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# Botryllamides: Natural Product Inhibitors of ABCG2

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**ABSTRACT** ABCG2 is a membrane-localized, human transporter protein that has been demonstrated to reduce the intracellular accumulation of substrates through ATP-dependent efflux. Highly expressed in placental syncytiotrophoblasts, brain microvasculature, and the gastrointestinal tract, ABCG2 has been shown to mediate normal tissue protection as well as limit oral bioavailability of substrate compounds. Development of ABCG2 inhibitors for clinical use may allow increased penetration of therapeutic agents into sanctuary sites and increased gastrointestinal absorption. Previously identified inhibitors have lacked potency or specificity or were toxic at concentrations needed to inhibit ABCG2; none are in clinical development. A previously developed high-throughput assay measuring inhibition of ABCG2-mediated pheophorbide a transport was applied to natural product extract libraries. Among the active samples were extracts from the marine ascidian *Botryllus tyreus*. Bioassay-guided fractionation resulted in purification of a series of botryllamides. Ten botryllamides were obtained, two of which (designated I and J) were novel. Activity against ABCG2 was confirmed by assessing the ability of the compounds to inhibit ABCG2-mediated BODIPY-prazosin transport in *ABCG2*-transfected HEK293 cells, compete with [$^{125}$I]-iodoarylazidoprazosin (IAAP) labeling of ABCG2, stimulate ABCG2-associated ATPase activity, and reverse ABCG2-mediated resistance.

The ATP-binding cassette (ABC) superfamily of genes comprise the largest family of transporter genes and encode membrane proteins that transport diverse substrates across biomembranes against a concentration gradient using energy derived from ATP hydrolysis (*1*). Of the 48 human ABC transporter genes, three are usually associated with multidrug resistance in cancer: *ABCB1* (*MDR-1*) encoding P-glycoprotein (P-gp), *ABCC1* encoding the multidrug associated protein 1 (MRP1), and *ABCG2* encoding the breast cancer resistance protein (BCRP or ABCG2) (*2*). P-gp was the first ABC transporter described and has been shown to transport a diverse range of substrates including anticancer drugs, antibiotics, and steroids (*2*). MRP1 was the second ABC transporter reported and was found to transport anticancer drugs as well as glucuronide and glutathione conjugates (*2*). ABCG2 is the most recent ABC transporter linked to multidrug resistance, counting chemotherapeutics, antibiotics, and HMG-CoA inhibitors among its substrates (*3*). Although its contribution to clinical drug resistance remains under investigation, ABCG2 is involved in modulating the oral availability of drugs and in forming normal protective barriers such as the maternal−fetal barrier and the blood−brain barrier (*4, 5*). ABCG2 has also been reported to be highly expressed in cancer stem cells (*6, 7*). Given these important roles, increased availability of modulators of ABCG2 activity would have significant research and clinical implications.

The search for ABCG2 inhibitors began with the observation that fumitremorgin C (FTC, produced by *Aspergillus fumigatus*) could reverse non-P-gp, non-MRP multidrug resistance in a drug-resistant cell line (later shown to be due to ABCG2 expression) (*8*). However, clinical development of FTC was never undertaken because of

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its neurotoxicity in various animal models (*9*). Compounds reported to be inhibitors of ABCG2 activity include drugs originally identified as P-gp inhibitors, protein tyrosine kinase inhibitors, steroids, flavonoids, FTC derivatives, and other natural products. However, these lack potency or specificity or have toxic effects, and none have been developed specifically as ABCG2 targeting agents (see refs 2−7 for lists and discussions of specific inhibitors). A potent and specific ABCG2 inhibitor would be of significant value for clarification of the physiological function(s) of ABCG2 for pharmacological applications such as improving drug penetration into sanctuary sites as well as to confirm its purported relevance in multidrug resistance. Therefore, a high-throughput screening (HTS) assay, based on accumulation of pheophorbide a (PhA), was developed (*10*) and used to identify novel inhibitors of ABCG2 (*11*).

To identify lead compounds for development as ABCG2 inhibitors, the ABCG2 HTS assay was applied to natural product extracts from the natural products repository of the National Cancer Institute (NCI). Natural products and derivatives have been and continue to be rich sources of novel therapeutics (*12, 13*) and comprise the majority of current cancer chemotherapeutics (*12, 14*). Many of the previously identified substrates and inhibitors of ABCG2 have also been natural products or derivatives, suggesting that this could be a fertile area for discovery of additional, unique inhibitors. After screening and bioassay-guided fractionation of active extracts, a series of botryllamides (*15, 16*) with variable activity against ABCG2 have been purified and characterized. Among these are two novel botryllamides. Confirmation and further characterization of the interaction of the botryllamides with ABCG2 included additional cell-based and biochemical assays. Specificity of the botryllamides for ABCG2 was assessed by determining their activity against MRP1 and P-gp.

## RESULTS AND DISCUSSION

To identify novel modulators of ABCG2, 89,229 natural product extracts from the NCI natural products repository were screened for ABCG2 inhibitory activity using a previously described high-throughput assay (*10*). Extracts were evaluated for their ability to increase intracellular accumulation of the ABCG2-specific substrate PhA in ABCG2-overexpressing NCI-H460 MX20 cells. Thirty-five confirmed hits (*i.e.*, reproducible activity of ≥50% of the activity of the positive control, FTC; see ref 10 for additional details) were obtained. Among these was a $CH_2Cl_2$−MeOH extract of the marine ascidian *Botryllus tyreus* that was collected along the coast of Papua New Guinea. Assay-guided fractionation of this extract by solvent partitioning and repeated chromatography on $C_{18}$ stationary phase yielded known compounds, botryllamide A−H (**1**−**8**). Figure 1 shows the structures of these compounds and the related botryllamides described below. Botryllamides A−H were previously isolated and characterized as a result of chemical studies of several *Botryllus* species (*15, 16*). The botryllamides have been reported to exhibit weak cytotoxicity to several tumor cell lines, and their biosynthesis appears to involve the conjugation of two tyrosine subunits. In the current investigation they were identified by comparison of their spectral data with published values (*15, 16*). In addition to the known botryllamides, two new compounds, designated botryllamide I (**9**) and J (**10**), were identified from the *B. tyreus* extract. See the Supporting Information for complete NMR spectroscopic and physical data for compounds **9** and **10**. In the course of assigning the structure of botryllamide J (**10**), it became apparent that the previously assigned structure of botryllamide H had to be revised to **11**.

Botryllamide I (**9**) was obtained as a glassy solid after final $C_{18}$ HPLC purification. Its molecular formula was established as $C_{19}H_{19}NO_4$ by HRESIMS measurements (obsd $[M - H]^-$ $m/z$ 324.1236, calcd for $C_{19}H_{18}NO_4$ 324.1241). Compound **9** was clearly related to the other botryllamides as its $^1$H NMR spectrum showed characteristic resonances for two methoxy groups ($\delta_H$ 3.74 and 3.76) and two pairs of (2H) aromatic doublets that were indicative of two *para*-substituted phenol rings. 2D NMR established the presence of two olefins in **9**, and HMBC correlations from H-3 ($\delta_H$ 6.05) and H-10 ($\delta_H$ 7.31) to C-1 ($\delta_C$ 163.9) confirmed they were connected through a carbonyl group. The $^1$H NMR spectrum acquired in $CD_3OH$ revealed the presence of an amide NH proton ($\delta_H$ 7.26) that was coupled (8.4 Hz) to H-10. HMBC correlations ($\delta_H$ 3.74/$\delta_C$ 149.3 and $\delta_H$ 3.76/$\delta_C$ 160.0) required that the methoxy groups were located at C-2 and C-15, respectively. The geometry of the C-10/C-11 double bond was *E* based on the 14.6 Hz coupling between H-10 and H-11. The geometry of the C-2/C-3 double bond in **9** could be inferred as *E* from the characteristic $^{13}$C NMR chemical shift of C-3 ($\delta_C$ 108.6). It was previously established with botryllamides A−D (**1**−**4**) that when $\Delta^{2,3}$ is *Z*, C-3 resonates downfield ($\delta_C$

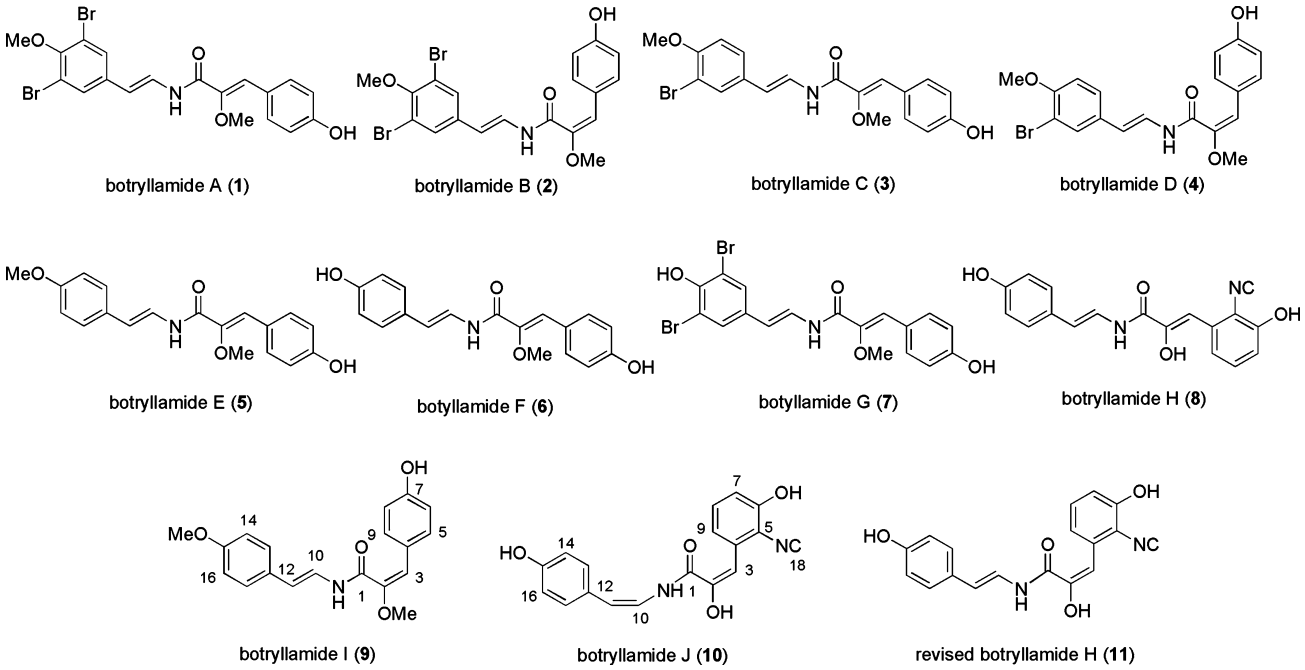


**Figure 1. Structures of botryllamides.**

> 120), and when $\Delta^{2,3}$ is *E*, C-3 is shifted upfield ($\delta_C <$ 110) (*15*). Therefore, the structure of botryllamide I (**9**) could be assigned as the $\Delta^{2,3}$ geometrical isomer of botryllamide E (**5**). This was confirmed by the observation that botryllamide I (**9**) could be irreversibly converted to botryllamide E (**5**) by exposure to sunlight.

Given this observation, in order to avoid the possibility of light-induced isomerization of botryllamides, dry compounds and stock solutions were light-protected during storage. Similarly, incubations were performed in the dark or under subdued light conditions.

Botryllamide J (**10**) was isolated as a pale yellow solid that was soluble in DMSO but not in MeOH. The molecular formula of **10** was established as $C_{18}H_{14}N_2O_4$ by HRESIMS ($[M - H]^-$ *m/z* 321.0879), and this formula was isomeric with botryllamide H (**8**). The $^1$H NMR spectrum in DMSO-$d_6$ was very similar to that obtained with **8** as it showed two doublet resonances ($\delta_H$ 6.79 and 7.35, each 2H) characteristic of a phenol and three resonances at $\delta_H$ 7.08 (d, 8.5 Hz), 7.38 (t, 8.5 Hz), and 7.58 (d, 8.5 Hz) consistent with a 1,2,3-trisubstituted aromatic ring. A three-proton spin system consisting of H-11 ($\delta_H$ 5.85), H-10 ($\delta_H$ 6.74), and an amide NH ($\delta_H$ 7.26) was apparent, and a 9.8 Hz coupling between H-10 and H-11 revealed that the geometry of the C-10/C-11 double bond in **10** was *Z*. HMBC correlations from H-3 ($\delta_H$ 7.45) to C-1 ($\delta_C$ 163.5) and C-4 ($\delta_C$ 124.1), from H-11 to C-13 ($\delta_C$ 130.1), and from the NH to C-2 ($\delta_C$ 156.9) helped establish the enamide amide and enol functionalities that connected the two aromatic rings. Similarity between the $^1$H and $^{13}$C resonances for the trisubstituted ring in **10** with those in botryllamide H (**8**) indicated that the substituents and substitution patterns were identical. All of the observed HMBC correlations about the ring were consistent with this assignment, and the presence of an isonitrile functionality was supported by an IR absorption at 2120 cm$^{-1}$ and a broadened $^{13}$C resonance at $\delta_C$ 162.3. The upfield shift of C-3 ($\delta_C$ 102.8) in **10** indicated that the $\Delta^{2,3}$ geometry was *E*. Since the chemical shift reported for C-3 in **8** was also upfield at $\delta_c$ 102.3, it appears that botryllamide H also has a $\Delta^{2,3}$ olefin with *E* geometry and that its structure should be revised to **11**. Thus, botryllamide J (**10**) was assigned to be the $\Delta^{10,11}$ geometric isomer of the revised structure of botryllamide H (**11**).

Figure 2 shows activities in the screening assay for each botryllamide obtained from fractionation of the original extract. Results are expressed as percent of activity of 1 μM of the known ABCG2 inhibitor FTC, which was set to 100%. Each purified botryllamide was resus-

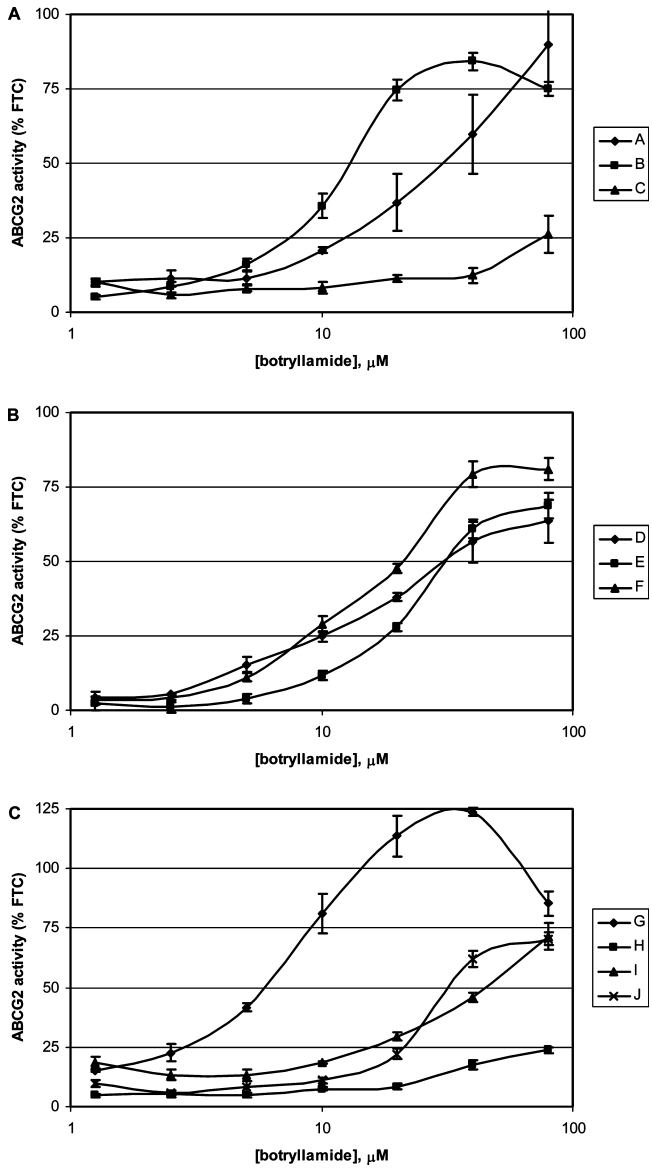


**Figure 2. Activity of botryllamides in screening assay. Botryllamides were assayed in the PhA accumulation assay (*10*). Serial 2-fold dilutions were prepared with a high concentration of 80 μM (final in assay). PhA accumulation was normalized to that obtained with 1 μM FTC (FTC signal = 100%). Error bars represent SEM for three independent experiments (each concentration run in duplicate for each experiment).**

pended in DMSO, and serial dilutions were prepared. Maximal activities and $IC_{50}$ values are shown in Table 1. As observed in Figure 2, all of the botryllamides except C and H had maximal activity at least 60% of that obtained with the positive control, FTC.

**TABLE 1. Effects of botryllamides on PhA accumulation[a]**

| Botryllamide | $IC_{50}$ (μM)[b] | Max activity (% FTC)[b] |
|---|---|---|
| A | 33.4 ± 6.7 | 89.7 ± 12.4[c] |
| B | 11.2 ± 0.6 | 84.3 ± 2.9[d] |
| C | nd | 26.2 ± 6.4 |
| D | 16.4 ± 2.3 | 63.5 ± 7.4[c] |
| E | 23.3 ± 1.2 | 68.8 ± 4.2[d] |
| F | 16.7 ± 0.8 | 81.0 ± 3.8[d] |
| G | 6.9 ± 0.5 | 123.7 ± 1.7[d] |
| H | nd | 23.8 ± 1.2[c] |
| I | 41.4 ± 6.1 | 71.3 ± 5.6[d] |
| J | 26.9 ± 1.6 | 70.5 ± 2.5[d] |
| FTC | 0.79 ± 0.01 | 100 |

[a]Apparent $IC_{50}$ values were calculated from dose–response data using SigmaPlot (SPSS, Inc., Chicago) 4-parameter logistic nonlinear regression analysis. Values shown are average ± SEM (3 plates, duplicate wells per plate). [b]$IC_{50}$ and maximal activities for inhibition of PhA accumulation were determined from dose–response curves (Figure 2); nd = could not be determined. [c]$p < 0.05$ compared to untreated control. [d]$p < 0.01$ compared to untreated control.

To confirm the activity of these compounds as ABCG2 inhibitors and to demonstrate that the activity was not linked to use of PhA as a substrate, an alternative flow-cytometry-based substrate efflux assay, two biochemical assays, and a cell-based assay for ABCG2 inhibition were applied. The flow-cytometry-based assay examined the ability of the botryllamides to inhibit efflux of the fluorescent ABCG2 substrate BODIPY-prazosin from *ABCG2*-transfected HEK293 cells (*18*). Cells were incubated with BODIPY-prazosin alone or in the presence of 10 μM botryllamide or FTC. As seen in Figure 3 (FTC panel), *ABCG2*-transfected cells incubated with BODIPY-prazosin alone demonstrate lower intracellular levels of the fluorescent compound (solid line) compared to cells incubated with BODIPY-prazosin in the presence of 10 μM concentration of the known ABCG2 inhibitor FTC (dashed line). The higher intracellular fluorescence of BODIPY-prazosin found in cells incubated with prazosin in the presence of inhibitor compared to cells incubated with prazosin alone, denoted by the difference between the solid histogram and the dashed histogram, is indicative of inhibition of ABCG2-

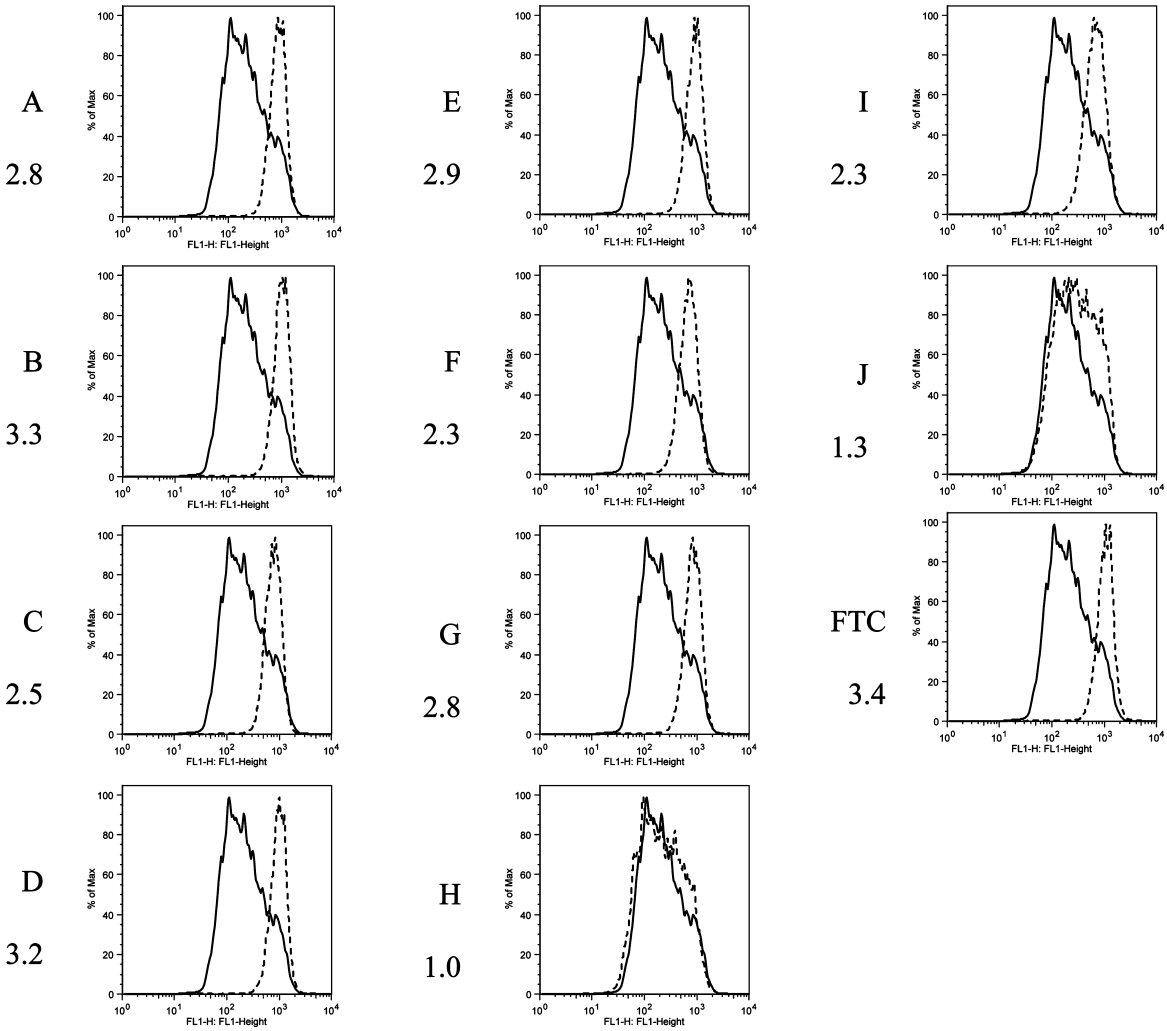


**Figure 3. Accumulation of BODIPY-prazosin. ABCG2-transfected HEK293 cells were incubated with the fluorescent ABCG2 substrate BODIPY-prazosin in the absence (solid line) or presence (dashed line) of 10 μM of the indicated botryllamides or FTC, and intracellular fluorescence (*i.e.*, accumulation of substrate) was assessed by flow cytometry according to the Methods. Letters to the left of the histograms identify the botryllamide (or control). The numbers indicate the fold increase in intracellular fluorescence increase in the presence of the botryllamide (or FTC) compared to DMSO control.**

mediated BODIPY-prazosin transport. Intracellular fluorescence of BODIPY-prazosin was 3.4-fold higher in the presence of FTC *versus* in the absence of inhibitor. All of the botryllamides except H and J showed substantial activity (2.3- to 3.3-fold increased fluorescence). On the basis of their ability to cause accumulation of ABCG2 substrates, botryllamides A−G, I, and J appear to be ABCG2 inhibitors. Botryllamide H was inactive in both the screening assay and the flow cytometry assay. Although C was inactive in the screening assay, it was active in the flow cytometry assay. Botryllamide J was found to be active in the screening assay but was marginally active in the BODIPY-prazosin assay. The combination of two different drug efflux assays thus identified seven ABCG2-active botryllamides.

Interaction with the ABCG2 protein by the botryllamides was also confirmed by two biochemical assays. First, the botryllamides were tested for their ability to inhibit the photolabeling of ABCG2 with [$^{125}$I]-IAAP (a prazosin analogue) in membranes isolated from

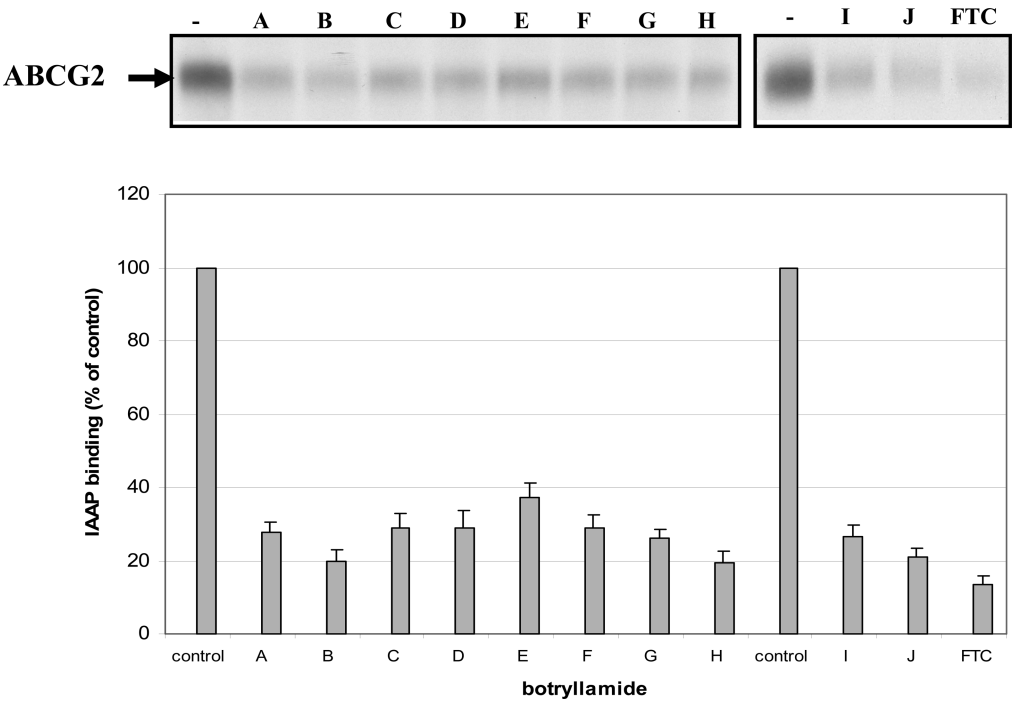


**Figure 4. Photoaffinity labeling of ABCG2 with [$^{125}$I]-IAAP. Crude membranes (1 mg protein mL$^{-1}$) from ABCG2-expressing MCF-7 FLV1000 cells were incubated with 20 μM of indicated botryllamides for 10 min at 21−23 °C in 50 mM Tris-HCl, pH 7.5. Then 3−6 nM [$^{125}$I]-IAAP (2200 Ci mmol$^{-1}$) was added and incubated for an additional 5 min under subdued light. The samples were illuminated with a UV lamp (365 nm) for 10 min at RT. The labeled ABCG2 was immunoprecipitated as described previously (*23*). Samples were separated on a 7% Tris-acetate gel at constant voltage, and gels were dried and exposed to X-ray film for 12−24 h at −80 °C. The incorporation of [$^{125}$I]-IAAP into the ABCG2 band was quantified using the STORM 860 phosphor imager system (Molecular Dynamics, Sunnyvale, CA) and the software ImageQuaNT, as described (*23*). Error bars represent SD.**

ABCG2-overexpressing MCF-7 FLV1000 cells. Previously, IAAP has been used as a photolabel to characterize the drug binding sites of ABC transporters such as ABCG2 (*23*). For this assay, membrane protein isolated from MCF-7 FLV1000 cells was incubated (under low light conditions) with [$^{125}$I]-IAAP in the presence or absence of test or control compounds. Exposure to a UV light source followed, to cross-link the IAAP with the ABCG2 protein. The labeled ABCG2 was then immunoprecipitated and separated by electrophoresis, yielding the band in the control lane in Figure 4. When membrane protein is incubated with a compound known to interact with ABCG2 at the IAAP binding site, such as FTC, it prevents IAAP labeling of the ABCG2 protein, as seen in the FTC lane (Figure 4). As seen in Figure 4, at 20 μM, all of the botryllamides, including those found to be inactive in one or both of the substrate accumulation assays, significantly reduced [$^{125}$I]-IAAP incorporation into ABCG2. [$^{125}$I]-IAAP binding was reduced to 19−37% of control, comparable to that obtained with FTC (14% of control). Although reduced IAAP binding could be due to conformational changes induced by the inhibitor, this assay is generally understood to indicate that decreased binding is due to competition of the inhibitor for the drug-binding site. Thus, all of the botryllamides were found to interact with ABCG2 at the IAAP binding site, regardless of whether they were active in the screening or flow cytometry assays.

The botryllamides were then tested for their ability to stimulate or inhibit ATP hydrolysis of ABCG2 in isolated membranes. All of the botryllamides stimulated ABCG2-mediated ATPase activity in this assay, indicating that all of the compounds directly interact with the protein (Figure 5). Many ABCG2 inhibitors, including FTC, inhibit ABCG2-associated ATPase activity while substrates often stimulate the ATPase activity (*23*). However, there is precedent for stimulation of ATP hydrolysis by ABCG2 inhibitors (for example, curcumin; see ref 24 for discussion). As a result, the stimulation of ATPase activity observed with the botryllamides does not preclude their tentative identification as ABCG2 inhibitors. The data obtained from the biochemical assays clearly indicate that the botryllamides directly interact with this transporter.

In a further strategy, the botryllamides were assessed for their ability to sensitize drug-resistant ABCG2-overexpressing cells to killing by the cytotoxic ABCG2 substrate mitoxantrone. To overcome drug resistance mediated by expression of ABC transporters such as P-gp, MRP1, or ABCG2, nontoxic inhibitors of the proteins are often used to inhibit the activity of the trans-

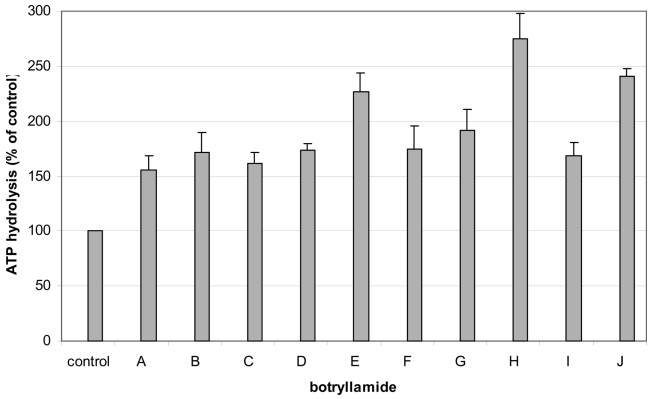


**Figure 5. ATPase assay. Crude membrane protein (100 μg protein $mL^{-1}$) from High-five cells expressing ABCG2 was incubated at 37 °C with 5 μM of indicated botryllamides in the presence and absence of BeFx (0.2 mM beryllium sulfate and 2.5 mM sodium fluoride) in ATPase assay buffer (50 mM KCl, 5 mM $NaN_3$, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT pH 6.8) for 10 min. The reaction was started by the addition of 5 mM ATP and incubated for 20 min at 37 °C. SDS solution (0.1 mL of 5% SDS) was added to terminate the reaction, and the amount of inorganic phosphate released was quantified with a colorimetric reaction, as described previously (*23*). The specific activity was recorded as BeFx-sensitive ATPase activity. Error bars represent SD. $p < 0.01$ *versus* untreated control for all botryllamides and for FTC.**

porters. NCI-H460 MX20 cells are resistant to the cytotoxic effects of mitoxantrone compared to ABCG2-negative parental NCI-H460 cells due to overexpression of ABCG2. As observed in Figure 6, when NCI-H460 MX20 cells were incubated with 10 μM mitoxantrone for 3 days, approximately 88% of the cells were alive compared to cells incubated with vehicle (DMSO). At this concentration of mitoxantrone, approximately 50% of the NCI-H460 parental cells survived compared to cells incubated with vehicle (data not shown). When the NCI-H460 MX20 cells were exposed to mitoxantrone in the presence of a nontoxic concentration of FTC (10 μM), sensitivity to mitoxantrone was restored, resulting in the survival of only 57% of the cells. Figure 6 shows the results of treating NCI-H460/MX20 (*i.e.*, ABCG2-overexpressing) cells with mitoxantrone in the presence or absence of 10 μM botryllamide. Each of the botryllamides, except C, H, and J, had an effect comparable to that of FTC and reduced the number of cells suviving in the presence of mitoxantrone to roughly half (45−55%) of control. As seen in the open bars, none of the botryllamides alone caused appreciable killing of these cells. Although this was not designed to be a stability study, the results also suggest that the botryllamides remain stable in cell culture medium for at least the 3-day period of the assay. The mitoxantrone sensitization experiments provide results with the botryllamides and a third ABCG2 substrate. Given the wide range of chemical classes known to be ABCG2 substrates, it is important to validate new inhibitors using multiple diverse substrates, since variation in the drug binding site has been previously observed for ABCG2 (*17*). PhA (a naturally occurring chlorophyll metabolite), prazosin (a quinazoline derivative), and mitoxantrone (a synthetic anthracenedione) provide this breadth.

Because some botryllamides have been previously reported to have cytotoxic activity and to evaluate their potential as ABCG2 substrates, parental NCI-H460 (ABCG2−) and NCI-H460/MX20 (ABCG2+) cells were exposed to concentrations of up to 100 μM of each botryllamide for 7 d. At 10 μM, none of the botryllamides reduced cell survival by more than 27% (see part A of the Supplementary Figure). At very high concentrations (100 μM; see part B of the Supplementary Figure), botryllamides A, B, D, F, and G showed significant cytotox-

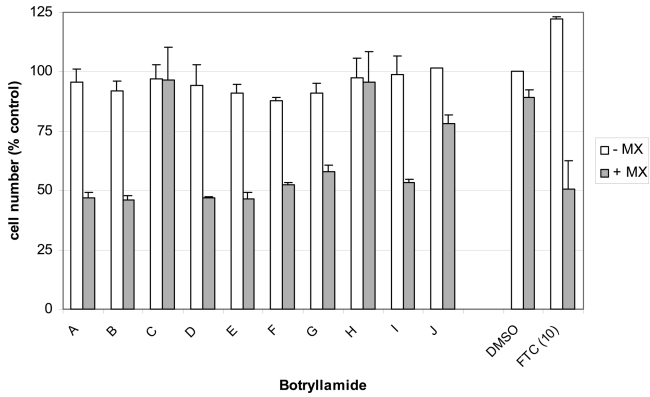


**Figure 6. Mitoxantrone sensitization assay. NCI-H460/MX20 (ABCG2-overexpressing) cells were incubated with 10 μM of the indicated botryllamide, 10 μM FTC, or vehicle (DMSO) in the absence (open bars) or presence (shaded bars) of 10 μM mitoxantrone (MX). Cell numbers were assessed after 3 days by the XTT assay as described (*10*) and presented as % of vehicle (DMSO) control. Bars represent average ± range of two assay plates. Each plate contained duplicate wells for each condition. $p < 0.01$ *versus* untreated control for all botryllamides.**

icity. However, unlike with other cytotoxics that are substrates of transporters, overexpression of ABCG2 had no protective effect, suggesting that, in addition to having relatively low cytotoxicity, the botryllamides are unlikely to be ABCG2 substrates.

On the basis of ABCG2-specific assays, it may be concluded that five of the active botryllamides are likely to be ABCG2 inhibitors. These compounds have the following approximate order of potency based on the PhA assay as confirmed by the BODIPY-prazosin assay and the mitoxantrone resistance reversal assay: G > B > D, F > A. Botryllamide H was found to be inactive in the PhA screen, BODIPY-prazosin, and mitoxantrone resistance reversal assays. Botryllamide C was not particularly active in the PhA screen or the mitoxantrone resistance reversal assay but was active in the BODIPY-prazosin assay. Botryllamide J was active in the PhA assay but was only marginally active in the BODIPY-prazosin and mitoxantrone resistance reversal assays. The flow-cytometry-based BODIPY-prazosin assay includes relatively short (≤2 h total) incubation times in contrast to the initial screening assay and mitoxantrone sensitization assay, which include much longer incubations (18 h to 3 d). It is not unreasonable to assume that the differential activity of some of the botryllamides observed in these assays may be due to relative stability of the compounds in culture medium or inside the cells. The longer incubation times as well as the more directly relevant mitoxantrone sensitization results suggest that only those compounds with activity in these assays may have practical applications in a cellular or *in vivo* environment.

Each of the botryllamides was also tested for its ability to inhibit P-gp and MRP1, other ABC transporters known to confer drug resistance. Many inhibitors of P-gp are also able to inhibit ABCG2 (*21*). Results are shown in Figure 7. P-gp- or MRP1-expressing HEK293 cells were incubated with the fluorescent substrates rhodamine or calcein AM alone (solid line), respectively, or in the presence of 50 μM botryllamide (dashed line). Only botryllamides A and C significantly inhibited P-gp-mediated rhodamine transport (17.6- and 46.4-fold change in fluorescence, compared to 29.7-fold for the positive control, valspodar). By contrast, all of the botryllamides had detectable activity against MRP1-mediated calcein transport (2.0- to 4.5-fold increase in cellular fluorescence). However, only botryllamides A (4.5-fold) and C (3.7-fold) approached the activity of the positive control (5.1-fold). Thus, two of the botryllamides (A and C) showed significant activity against both P-gp and MRP1. Since it had no effect on ABCG2-mediated transport, botryllamide H may be relatively MRP1-selective, although it appears to have low potency. However, it must be noted that high concentrations (50 μM) of botryllamide were required for their inhibitory effects on P-gp or MRP1 and that when the experiment was attempted at lower concentrations (5 μM or less; data not shown) the effect was almost always lost, suggesting that the botryllamides are generally more specific for ABCG2 than for P-gp or MRP1. None of the compounds showed significant specificity for P-gp. Botryllamides E and G appear to be ABCG2-selective since they had minimal effects on P-gp and MRP1.

The botryllamides have several notable structural features, including variations in the bromination patterns, double bond geometry, and methylation. However, none of these features, except possibly the isonitrile group on botryllamides H and J, appear to consistently correlate with activity against ABCG2 and none are predictive of their ability to interact with the ABCG2 protein. In contrast to the lack of structural features associated with ABCG2 activity, an inhibitory effect on P-gp (observed only with botryllamides A and C) appears to require bromination and O-methylation at C-15 as well as *trans* configurations around both double bonds. For example, botryllamide G differs from A and C only in lacking the O-methylation, whereas D differs from C in configuration around the $\Delta^{2,3}$ double bond, and E lacks bromination. Botryllamides D, E, and G were inactive against P-gp. Although these results are only suggestive, it may be possible to isolate or synthesize additional botryllamide analogues with variable relative selectivity and potency to test these hypotheses.

The structures of botryllamides I and J have not been described previously, and thus they represent new members of this class of ascidian metabolites. Botryllamide I is a geometrical isomer of botryllamide E and the only structural difference is the $\Delta^{2,3}$ *E* double bond configuration in I *versus* a $\Delta^{2,3}$ *Z* geometry in E. Botryllamide J is structurally related to botryllamide H. Unlike the other botryllamides, they both lack methylation of the C-2 enol group and contain a relatively rare isonitrile substituent. The $\Delta^{9,10}$ enamine double bond in botryllamide J was assigned as *Z* on the basis of the $^1H-^1H$ coupling constant and NOE evidence. This is the first compound in the botryllamide family with *cis* geometry at this position. The $\Delta^{2,3}$ double bond in botryllamide J

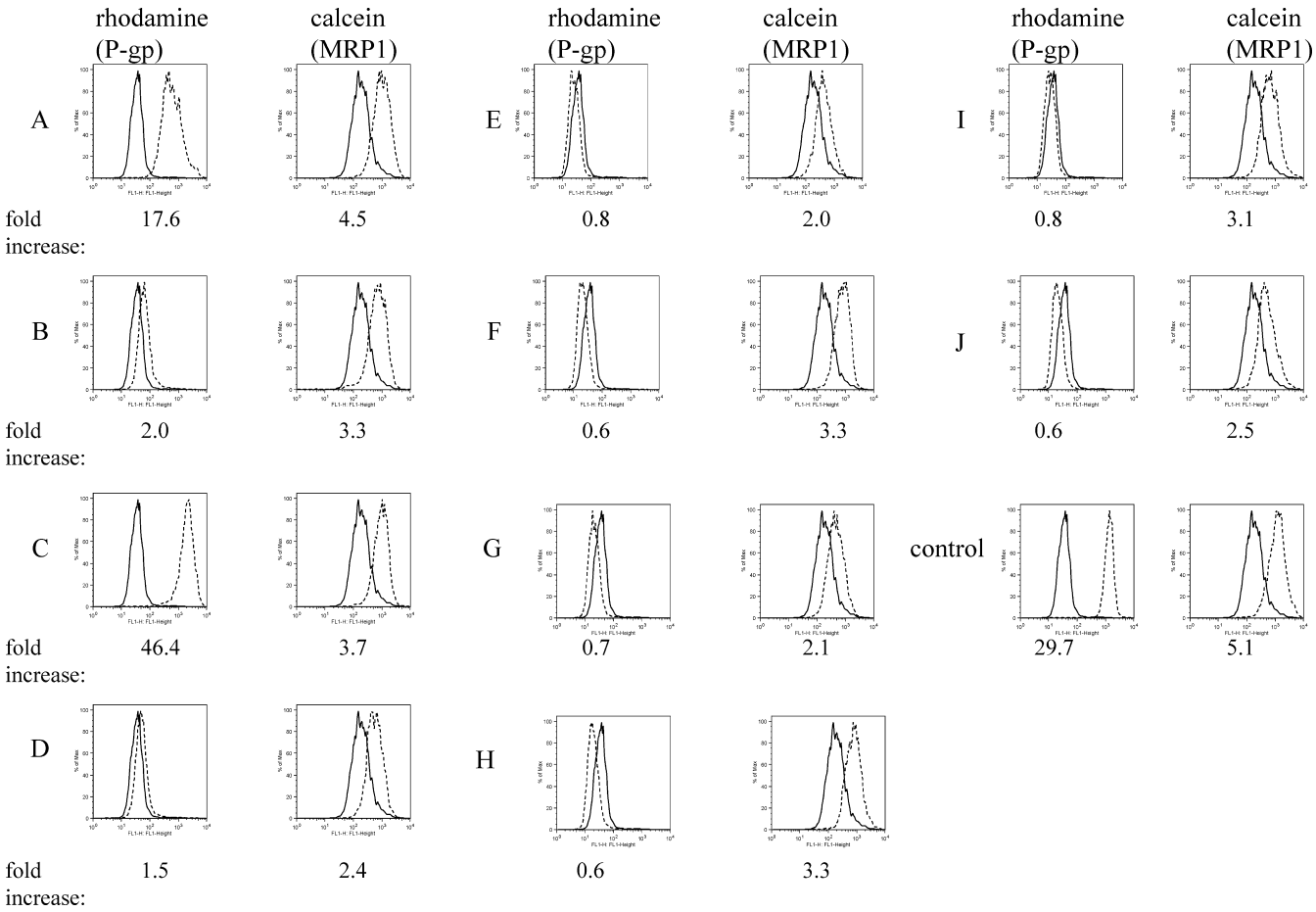


**Figure 7. Assays of botryllamide effects on P-gp and MRP1: P-gp-transfected or MRP1-transfected HEK293 cells were incubated with rhodamine 123 or calcein AM, respectively, in the absence (solid lines) or presence (dashed lines) of 50 μM of the botryllamides according to the Methods section. Intracellular fluorescence was assessed by flow cytometry as described in the Methods section. Botryllamides and positive controls for inhibition of P-gp (valspodar) and MRP1 (MK-571) are identified. The numbers indicate the fold increase in fluorescence in the presence of the botryllamide (or control inhibitor) *versus* in the absence of compound.**

was assigned as *E* on the basis of well established $^{13}C$ chemical shift evidence for C-3 (δ 102.8). Careful analysis of the published spectral data for botryllamide H (*16*) revealed that the chemical shift of C-3 in H (δ 102.3) was characteristic of a $\Delta^{2,3}$ double bond with *E* geometry. The original botryllamide H publication did not provide a rationale for assigning the $\Delta^{2,3}$ geometry, and it now appears that the initial structure of botryllamide H (**8**) needs to be revised to **11**. With regard to biological activity, however, there is nothing noteworthy about either of these compounds in the assays described here.

In summary, novel ABCG2 inhibitors have been identified by screening natural product extracts from the NCI natural products repository. Among the ABCG2 inhibitors that have been previously identified (including tyrosine kinase inhibitors, estrogen agonists and antagonists, curcumins, and other classes of synthetic and natural compounds), the majority lack potency or selectivity or exhibit toxicity. None of the ABCG2 inhibitors identified to date have proven effective in clinical applications. Botryllamides form a unique structural class of diverse inhibitors of drug transport proteins. Synthesis and/or purification of additional members of this class of compounds may lead to a better understanding of structure−activity relationships and the potential for designing botryllamide derivatives with defined specificity

patterns and potency. Given the aforementioned problems with ABCG2 inhibitors, including FTC (*9*), the availability of a new compound class should prove to be very useful for increasing understanding of ABCG2 function and potential as a therapeutic target. Botryllamides could also prove useful in normalizing oral bioavailability of ABCG2 substrate drugs, which in turn could reduce intra- and interpatient variability, thus improving clinical efficacy of ABCG2 substrate drugs. It should be noted that all of the active botryllamides were able to inhibit BODIPY-prazosin efflux and to sensitize ABCG2-overexpressing cells to mitoxantrone to the same extent and at the same concentrations as observed with FTC. Botryllamide G is the most potent and specific of these compounds as an ABCG2 inhibitor and will serve as a starting point for further analogue synthesis. Until such analogues are developed, botryllamide G itself may prove useful in further studies of ABCG2 function and for probing the potential uses of botryllamides as modulators of ABCG2. Although the active botryllamides had relatively low cytotoxicity at concentrations at which they were active, analogue development may allow for further reduction in toxicity. Similarly, the results suggest that it may be possible to discover or develop botryllamides or analogues with MRP1 and/or P-gp selectivity. Given that these compounds showed relatively low cytotoxicity, they may also provide a starting point for development of clinically useful derivatives.

## METHODS

**Materials.** PhA was obtained from Frontier Scientific (Logan, UT). FTC was from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program (DTP), Division of Cancer Treatment and Diagnostics, NCI (Bethesda, MD). Natural product extracts were obtained from the Natural Products Branch of the DTP. Cell culture media were from Invitrogen (Carlsbad, CA), and fetal bovine serum (FBS) was from Hyclone (Kansas City, MO).

**Cell Culture.** NCI-H460 human lung non-small-cell carcinoma cells (NCI, Frederick, MD) were selected for overexpression of ABCG2 by maintenance in RPMI1640/10% FBS supplemented with 20 nM mitoxantrone (MX) and were designated NCI-H460/MX20 (*18*). ABCG2-transfected, ABCB1-transfected (*i.e.*, P-gp-expressing), and ABCC1-transfected (*i.e.*, MRP1-expressing) HEK293 cells were maintained as previously described (*19*).

**Screening Assay for ABCG2 Inhibitors.** Accumulation of PhA, a fluorescent ABCG2 substrate (*20, 21*), formed the basis of the assay for inhibitors of ABCG2 activity (*10*). Briefly, PhA (1 μM final) was added to NCI-H460/MX20 cells immediately followed by extracts (10 μg $mL^{-1}$), compound, or vehicle (DMSO/PBS) control and incubated (18 h). After removal of medium and washing with PBS containing $Ca^{2+}$ and $Mg^{2+}$, fluorescence intensity was read on a fluorescence plate reader in bottom read mode (395 nm excitation, 670 nm emission). Each plate had control wells containing vehicle (DMSO) or 10 μM (final concentration) of FTC. Data were normalized to FTC-treated cells on the same plate and reported as % of FTC fluorescence. Final DMSO concentrations were below 0.2% (v:v).

**Flow Cytometry.** Purified compounds were confirmed for their ability to inhibit ABCG2-mediated transport in a flow cytometry assay using BODIPY-prazosin as a substrate (*19*). The ability of the botryllamides to inhibit P-gp-mediated rhodamine 123 efflux and MRP1-mediated calcein efflux was assessed as previously described (*19, 22*). Briefly, transfected HEK293 cells expressing ABCG2, P-gp, or MRP1 were trypsinized and incubated in complete medium (phenol red-free Richter's medium with 10% FCS and penicillin/streptomycin) containing 200 nM of BODIPY-prazosin, 0.5 μg $mL^{-1}$ of rhodamine 123, or 200 nM of calcein AM, respectively, in the presence or absence of the desired concentration of inhibitor for 30 min at 37 °C. Cells were then washed and incubated in substrate-free medium continuing with or without inhibitor for 1 h. Positive controls for inhibition of ABC transporters were 10 μM FTC for ABCG2, 3 μg $mL^{-1}$ valspodar for P-gp, and 50 μM MK- 571 for MRP1.

Intracellular fluorescence of BODIPY-prazosin, rhodamine 123, or calcein was detected with a FACSort flow cytometer equipped with a 488 nm argon laser and 530 nm bandpass filter. At least 10,000 events were collected. Dead cells were eliminated on the basis of propidium iodide staining.

**Cytotoxicity Assays.** Botryllamides were assessed for their ability to affect NCI-H460 and NCI-H460/MX20 cell growth in the presence or absence of 10 μM mitoxantrone as described (*10*). After 2 days of treatment, cell numbers were assessed using an XTT assay as described (*10*).

**Photoaffinity Labeling of ABCG2 with [$^{125}$I]-IAAP.** ABCG2 expressed in MCF-7 FLV1000 cells was photolabeled with [$^{125}$I]-IAAP as described (*23*). Briefly, crude membranes (1 mg protein $mL^{-1}$) of MCF-7 FLV1000 cells were incubated with 20 μM of the indicated compound for 10 min at RT in 50 mM Tris-HCl, pH 7.5. After addition of 3−6 nM [$^{125}$I]-IAAP (2200 Ci $mmol^{-1}$) (PerkinElmer Life Sciences, Wellesley, MA) samples were incubated for 5 min under subdued light and then exposed to UV (365 nm) light for 10 min. Labeled ABCG2 was immunoprecipitated using BXP-21 antibody. The radioactivity incorporated into the ABCG2 band was quantified using the STORM 860 PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) and ImageQuaNT software (Molecular Dynamics).

**ABCG2-Associated ATPase.** Crude membrane protein (100 μg protein $mL^{-1}$) from High-five cells expressing ABCG2 was incubated at 37 °C with compound (5 μM) in the presence and absence of BeFx (0.2 mM beryllium sulfate and 2.5 mM sodium fluoride) in ATPase assay buffer (50 mM KCl, 5 mM $NaN_3$, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT pH 6.8) for 10 min. The reaction was started by the addition of 5 mM ATP, incubated for 20 min at 37 °C, and terminated by addition of SDS solution (0.1 mL of 5% SDS). Release of inorganic phosphate was quantified with a colorimetric reaction (*23*). The specific activity was recorded as BeFx-sensitive ATPase activity.

**Data Analysis/Statistical Significance.** In each case quantitative data were compared to controls present in the same experiment. $P < 0.05$ was considered to be significant.

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*Supporting Information Available:* This material is available free of charge *via* the Internet at http://pubs.acs.org.

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