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Inhibition of ABCG2-mediated drug efflux by naphthopyrones from marine crinoids

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ABSTRACT

Five new naphthopyrones (**1**–**5**) along with the known compounds TMC-256A1, 5,8-dihydroxy-6-methoxy-2-propyl-4*H*-naphtho[2,3-*b*]pyran-4-one, TMC-256C1, comaparvin, 6-methoxycomaparvin, and 6-methoxycomaparvin 5-methyl ether (**6**–**11**) were isolated from crinoids of the family Comasteridae. All compounds were tested for their ability to inhibit the multidrug transporter ABCG2, which plays a role in drug resistance. Six of the seven angular naphthopyrones showed moderate activity with <60% inhibition of ABCG2-mediated transport as compared to the positive control fumitremorgin C. None of the linear naphthopyrones inhibited ABCG2-mediated efflux.

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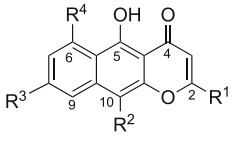
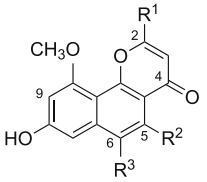
ABCG2 (BCRP/breast cancer resistance protein) is a multidrug transporter belonging to the ATP-binding cassette (ABC) superfamily of membrane proteins. It is one of several cellular transporters that are implicated in the multiple drug resistance (MDR) phenotype which is at least partially responsible for chemotherapeutic drug resistance. ABCG2 is expressed in the liver, small intestine, kidney, colon, brain endothelial cells, hematopoietic stem cells and placenta, which suggests a major role in limiting the bioavailability and penetration of xenobiotics, as well as protecting the blood–brain barrier.[1,2] It is able to efflux a wide range of structurally and mechanistically diverse drugs from cells. Therefore developing inhibitors of ABCG2 potentially offers an opportunity to reverse drug resistance, alter uptake of substrate drugs, improve oral bioavailability, and target stem cells. Many of the known inhibitors of ABCG2 are derived from natural products and the recent identification in our laboratory of a series of botryllamides,[3] a new compound class of ABCG2 inhibitors, prompted us to screen for additional inhibitors of ABCG2.



1: R^1=CH_3, R^2=R^3=OCH_3, R^4=OH
2: R^1=$CH_2CH_2CH_3$, R^2=R^3=OCH_3, R^4=OH
6: R^1=CH_3, R^2=H, R^3=OH, R^4=OCH_3
7: R^1=$CH_2CH_2CH_3$, R^2=H, R^3=OH, R^4= OCH_3



3: R^1=CH_3, R^2=OH, R^3=OCH_3
4: R^1=CH_3, R^2=R^3=OCH_3
5: R^1=$CH_2CH_2CH_3$, R^2=OCH_3, R^3=H
8: R^1=CH_3, R^2=OH, R^3=H,
9: R^1=$CH_2CH_2CH_3$, R^2=OH, R^3=H
10: R^1=$CH_2CH_2CH_3$, R^2=OH, R^3=OCH_3
11: R^1=$CH_2CH_2CH_3$, R^2=R^3=OCH_3

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A high throughput assay measuring accumulation of the ABCG2 specific substrate pheophorbide a (PhA) in ABCG2-overexpressing NCI-H460 MX20 cells was developed[4] and used to screen extracts from the National Cancer Institute's natural products repository. Three of the thirty-five extracts that showed activity in the assay were from the marine crinoids *Capillaster multiradiatus*, *Comanthus parvicirrus* and an unidentified crinoid of the family Comasteridae.[5] While there are no reports in the literature describing chemical constituents of the sea star *Capillaster multiradiatus*, *Comanthus parvicirrus* has been known to produce naphthopyrone compounds.[6] Bioassay-guided fractionation of the three active extracts led to the isolation of five new naphthopyrones (**1–5**) along with the known compounds TMC-256A1 (**6**),[7] 5,8-dihydroxy-6-methoxy-2-propyl-4*H*-naphtho[2,3-*b*]pyran-4-one (**7**),[8] TMC-256C1 (**8**),[7] comaparvin (**9**),[9] 6-methoxycomaparvin (**10**),[6] and 6-methoxycomaparvin 5-methyl ether (**11**).[6] The known compounds were identified by analysis of their spectroscopic data along with comparisons to literature values.

Each crinoid sample was sequentially extracted with 1:1 CH_2Cl_2–MeOH and 100% MeOH to provide the organic solvent extracts. A 2.1 g aliquot of the extract from the as yet taxonomically undefined crinoid was subjected to a solvent–solvent partitioning scheme that concentrated the ABCG2 inhibitory activity into the EtOAc and MeOtBu soluble fractions. The EtOAc fraction was chromatographed on Sephadex LH-20 with 2:5:1 hexane–CH_2Cl_2–MeOH and the active fractions were subjected to C_{18} reversed-phase HPLC eluting with a linear gradient from 75:25 MeOH/H_2O to 100% MeOH, to yield compounds **1** (1.8 mg), **2** (3.2 mg), **3** (2.0 mg), **4** (5.6 mg), **5** (5.8 mg), **8** (2.6 mg), **9** (2.2 mg), and **10** (5.3 mg). The MeOtBu fraction was chromatographed on Sephadex LH-20 with 1:1 CH_2Cl_2–MeOH and further purified by HPLC as described above to yield compound **11** (1.1 mg).

One gram of the crude organic extract of *Comanthus parvicirrus* was subjected to the same partitioning scheme as above with the active EtOAc fraction yielding an additional 5 mg of compound **3**. The MeOtBu fraction was chromatographed on Sephadex LH-20 with 1:1 CH_2Cl_2–MeOH, followed by hexane–CH_2Cl_2–MeOH 2:5:1 to give compounds **8** (12 mg) and **9** (40 mg).

A 1 g aliquot of the extract from *Capillaster multiradiatus* was also subjected to a solvent–solvent partitioning scheme that concentrated the ABCG2 inhibitory activity into the EtOAc soluble fraction. This fraction was chromatographed on Sephadex LH-20 with 1:1 CH_2Cl_2–MeOH. Final purification by C_{18} reversed-phase HPLC as above provided compounds **6** (1.0 mg) and **7** (2.1 mg).

HRFABMS of compound **1**[10] showed a peak at *m/z* 303.0895 $[M+H]^+$ indicative of a molecular formula of $C_{16}H_{14}O_6$ and consistent with 10 double bond equivalents. The ^{13}C NMR (Table 1) and HSQC spectra showed 16 carbon signals including one methyl, two methoxyl groups, three sp^2 methines, and ten quaternary sp^2 carbons, one of which was a carbonyl. The ^1H NMR spectrum showed signals for one singlet methyl (δ 2.41), two methoxyls (δ 3.84, 3.85), one singlet aromatic proton (δ 6.14), two *meta*-coupled aromatic protons (δ 6.45, 6.87, J = 2.0 Hz), and two phenolic hydroxyl protons (δ 10.41, 14.56), one of which was intramolecularly hydrogen-bonded. The NMR spectra of **1** were very similar to those of compound **6** which was identified as the known naphthopyrone TMC-256A1 by comparison with literature values.[7]

The difference in the NMR spectra between **1** and **6** was the observation of ^{13}C and ^1H resonances (δ 60.6 and δ 3.84) in **1** which were consistent with an additional methoxyl substituent. Analysis of the HMBC spectrum of **1** established the location of the hydroxyl and methoxyl groups. An HMBC correlation from the hydroxyl proton at δ 14.56 to C-4a (δ 102.2), C-5 (δ 157.9) and C-5a (δ 106.5), along with the sharp ^1H signal, established that a hydroxyl group was substituted at C-5 and that it was hydrogen-bonded to a nearby carbonyl. A methoxyl group at δ 3.85 was positioned on C-8 by HMBC correlations to C-8 (δ 161.1) and a four-bond correlation to C-7 (δ 97.7). An HMBC correlation from δ 3.84 to C-10 (δ 130.2) placed the remaining methoxyl group on C-10. A NOE interaction between the protons at δ 14.56 and δ 10.41 placed the remaining hydroxyl group on C-6 (δ 160.3).

The molecular formula of compound **2**[11] was established as $C_{18}H_{18}O_6$ by HRFABMS (*m/z* 331.1174 $[M+H]^+$). The NMR and UV spectra were very similar to the naphthopyrone core of compound **1** and additional ^{13}C (δ 13.3 and δ 19.6) and ^1H methylene resonances (δ 0.98 and δ 1.73) were attributed to a propyl substi-

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR spectral data for compounds **1–5** (DMSO-d_6, δ in ppm)

Position	1		2		3		4		5	
	^{13}C δ	^1H δ, (mult, J Hz)	^{13}C δ	^1H δ, (mult, J Hz)	^{13}C δ	^1H δ, (mult, J Hz)	^{13}C δ	^1H δ, (mult, J Hz)	^{13}C δ	^1H δ, (mult, J Hz)
2	168.2		170.9		167.5		163.1		165.5	
3	106.5	6.14, s	106.1	6.13, s	109.2	6.42, s	111.6	6.15, s	112.0	6.11, s
4	183.7		183.7		182.5		175.7		175.7	
4a	102.2		102.4		107.6		113.3		110.9	
5	157.9		157.9		145.8		146.0		155.8	
5a	106.5		106.5							
6	160.3		160.3		133.5		142.6		101.5	6.90, s
6a					135.4		134.2		139.4	
7	97.7	6.45 (d, 2.0)	97.7	6.44 (d, 2.0)	95.3	6.91 (d, 1.5)	96.3	6.97 (d, 2.0)	101.5	6.68 (d, 2.0)
8	161.1		161.1		160.2		159.7		159.4	
9	94.9	6.87 (d, 2.0)	94.9	6.87 (d, 2.0)	97.6	6.52 (d, 1.5)	99.6	6.63 (d, 2.0)	97.8	6.48 (d, 2.0)
9a	135.4		135.4							
10	130.2		130.3		159.5		159.3		158.8	
10a	143.4		143.4		103.1		106.6		104.2	
10b					151.5		152.7		156.8	
2-CH_2	20.2	2.41	35.2	2.66 (t, 7.5)	20.0	2.48, s	19.2	2.36, s	13.3	2.60 (t, 7.5)
2-CH_2			19.6	1.73 (hex, 7.5)					19.0	1.78 (hex, 7.5)
2-CH_3			13.3	0.98 (t, 7.5)					34.6	0.95 (t, 7.5)
5-OH		14.56		14.55, s		13.08				
6-OH		10.41		10.39, s						
8-OH						10.4		10.30, s		10.20, s
5-OCH_3							61.4	3.77, s	55.6	3.83, s
6-OCH_3					59.5	3.84, s	60.8	3.85, s		
8-OCH_3	55.8	3.85	55.8	3.85, s						
10-OCH_3	60.6	3.84	60.7	3.84, s	56.1	3.94, s	56.2	3.92, s	55.9	3.90, s

Table 2
Effects of naphthopyrones (**1**–**11**) on PhA accumulation

Compounds	Maximum activity, % FTC[a]	IC_{50}[b], μM
3	59.3 (±1.4)	11.9 (±1.8)
5	46.3 (±1.2)	5.9 (±0.5)
8	31.0 (±1.0)	12.8 (±0.4)
9	54.3 (±2.3)	9.0 (±0.8)
10	27.5 (±3.5)	>20[c]
11	44.0 (±8.0)	16.6 (±4.1)
1, **2**, **4**, **6**, **7**	na	na

[a] Maximal activities and IC_{50} values for inhibition of PhA accumulation were determined from dose–response curves (na = not active).
[b] 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 (three plates, duplicate wells per plate).
[c] Highest concentration tested.

tution on C-2 (δ 170.9). This was supported by an HMBC correlation from H-3 (δ 6.13) to the methylene carbon at δ 35.2, and from the methylene proton at δ 2.66 to C-2 (δ 170.9) and C-3 (δ 106.1).

Compound **3**[12] had the same molecular formula of $C_{16}H_{14}O_6$ (*m/z* 303.0868 $[M+H]^+$) as compound **1** by HRFABMS but differed in the UV, ^{13}C and ^1H spectra. UV differences between linear and angular naphthopyrones are well documented[13] and absorptions at 245, 285 and 370 nm for **3** indicated an angular naphthopyrone. This was also shown by the upfield shift of the C-5 OH proton signal at δ 13.08 compared to δ 14.56 for the linear naphthopyrone **1**. The hydroxyl group was positioned on C-5 by HMBC correlations to C-5 (δ 145.8) and C-4a (δ 107.6), and an HMBC correlation between the methoxyl resonance at δ 3.84 to C-6 (δ 133.5) established the presence of a methoxyl group at C-6. A literature search allowed us to determine that **3** differed from the known compound **10** by the substitution of a methyl group on C-2 (δ 167.5).[6]

The molecular formula for compound **4**[14] was established as $C_{17}H_{16}O_6$ by a HRFABMS measurement of *m/z* 317.1092 $[M+H]^+$ which differed from **3** by the addition of 14 Da. The NMR and UV data for **4** corresponded closely to those of **3** except for the loss of a signal at δ 13.08 characteristic of a hydroxyl proton and the appearance of an additional methoxyl (δ 3.77 and δ 61.4). An HMBC correlation between the methoxyl resonance at δ 3.77 to C-5 (δ 146.0) established the presence of the methoxyl group at this position.

Compound **5**[15] also showed characteristic UV signals at 240, 275 and 360 nm indicative of an angular naphthopyrone and had NMR data similar to **9**. HRFABMS measurement of a peak corresponding to $[M+H]^+$ (*m/z* 315.1199) established a molecular formula of $C_{18}H_{18}O_5$ for compound **5**. This differed from the known compound **9** by the addition of 14 Da. An HMBC correlation from an additional methoxyl group (δ 3.83 and δ 55.6) to C-5 (δ 155.8) showed the replacement of a hydroxyl group at this position and accounted for the 14 amu difference.

A high throughput assay measuring accumulation of the ABCG2 substrate PhA in ABCG2-overexpressing NCI-H460 MX20 cells was used for bioassay-guided isolation of the compounds.[4] The known inhibitor, fumitremorgin C (FTC, 1 μM) was used as a positive control and data were normalized to FTC and reported as the percentage of FTC induced fluorescence (Table 2).

Although none of the linear naphthopyrones tested inhibited ABCG2-mediated transport, inhibition of Pgp-mediated drug efflux by linear naphthopyrones has been previously reported.[16] Of the seven angular naphthopyrones tested, six showed weak to moderate activity varying from 27% to 59% of that obtained with the positive control, FTC. Compound **4** did not show activity even though it only differs from **11** by the substitution of a methyl instead of propyl group on C-2. This loss of activity due to a minor substitution was also observed in the botryllamide class of compounds.[3] Although the naphthopyrones did not show strong inhibition of ABCG2-mediated transport they reinforced the emerging role of natural products in the development of inhibitors/modulators of multidrug resistance.

Acknowledgments

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10. Compound (**1**): $[\alpha]_D^{25}$ −36.6 (*c* 0.18, MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (3.95), 280 (4.38), 415 (3.58) nm; ^1H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 303.0895 $[M+H]^+$ (calcd for $C_{16}H_{15}O_6$, 303.0869).
11. Compound (**2**): $[\alpha]_D^{25}$ −32.9 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (4.32), 280 (4.38), 415 (3.58) nm; ^1H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 331.1174 $[M+H]^+$ (calcd for $C_{18}H_{19}O_6$, 331.1182).
12. Compound (**3**): $[\alpha]_D^{25}$ −7.88 (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ϵ) 245 (4.04), 285 (3.81), 370 (3.21) nm; ^1H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 303.0868 $[M+H]^+$ (calcd for $C_{16}H_{15}O_6$, 303.0869).
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14. Compound (**4**): $[\alpha]_D^{25}$ −62.0 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ϵ) 234 (4.23), 275 (4.15), 365 (3.68) nm; ^1H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 317.1092 $[M+H]^+$ (calcd for $C_{17}H_{17}O_6$, 317.1026).
15. Compound (**5**): $[\alpha]_D^{25}$ −16.8 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ϵ) 240 (4.78), 275 (4.63), 360 (4.04) nm; ^1H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 315.1199 $[M+H]^+$ (calcd for $C_{18}H_{19}O_5$, 315.1232).
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