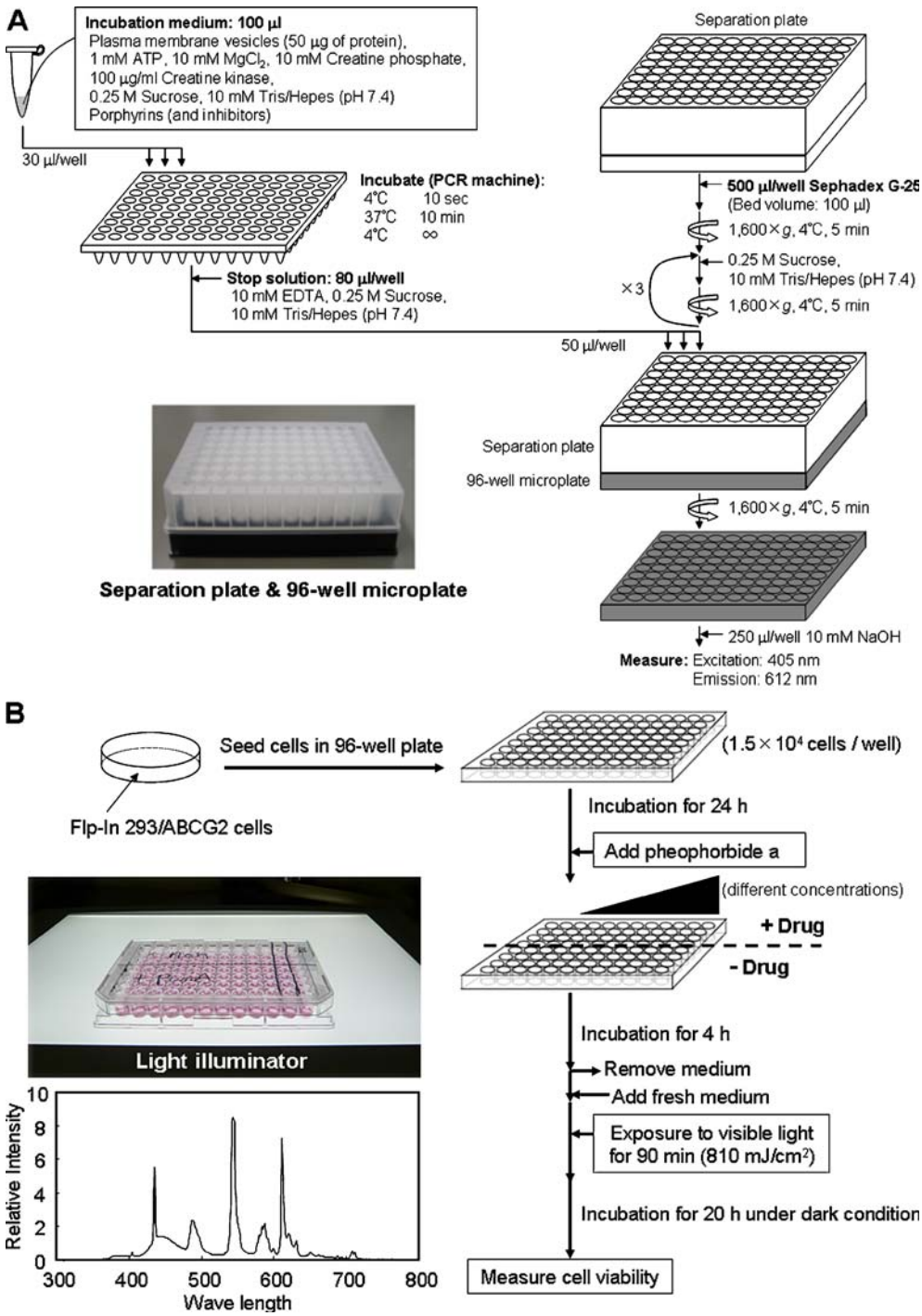

Fig. 2. Schematic illustrations of the detection of ATP-dependent hematoporphyrin transport into plasma membrane vesicles by using a 96-well separation plate (**A**) and evaluation of the phototoxic effects of drugs on Flp-In-293/ABCG2 cells expressing human ABCG2 (**B**). Experimental procedures are described in MATERIALS AND METHODS. The visible light spectrum of the light viewer (Hakuba Model 7000PRO) was measured with the multi-channel analyzer Model C7473 (Hamamatsu Photonics K.K., Japan). The light intensity (0.15 mJ s^{-1} cm^{-2}) at the 96-well plate placed on the light viewer was measured with a laser power meter (Model NOVA-10A-P-SH, Ophir Optromics, Ltd., UT, USA).

When mock and ABCG2-expressing Flp-In-293 cells were treated with pheophorbide a, the mock cells were about ten times more sensitive to light at low concentrations of pheophorbide a than were the ABCG2-expressing cells. Figure 5 demonstrates the effect of purvalanol A, WHI-P180, bohemine, and roscovitine on the photosensitivity of ABCG2-expressing Flp-In-293 cells. It is important to note that 2.5 µM purvalanol A greatly enhanced the photosensitivity of ABCG2-expressing Flp-In-293 cells, suggesting that the inhibition of ABCG2-mediated pheophorbide a transport by

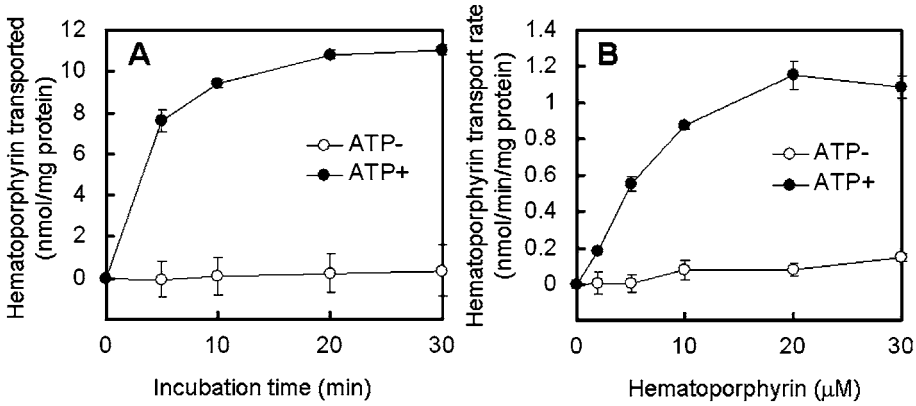

Fig. 3. **A** Time courses of ATP-dependent transport of hematoporphyrin into plasma membrane vesicles prepared from ABCG2-expressing Sf9 cells. Plasma membrane vesicles (50 μg of protein) were incubated with 100 μM hematoporphyrin in the presence or absence of 1 mM ATP in the standard incubation medium at 37°C for different periods, as indicated. The amount of hematoporphyrin transported into membrane vesicles was detected as described in "Materials and Methods". **B** Effect of hematoporphyrin concentration on the rate of ATP-dependent transport of hematoporphyrin into plasma membrane vesicles. ABCG2-expressing plasma membrane vesicles (50 μg of protein) were incubated with hematoporphyrin at different concentrations (0, 10, 20, 50, 100, and 200 μM) in the presence or absence of 1 mM ATP in the standard incubation medium at 37°C for 10 min. The amount of hematoporphyrin transported into membrane vesicles was spectrophotometrically detected. Data are expressed as mean values±SD (n=8).

this CDK inhibitor plays a significant role. The other CDK inhibitors (WHI-P180, bohemine, roscovitine, and olomoucine) exhibited little effect on the photosensitivity under the same experimental conditions.

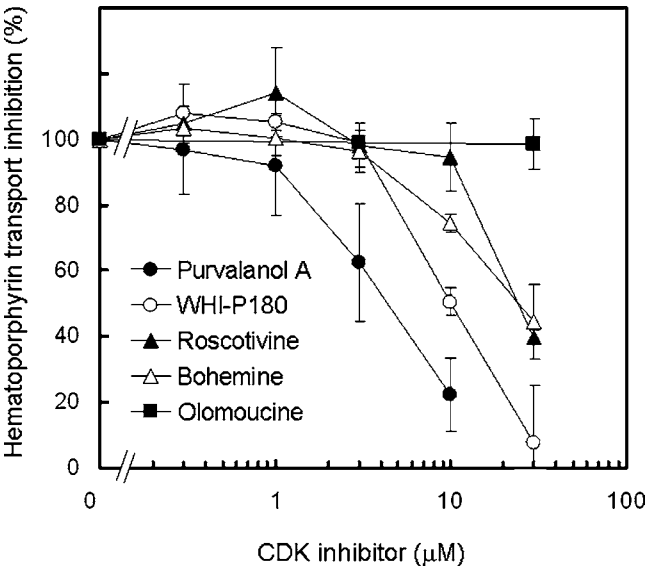

Fig. 4. Inhibition of ABCG2-mediated hematoporphyrin transport by purvalanol A, WHI-P180, roscovitine, bohemine, or olomoucine. ABCG2-expressing plasma membrane vesicles (20 μg of protein) were incubated with 20 μM hematoporphyrin in the presence of purvalanol A, WHI-P180, roscovitine, bohemine, or olomoucine (final concentration: 0, 0.3, 1, 3, 10, or 30 μM) in the standard incubation medium (0.25 M sucrose and 10 mM Tris/HEPES, pH 7.4, 1 mM ATP, 10 mM creatine phosphate, 100 μg/ml of creatine kinase, 10 mM $MgCl_2$) at 37°C for 10 min. Hematoporphyrin transported into membrane vesicles was detected as described in "Materials and Methods". Data are expressed as mean values±SD (n=5).

DISCUSSION

Inhibition of ABCG2 by Protein Kinase Inhibitors

Protein kinases are potential drug targets for the treatment of a variety of diseases, including cancer (26). In particular, specific tyrosine kinase inhibitors are rapidly being

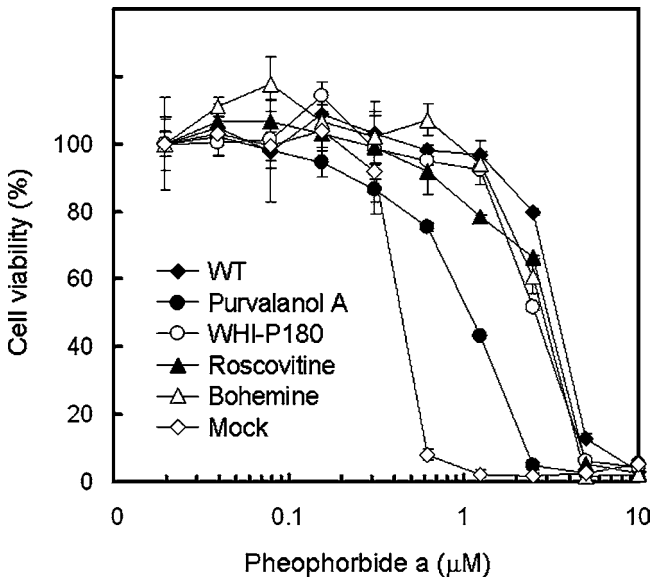

Fig. 5. Photosensitization of Flp-In-293/ABCG2 cells by inhibiting ABCG2 with purvalanol A, WHI-P180, roscovitine, or bohemine. Flp-In-293 cells expressing ABCG2 WT were incubated with pheophorbide a in the presence of 2.5 μM purvalanol A, 5 μM WHI-P180, 10 μM roscovitine, or 10 μM bohemine at 37°C for 4 h. Thereafter, the incubation medium was replaced with fresh medium, and cells were exposed to light for 90 min, in the same manner as described in Fig. 2B. Cell viability was measured by the MTT assay. Data are expressed as mean values±SD (n=4).

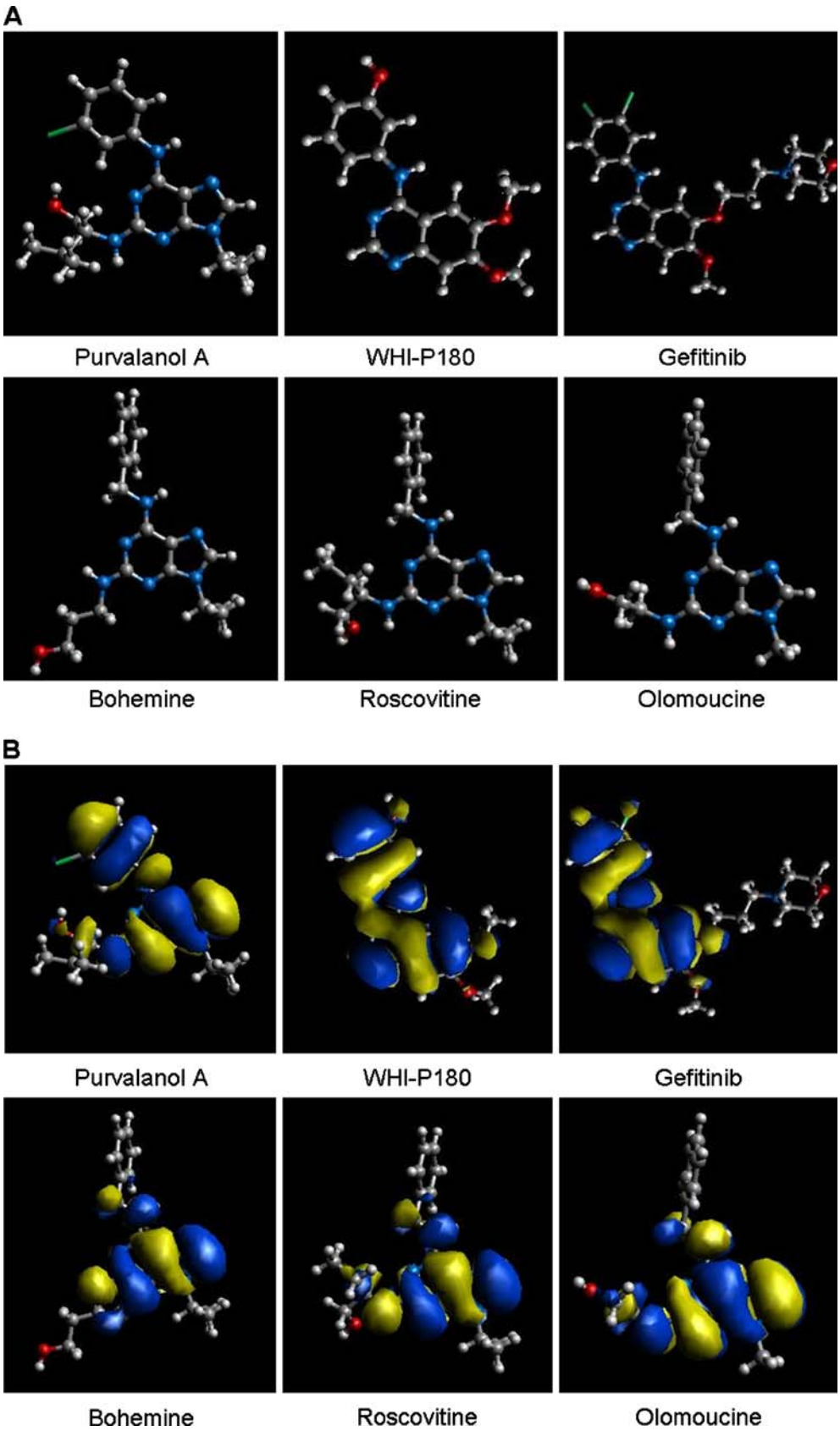

Fig. 6. Putative three-dimensional (3D) structures of purvalanol A, WHI-P180, gefitinib, roscovitine, bohemine, roscovitine, and olomoucine. The 3D structures (**A**) and HOMO profiles (**B**) of those CDK inhibitors were generated by ab initio MO calculation as described previously (29).

developed as new drugs for the inhibition of malignant cell growth and metastasis formation. Most of these newly developed tyrosine kinase inhibitors are hydrophobic, and thus rapidly penetrate the cell membrane to reach intracellular targets. Recently we have reported that gefitinib and imatinib are potent inhibitors for human ABCG2 (4,18). Since porphyrins are regarded as endogenous substrates for ABCG2, it was suggested that functional impairment owing to inhibition of the transport function of ABCG2 by drugs could result in a disruption of cellular porphyrin homeostasis. In this regard, we have provided evidence that imatinib inhibited the porphyrin transport activity of ABCG2 and caused *in vitro* phototoxicity resulting from cellular accumulation of porphyrin (4). In the clinic, side-effects related to photosensitization have been reported in patients treated with imatinib mesylate (15,16). The reported cutaneous side-effects include skin rash, hypopigmentation, and depigmentation; and those adverse effects were observed in long-term-treated patients, suggesting that cumulative dosages of imatinib represent the unique determinant for cutaneous photosensitization (16). The mechanism of those side-effects is not known at present, but our data suggest a possible involvement of ABCG2 inhibition.

By using the recently developed high-speed screening system, we investigated the interaction of ABCG2 with a variety of test compounds and drugs to gain insight into the reactions between their structural components and the inhibition of ABCG2 (18). We performed a QSAR analysis by introducing the chemical fragmentation codes as a structure-indexing language. The QSAR analysis revealed that the structural components represented by the chemical fragmentation codes of H121, H122, D013, D023, and H441 positively contributed to the inhibition. As shown in Fig. 1, those chemical fragmentation codes were found in the chemical structures of purvalanol A, WHI-P180, bohemine, roscovitine, and olomoucine. The previous QSAR analysis data appeared to be consistent, in part, with our present observation that purvalanol A, WHI-P180, bohemine, and roscovitine inhibited ABCG2-mediated hematoporphyrin transport (Fig. 4).

Relationship Between the Structure of CDK Inhibitors and the Photosensitization Activity

To gain further insights into drug-ABCG2 interactions, the three-dimensional (3D) structures of those CDK inhibitors were generated by ab initio MO calculation. Figure 6 depicts the 3D structures (A) and HOMO profiles (B) of purvalanol A, WHI-P180, bohemine, roscovitine, and olomoucine as well as gefitinib. It has become clear that purvalanol A and WHI-P180 have a planar structure, whereas bohemine, roscovitine, and olomoucine do not. In the latter CDK inhibitors, the aromatic ring is orthogonal to the purine ring (Fig. 6A). As compared with bohemine, roscovitine, and olomoucine, both purvalanol A and WHI-P180 were stronger inhibitors for ABCG2-mediated methotrexate transport in membrane vesicles (Saito H and Ishikawa T, unpublished data), being consistent with the present observations in terms of the inhibition of hematoporphyrin transport (Fig. 4). It is suggested that the planar structure is an important factor for interactions with the active site of ABCG2. Interestingly, even though WHI-P180 inhibited ABCG2-mediated hematoporphyrin transport in membrane vesicles (Fig. 4), it did not evoke the photosensitivity of ABCG2-expressing Flp-In-293 cells (Fig. 5). WHI-P180 is thought to be transported out of cells as a substrate of ABCG2, since both the molecular structure and the HOMO profile of WHI-P180 are quite similar to those of gefitinib (Fig. 6B). Indeed, gefitinib is a substrate for ABCG2 (27). The inhibition kinetics strongly suggest that both gefitinib and WHI-P180 are bound to an ABCG2-ATP complex as substrates (18). On the other hand, purvalanol A, that exhibits a different HOMO profile (Fig. 6B), does not appear to be a substrate for ABCG2, but it binds to both ABCG2 and the ABCG2-ATP complex to inhibit porphyrin transport.

CONCLUSION

At present, several CDK inhibitors are in the development stage; i.e., roscovitine (phase II), flavopiridol/HMR-1275 (phase II), E7070 (phase I), and BMS-387032 (phase I) (19,28). The phototoxicity of those drug candidates has not yet been published because of regulatory limitations in the development stage. In general, the photosensitivity risk associated with the use of drugs is considered to depend on both environmental and genetic factors. It is of great interest and importance to analyze potential links between the photosensitivity risk and genetic/environmental factors. If

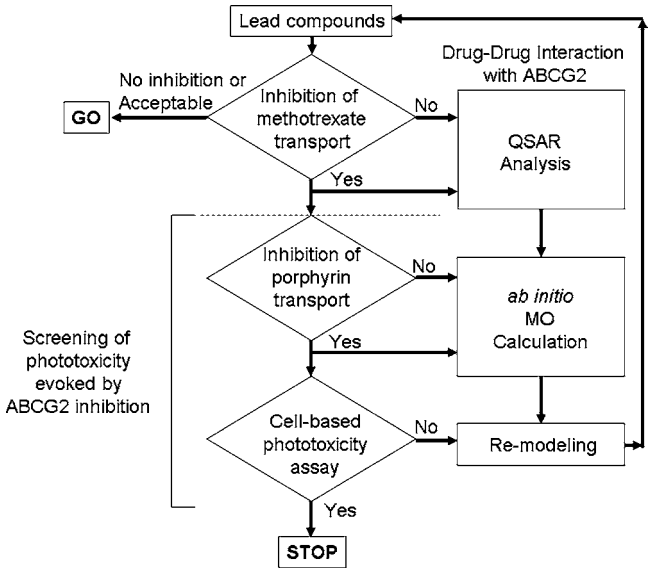

Fig. 7. Flow chart to circumvent the drug-induced phototoxicity evoked by ABCG2-inhibition. Lead compounds will be tested in the high-speed screening to discriminate inhibitors or non-inhibitors for ABCG2-mediated methotrexate transport (18). Based on the inhibition data, the QSAR analysis with chemical fragmentation codes (18) is performed to identify the chemical groups that are important for inhibiting the function ABCG2. Potential inhibitors are further tested in the membrane vesicle-based screening for inhibition of porphyrin transport, as shown in this study. Based the data from those two screenings, we perform ab initio MO calculation to get insight into structural and/or molecular orbital requirements involved in the inhibition of ABCG2-mediated transport. Inhibitors, thus identified, will be tested in cell-based phototoxicity assay (shown in this study) to decide "STOP" or re-modeling of their chemical structure.

the photosensitizing effect of a chemical or drug is known before its exposure to patients, appropriate clinical management may help to control the photosensitive reactions. The bias and inaccuracy of the reporting procedure for these adverse reactions, however, derives from the difficulty in distinguishing between sunburn and a mild drug photosensitivity reaction, together with the patient's ability to control the incidence by taking protective action. For many drugs that have been implicated in adverse photosensitivity reactions, it would be possible to demonstrate by *in vitro* experiments a level of photochemical activity that relates to the severity of the observed *in vivo* effects. There is a strong requirement that screening for photochemical reactivity should be included in the preclinical stage or the development phase for new drugs.

In the present study, we have developed simple methods to quantitatively measure the effect of drugs on cellular photosensitivity *in vitro*. Such technologies may offer a great advantage for predicting photosensitive reactions before drugs are introduced in therapy or drug products are made available on the market. High-speed screening methods for the detection of drug-induced photosensitivity *in vitro* would provide a better assessment of the risk of photosensitivity. In Fig. 7, we propose a strategy to circumvent the drug-induced phototoxicity evoked by ABCG2-inhibition. The relevant studies are ongoing in our laboratory, and those results will be reported elsewhere.

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