European Journal of Medicinal Chemistry 122 (2016) 408–418

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

2-Indolylmethylenebenzofuranones as first effective inhibitors of ABCC2

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ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form
18 June 2016
Accepted 20 June 2016
Available online 27 June 2016

Keywords:
ABC transporters
ABCC2 inhibitors
Indolylmethylenebenzofuranone
Aurones
Drug interactions

ABSTRACT

ABC-transporters play a vital role in drugs bioavailability. They prevent intracellular accumulation of toxic compounds, rendering them a major defense mechanism against harmful substances. In this large family, ABCC2 is an apical efflux pump representing about 10% of all membrane proteins in liver and small intestine, and up to 25% in colon. In these tissues, ABCC2 plays a major role in the pharmacokinetics and pharmacodynamics of endo- and xenobiotics. To gain insight in the function of this crucial protein, we have investigated and developed the first effective inhibitors of this pump. Firstly, we set up a cellular flow cytometry assay for monitoring the drug efflux carried out by ABCC2, and used it for the screening of chemical libraries derived from several chemical classes. We found that 2-indolylmethylenebenzofuranone derivatives as promising candidates. Optimization of the hits provided new compounds that inhibit ABCC2 in the micromolar range, making them the first potent ABCC2 inhibitors reported so far. Such compounds would constitute valuable tools to further investigate the role of ABCC2 in the pharmacokinetics and pharmacodynamics of drugs.

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1. Introduction

ABC (ATP-binding cassette) transporters are responsible for the ATP-dependent movement of a wide variety of xenobiotics, including drugs, lipids and metabolic products across the plasma and intracellular membranes [1,2]. They are highly expressed in major pharmacological barriers [3] and exercise an important influence on the absorption, distribution, and/or elimination of drugs and other xenobiotics [4]. A well-defined role in the transport of clinically relevant drugs was especially described for the ABCB1 (P-gp), ABCC1-5 (MRP 1–5) and ABCG2 (BCRP) [5]. Their contribution to multidrug resistance (MDR) in tumor cells is well documented [6], making them privileged targets to tackle MDR. These efflux pumps are also involved in drug-drug interactions. Numerous examples of co-administration of an ABCB1 inhibitor with an ABCB1 substrate showed that there is a risk of increased blood level of the latter and serious side effects. Such drug-drug interactions are known for verapamil (dronedarone, quinidine, ranolazine), loperamide (tipranavir, ritonavir), saquinavir (tipranavir, ritonavir) and interactions with elacridar (GF120918) [7].

Initially identified as the first canalicular multispecific organic

Abbreviations: ABCC2, second member of the C subfamily of the ABC pumps; DDI, drug-drug interactions; P-gp, P-glycoprotein; MRP, multidrug resistance protein; BCRP, breast cancer resistance protein; MDR, multidrug resistance; CCR2, CC chemokine receptor 2; CCL2, CC chemokine ligand 2; CCR5, CC chemokine receptor 5.

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http://dx.doi.org/10.1016/j.ejmech.2016.06.039
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anion transporter, cMOAT, ABCC2 is the second member of the ABC C-subfamily. It plays an important role in the export of organic anions, unconjugated bile acids and xenobiotics into the bile. Localized on the apical side of polarized cells, ABCC2 is considered the ideal efflux pump in the terminal phase of detoxification [8]. Mutations of the ABCC2 gene are associated with Dubin–Johnson syndrome, a condition in which the lack of hepatobiliary transport of non-bile salt organic anions results in conjugated hyperbilirubinaemia [8,9]. Recent studies showed that ABCC2 is one of the ABC pumps with the highest expression level in organs that are important for the metabolism of endo- and xenobiotics, such as the liver, kidneys and intestine. The high abundance of ABCC2 has been quantified using quantitative proteomics and led to an estimation of about 25% of membrane proteins in colon and10% in small intestine and liver [10].

The high ABCC2 expression suggests that it plays a major role in drug pharmacokinetics and pharmacodynamics that is essential to be investigated. This could be achieved by using efficient inhibitors as preconized by FDA (Food and Drug Administration) and EMA (European Medicines Agency) [11,12]. Up to date, only few ABCC2 modulators, poorly efficient and non-specific are reported [8]. The most efficient ABCC2 inhibitor is cyclosporine A with an IC_{50} of 20 μM, ut it is non selective as it also inhibits ABCB1 and ABCC1 [13]. Therefore, it is mandatory to explore new ways to discover better inhibitors.

Hence, we firstly set up a cellular assay using cell lines overexpressing ABCC2 and flow-cytometry in order to screen chemolibraries. After the evaluation of a library of more than 500 compounds belonging to diverse chemical libraries, we found 2-indolylmethylenebenzofuran-3(2*H*)-ones among the most promising scaffolds (Fig. 1). In the present work, we report the cellular test and the optimization of the hits. The ultimate goal is to set up the structural bases needed for the development of active and selective inhibitors of ABCC2, needed to explore the pathological and clinical role of this protein.

2. Results

2.1. Chemistry

The synthesis of 2-indolylmethylenebenzofuran-3(2*H*)-ones was performed using a previously reported method [14] [15] involving an aldol condensation between indolecarboxaldehydes **2a-s** and 4,6-dihydroxybenzofuran-3(2*H*)-one **1** under basic conditions (Scheme 1) [16].

The 4,6-dihydroxybenzofuran-3(2*H*)-one **1** was first synthesized with 84% yield in two steps, starting from phloroglucinol and chloroacetonitrile in HCl/Et_2O (Scheme 1). The indolecarboxaldehydes **2a**–**s** were obtained by the mean of an alkylation in the presence of sodium hydride 60% and alkyl halide (44–93%). The synthesis and characterization of compounds **3a**, **b**, **d**–**l** and **n** were already described by our group in a precedent study [16].

2.2. Biological evaluation

2.2.1. ABCC2-mediated calcein efflux inhibition

We set up a high-throughput flow-cytometry method to evaluate over 500 compounds of various chemical families covering diverse classes of compounds, some of which were previously shown to be inhibitors of ABC proteins (ABCB1, ABCG2, and ABCC1). The activity of ABCC2 *in cellulo* was monitored by using the ABCC2-transfected MDCKII cells (Madin-Darby Canine Kidney II cells), relative to MDCKII control cells [17] and selected calcein as substrate of the pump as previously reported. The evaluation was conducted in the presence of 1 μM GF 120918 and 25 μM MK-571 as inhibitors of ABCB1 and ABCC1 respectively, since these ABC pumps are also expressed in MDCKII cells and transport calcein [18,19]. Used at 25 nM, calcein accumulation in ABCC2-MDCKII cells was not modified in the presence of GF120918 and MK-571, showing that the difference in calcein accumulation with parental MDCKII cells is only due to the ABCC2 efflux activity [17].

2.2.2. Libraries screen

We tested over 500 compounds belonging to more than 20 families including flavonoid [20], flavones, flavonols, flavanones, flavanols, chromones [21], aurones, chalcones [22], xanthones, flavonoid dimers, quinolones, alkaloids, acridones, thiourea derivatives, diphenyloxazolines, biphenyl imines, steroids and peptide analogues [23], indenoindoles, benzofurans, phthalimides, carbazoles, indazoles, carboxylic acids carbamazones. General classes of compounds included in this study are given in Fig. 2.

These classes of compounds were chosen mostly because most of the chemical scaffolds were previously shown to interact with ABC proteins. Used at 5 μM, the compounds were compared to the reference inhibitor, cyclosporine A used at 25 μM, which corresponds to its IC_{50}. From this large screen, summarized in Fig. 3, it turned out that compounds from the indolylmethylenebenzofuranone family (Fig. 1) displayed the highest inhibition of calcein ABCC2-mediated efflux (orange bars). The structurally close aurones and azaurones did not display any efficacy suggesting a critical role of the indole moiety. Therefore, further investigations were solely focused on indolylbenzofuranone class.

2.2.3. Inhibition efficacy of indolylmethylenebenzofuranone derivatives

The primary screen showing that indolylmethylenebenzofuranones were the most promising compounds prompted us to synthesize a series of analogs and to study their influence on the accumulation of calcein in the MDCKII-ABCC2 cells. The structures and measured IC_{50} of the compounds set are shown in Table 1.

As shown in Table 1, compounds tested at 5 μM were much more efficient inhibitors of ABCC2-mediated calcein efflux than cyclosporine A used at 25 μM. As shown through the IC_{50} values, the compounds efficacies ranged from 4 μM for the most active compounds (**3i**, **3j**, **3m** and **3p**) to 20 μM for the less active ones. The SAR analysis revealed that the substitution at the indolic nitrogen may

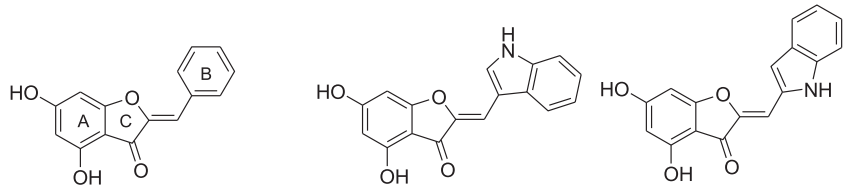
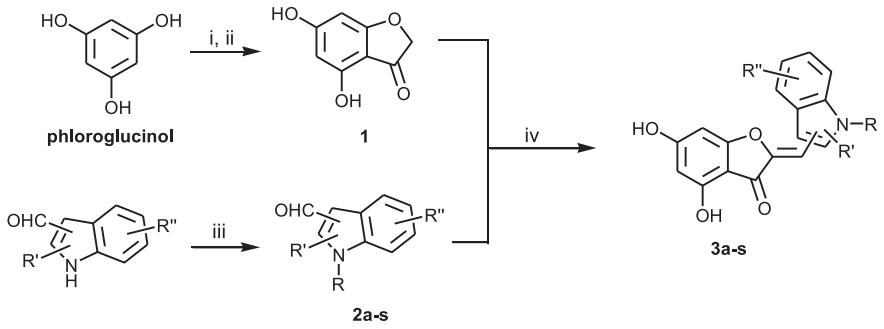


Fig. 1. Structures of aurones and 2-indolylmethylenebenzofuran-3(2*H*)-ones studied as inhibitors of ABCC2.



Scheme 1. General pathway for the synthesis of 2-indolylmethylenebenzofuran-3(2*H*)-ones. Reagents and conditions: (i) $ClCH_2CN$, HCl, $ZnCl_2$, Et_2O, 0 °C; (ii) H_2O, 100 °C, 5 h, 84% (for 2 steps); (iii) RX (X = Br or I), NaH, DMF, rt, 18–72 h, 44–93%; (iv) KOH 50% in H_2O, EtOH, reflux, 2–15 h (see detailed structures in Table 1).

tolerate a panel of substituents with equivalent activity (**3d**, **3e**, **3f**) but some adequacy of size and/or hydrophobicity should be respected (**3f** *vs* **3g** and **3h**). However, the substitution by alkyl chains is more favorable than with aryl groups. Also, we observe that the linkage of the indole to the benzofuranone through its C-3 is slightly more advantageous than C-2. A marked impact of the substitution pattern of the phenyl ring of indole was observed: a substitution at C-6 is more advantageous over C-8 (**3n** *vs* **3l**). It is noteworthy that the influence of the substitution at the phenyl ring of indole is dependent on the substituents at the N-1 (**3p** *vs* **3q-s**). The latter is confirmed by comparing the most active compound of the series **3m** to **3l**.

In order to ensure the effectiveness of our best inhibitors, we checked them in another cell system, using Flp-InTM-293 cells transfected ABCC2 gene, which confirmed the above results (Fig. 4).

2.2.4. Selectivity for ABCC2 versus ABCB1 and ABCC1

In the case of ABC transporters, it is not trivial to address the specificity issues, since this family of proteins is characterized by substrates and inhibitors overlapping. This is particularly exacerbated in the case of ABCC2 for which the best reported inhibitor so far, cyclosporine A, is not selective and fully inhibits much more efficiently ABCB1- and ABCC1-mediated calcein efflux, at lower concentrations (2.5 μM and 25 μM, respectively) (Fig. 5).

We tested our compounds series on both transporters. As shown in Fig. 5, among the pool of the most efficient ABCC2 inhibitors, **3m**, **3i**, **3p** and **3j**, displayed a higher selectivity towards calcein ABCC2-mediated efflux, with **3p** being the more selective. Three other compounds, **3c**, **3b** and **3r**, although less efficient towards ABCC2 than **3p**, displayed a higher selectivity towards ABCC2 versus ABCB1 and ABCC1. At this point, it is however not possible to draw a clear and rational structure-activity relationship to address the selectivity issues. Such RSA could be performed in a further study by analyzing large series and diversely substituted compounds, especially those bearing concomitant substituents at the indolic nitrogen and at the indole phenyl ring.

2.2.5. Innocuity of ABCC2 inhibitors on cell survival

The cellular cytotoxicity of the most potent ABCC2 inhibitors was evaluated using the MTT assay. As shown in Fig. 6, compounds, **3m**, **3i**, **3p** and **3j** were not at all -or poorly-cytotoxic for MDCKII WT and MDCKII ABCC2 cells, displaying $IC_{50} > 50$ μM. Particularly, the most promising compound, **3p**, displayed no visible cytotoxicity over the range of tested concentrations.

3. Discussion and conclusion

Despite the high abundance of ABCC2 in apical membranes, its precise role remains unclear [38]. This is partly because only few validated cells expressing a functional form of the pump [39] are available, and to the lack of potent and specific ABCC2 inhibitors [8,40]. In order to achieve the latter goal, we set up a cell-based model using the MDCKII cells to evaluate ABCC2-mediated transport and adapted it for high-throughput flow-cytometry screening, then screened a chemolibrary of more than 500 compounds that regroups various classes of candidates and some of which proved to be modulators for other ABC transporters, such as ABCB1, ABCC1 and ABCG2 [21,41,42].

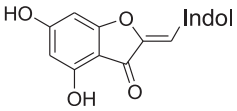
Our initial screen of different chemolibraries allowed us to identify few indolylmethylenebenzofuranones as potential inhibitors of ABCC2. These compounds are considered as structural analogs of the naturally occurring aurones (*Z*)-2-benzylidenebenzofuran-3-(2*H*)-ones. Aurones are flavone isomers found in vegetables and especially in fruits and flowers, where they contribute to the bright yellow color. Probably due to their scarce occurrence in nature, the medicinal chemistry of aurones is quite recent but in constant progress [43–45].

Regarding selectivity for ABCC2 inhibitors, three inhibitors and particularly **3p**, displayed a higher inhibition selectivity towards ABCC2 than ABCC1 and ABCB1, showing that ABCC2 can be specifically targeted although the substrate and inhibitor overlaps with the two other pumps. However, this does not exclude a reactivity of these compounds towards other transporters not tested in the present study. The selectivity is not yet complete but this result is encouraging for the development of a next generation of compounds. The low toxicity of the identified inhibitors is also encouraging and stimulates further investigations in order to enhance efficacy and selectivity. The robust cellular assay designed within this work and the easy access to indolylmethylenebenzofuranones are precious elements to pursue the investigation of ABCC2 inhibitors.

As chemical tools, these inhibitors can help to gain insight into the role of this highly expressed transporter in liver, kidneys and intestine. They can be used to investigate drug pharmacokinetics and pharmacodynamics for which the role of ABCC2 transport is documented. Used in this context, to avoid clinical complications, the purpose of the inhibitors is not to block long-term the protein, but rather in controlled rounds.

While ABCC2 is highly expressed in organs playing key roles in drug metabolism, surprisingly little is known about this protein and its impact on drug pharmacokinetics, pharmacodynamics. Being highly expressed in liver and knowing that more than 700 drugs are associated with drug-induced liver injury [46], our ABCC2 inhibitors can serve as tools to build more convenient and practical approaches to predict drug-induced liver injury and drug-drug interactions due to the implication of this pump.

Table 1
ABCC2-mediated calcein transport inhibition efficacy (IC_{50}) of indolylmethylene-benzofuranone for the ABCC2 inhibitors in MDCKII cells.



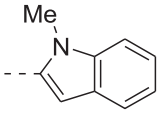
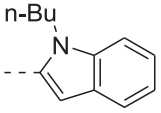
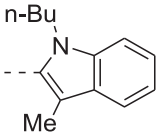
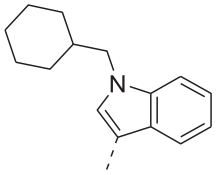
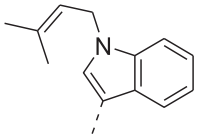
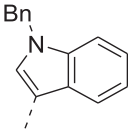
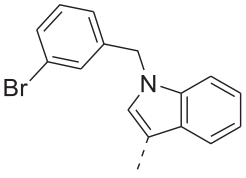
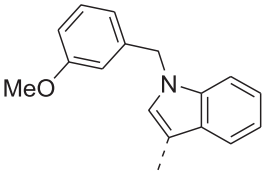
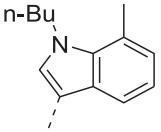
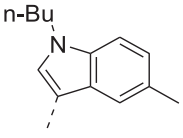
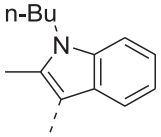
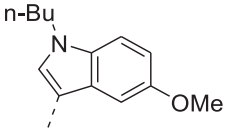
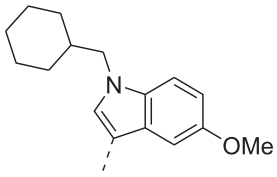
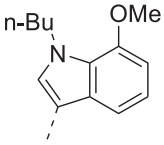
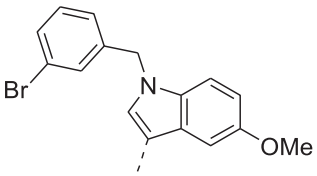
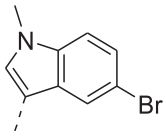
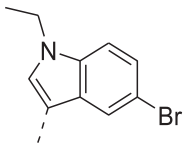
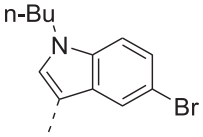
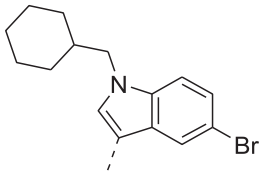
Entry	Compound[a]	Indol moiety	IC_{50} (μM)
1	**3a**		11.1 ± 0.7
2	**3b**		9 ± 2.2
3	**3c**		8.2 ± 0.9
4	**3d**		10.5 ± 1.1
5	**3e**		10.6 ± 1.2
6	**3f**		12.2 ± 1.2
7	**3g**		14.4 ± 1.4
8	**3h**		17.5 ± 1.8
9	**3i**		4.5 ± 0.9
10	**3j**		4.8 ± 0.6

Table 1 (*continued*)

Entry	Compound[a]	Indol moiety	IC_{50} (μM)
11	**3k**		11.4 ± 0.7
12	**3l**		13.9 ± 1.6
13	**3m**		4.1 ± 0.8
14	**3n**		6.3 ± 0.2
15	**3o**		7.3 ± 0.7
16	**3p**		4.5 ± 0.58
17	**3q**		17.8 ± 1.8
18	**3r**		15.4 ± 2
19	**3s**		15.8 ± 1.6
20	Cyclosporine A		25.6 ± 1.4

[b] IC_{50} (mean) = mean value of compound concentration needed to inhibit 50% of calcein transport. Data are the mean ± SD of 3 independent experiments.
[a] Dashed line indicates the site of linkage to the benzofuranone moiety.

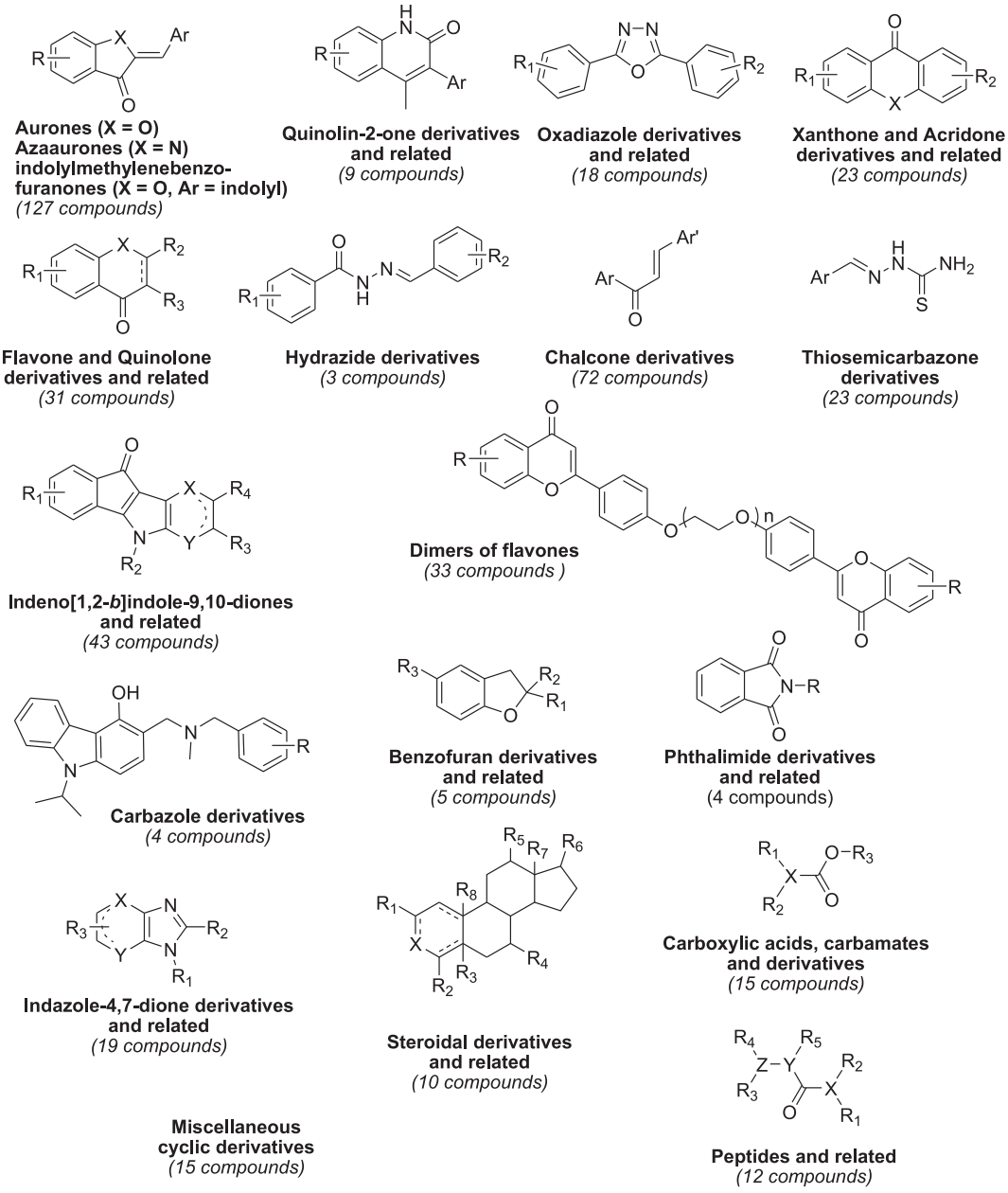


Fig. 2. Main classes of compounds tested in the study. The graphic representation includes the general structure, the name of the class and the number of compounds screened. We included literature references for molecules that were previously published [24–37].

4. Experimental section

4.1. Chemistry

Commercially available reagents and solvents were used without further purification. Reactions were monitored by thin-layer chromatography (plates coated with silica gel 60 F_{254} from Merck). Products were purified with column chromatography on Silica gel 60 (230–400 mesh from Macherey-Nagel) or by automatic flash chromatography with the Grace device: Reveleris X2. Melting points were measured on a Büchi B540 apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded at room temperature in deuterated solvents on a Brüker AC-400 instrument (400 MHz). Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS as internal standard or relative to the solvent [^{1}H: δ(DMSO-d_6) = 2.50 ppm, δ($CDCl_3$) = 7.24 ppm, δ(acetone-d_6) = 2.05 ppm; ^{13}C: δ(DMSO-d_6) = 39.51 ppm, δ($CDCl_3$) = 77.23 ppm, δ(acetone-d_6) = 29.84 ppm]. Electrospray ionization ESI mass spectra were acquired by the Analytical Department of Grenoble University on an Esquire 3000 Plus Bruker Daltonis instrument with a nanospray inlet. Accurate mass measurements (HRMS) were carried out on a ESI/QTOF with the Waters Xevo G2-S QTof device. The purity of compounds was determined by HPLC (Agilent 1100 Series HPLC): all tested compounds have a purity $\geq$ 95%.

4.1.1. General procedure A for the synthesis of N-alkyl-indole-3-carboxaldehyde (**3c**, m, o–s)

To a suspension of NaH 60% in oil (2.25 equiv.) in dry DMF (0.8 mL/mmol) was added, at 0 °C and under nitrogen atmosphere,

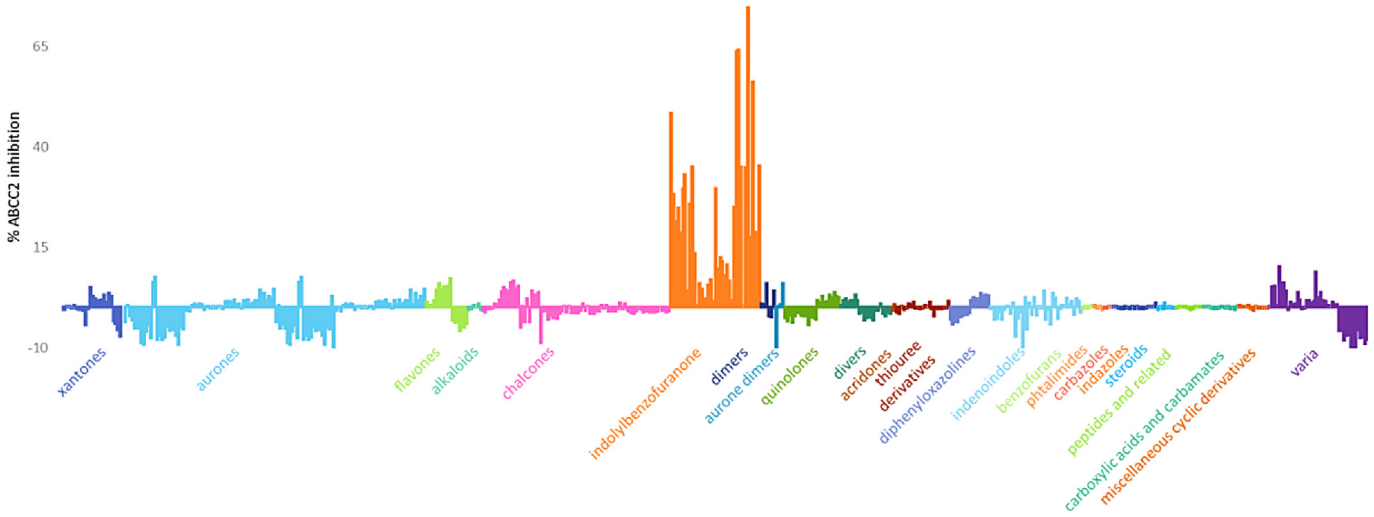


Fig. 3. Initial screening of ABCC2-mediated calcein transport inhibitors. MDCKII WT and ABCC2 cells were incubated with 5 μM compounds. Inhibition values were calculated as described in materials and methods. Negative values are due to normal accumulation differences in the ABCC2-transfected cells (less calcein accumulated after treatment with compounds).

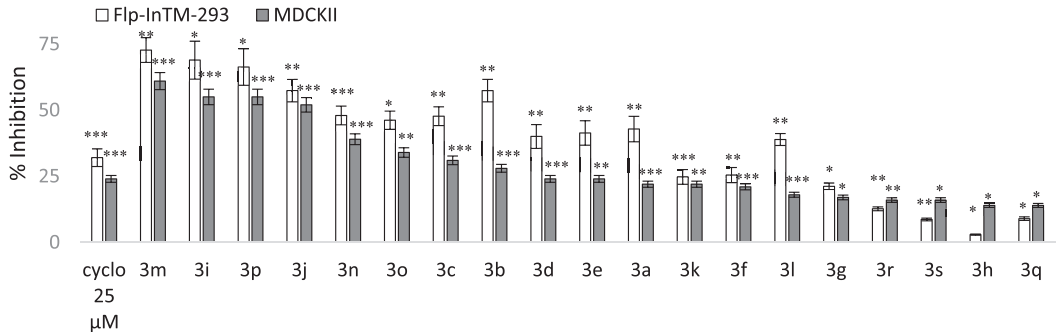


Fig. 4. Calcein transport inhibition (%) by 5 μM compounds of ABCC2 expressed in Flp-InTM-293 (white bars) and MDCKII (grey bars) cells.

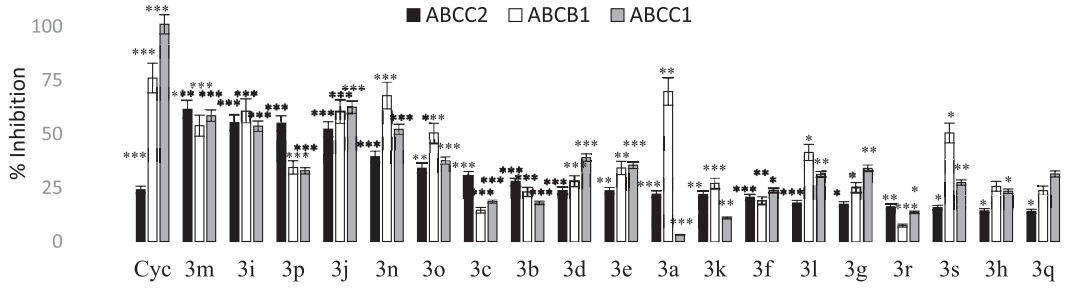


Fig. 5. Calcein transport inhibition of the human MDR ABC transporters, ABCB1 and ABCC1, by compounds of the study. Compounds were tested at 5 μM on cells expressing each transporter and the corresponding control: ABCC2 MDCKII WT and MDCKII ABCC2 (black bars), NIH3T3 WT and NIH3T3 ABCB1 (white bars), BHK21 WT and BHK21 ABCC1 (grey bars). Cyclosporine A (cyc) was used as reference compound, at 25 μM for ABCC2 and ABCC1 and 2.5 μM for ABCB1 (*** – $p < 0.001$; ** – $p < 0.01$; * – $p < 0.05$).

a solution of indolecarboxaldehyde (1 equiv.) in dry DMF (2.5 mL/mmol). After stirring for 30 min at rt, alkyl halide (1.0–3.0 equiv.) was slowly added. After stirring overnight, the reaction was quenched by addition of water and the product was extracted with diethyl ether. The organic layer was dried over $MgSO_4$, filtered off and concentrated under vacuum. The crude product was purified by column chromatography on silica gel.

*4.1.1.1. N-Butyl-3-methylindole-2-carboxaldehyde (**2c**).* The crude product was prepared according to general procedure A starting from 3-methylindole-2-carboxaldehyde (199 mg, 1.25 mmol) and bromobutane (377 mg, 2.75 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 98:2 to 96:4), the pure product (120 mg, 0.56 mmol, 44%) was obtained as a yellow oil. $R_f = 0.17$ (95:5 cyclohexane/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 10.14 (s, 1H), 7.68 (dd, $J = 8.0$, 1.0 Hz, 1H), 7.39 (ddd, $J = 8.5$, 6.8, 1.0 Hz, 1H), 7.33 (dd, $J = 8.5$, 1.1 Hz, 1H), 7.13 (ddd, $J = 8.0$, 6.8, 1.1 Hz, 1H), 4.50 (t, $J = 7.4$ Hz), 2.62 (s, 3H), 1.67–1.77 (m, 2H), 1.29–1.40 (m, 2H), 0.93 (t, $J = 7.4$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 181.3 (CH), 139.3 (C), 130.9 (C), 127.3 (CH), 127.1 (C),

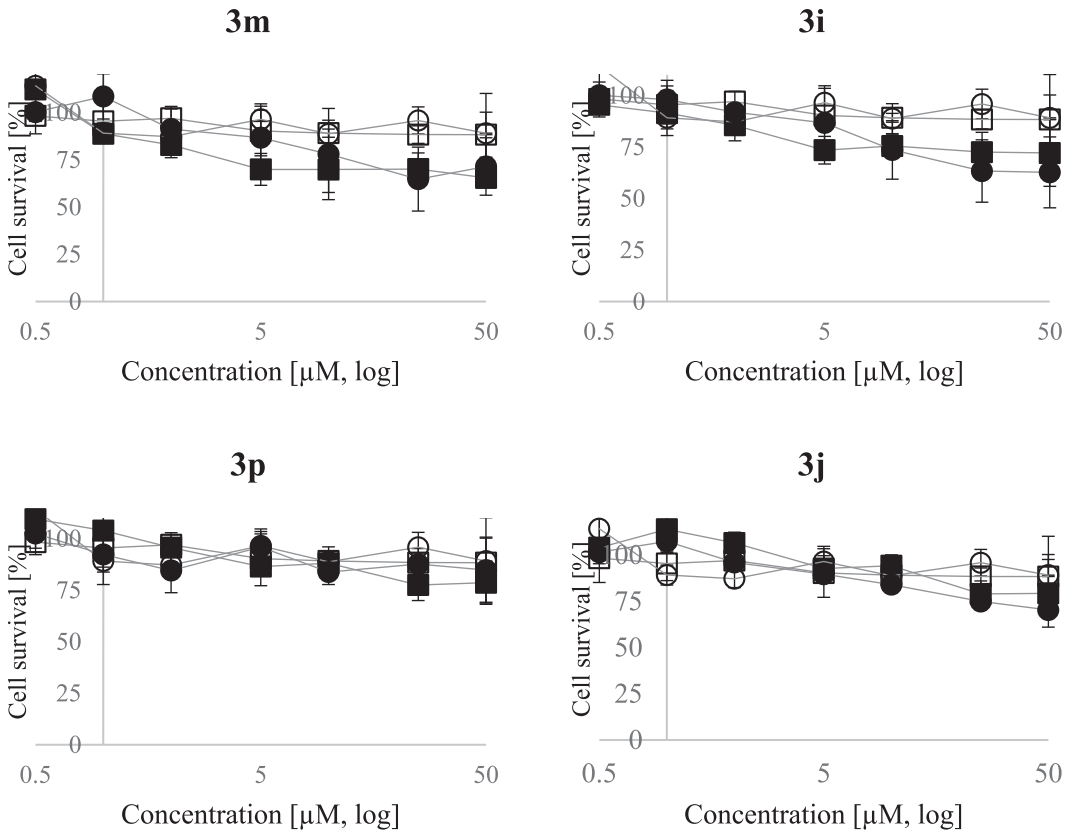


Fig. 6. Safety of compounds towards MDCKII WT and MDCKII ABCC2 cells (black symbols), added to WT (circles) or ABCC2 (squares) cells. Negative controls were carried out in parallel by replacing compounds by DMSO (open symbols) at the corresponding compound concentrations.

126.8 (C), 121.5 (CH), 120.1 (CH), 110.6 (CH), 44.6 (CH_2), 32.9 (CH_3), 20.3 (CH_2), 14.0 (CH_2), 8.7 (CH_3); LRMS (ESI+) *m/z* (%) 216 (100) $[M+H]^+$; HRMS (ESI+) *m/z* calc.for $C_{14}H_{18}NO$ 216.1388 $[M+H]^+$, found 216.1388.

*4.1.1.2. N-(Cyclohexylmethyl)-5-methoxyindole-3-carboxaldehyde (**2m**).* The crude product was prepared according to general procedure A starting from 5-methoxyindole-3-carboxaldehyde (240 mg, 1.37 mmol) and bromomethylcyclohexane (487 mg, 2.75 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 7:3), the pure product (344 mg, 1.27 mmol, 93%) was obtained as a white solid. R_f = 0.39 (7:3 cyclohexane/EtOAc); m.p. 69–71 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.92 (s, 1H), 7.77 (d, J = 2.5 Hz, 1H), 7.58 (s, 1H), 7.22 (d, J = 8.9 Hz, 1H), 6.93 (dd, J = 8.9, 2.5 Hz, 1H), 3.92 (d, J = 7.2 Hz, 2H), 3.87 (s, 3H), 1.78–1.90 (m, 1H), 1.56–1.76 (m, 5H), 1.10–1.24 (m, 3H), 0.92–1.04 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 184.6 (CH), 156.8 (C), 139.2 (CH), 132.6 (C), 126.3 (C), 117.8 (C), 114.6 (CH), 111.4 (CH), 103.5 (CH), 56.0 (CH_3), 54.2 (CH_2), 38.6 (CH), 31.1 (2xCH_2), 26.3 (CH_2), 25.8 (CH_2); LRMS (ESI+) *m/z* (%) 294 (20) $[M+Na]^+$, 272 (100) $[M+H]^+$; HRMS (ESI+) *m/z* calc. for $C_{17}H_{22}NO_2$ 272.1651 $[M+H]^+$, found 272.1654.

*4.1.1.3. N-(3-Bromobenzyl)-5-methoxyindole-3-carboxaldehyde (**2o**).* The crude product was prepared according to general procedure A starting from 5-methoxyindole-3-carboxaldehyde (100 mg, 0.57 mmol) and 3-bromobenzyl bromide (285 mg, 1.14 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 7:3), the pure product (133 mg, 0.39 mmol, 68%) was obtained as a yellow oil. R_f = 0.32 (7:3 cyclohexane/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.91 (s, 1H), 7.78 (d, J = 2.5 Hz, 1H), 7.61 (s, 1H), 7.38–7.42 (m, 1H), 7.28 (dd, J = 1.6, 1.6 Hz, 1H), 7.15 (dd, J = 7.8, 7.8 Hz, 1H), 7.11 (d, J = 8.9 Hz, 1H), 6.99–7.03 (m, 1H), 6.89 (dd, J = 8.9, 2.5 Hz, 1H), 5.22 (s, 2H), 3.84 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 184.7 (CH), 156.9 (CH), 138.7 (C), 137.9 (CH), 132.2 (C), 131.6 (CH), 130.8 (CH), 130.2 (CH), 126.4 (C), 125.7 (CH), 123.3 (C), 118.5 (C), 114.9 (C), 111.3 (CH), 103.6 (CH), 55.9 (CH_2), 50.5 (CH_3); LRMS (ESI+) *m/z* (%) 346 (100) $[M(^{81}Br)+H]^+$, 344 (100) $[M(^{79}Br)+H]^+$; HRMS (ESI+) *m/z* calc. for $C_{17}H_{15}BrNO_2$ 344.0286 $[M+H]^+$, found 344.0278.

*4.1.1.4. N-Methyl-5-bromoindole-3-carboxaldehyde (**2p**).* The crude product was prepared according to general procedure A starting from 5-bromoindole-3-carboxaldehyde (100 mg, 0.45 mmol) and iodomethane (127 mg, 0.89 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 6:4 to 5:5), the pure product (86 mg, 0.36 mmol, 81%) was obtained as a yellowish solid. R_f = 0.31 (5:5 cyclohexane/EtOAc); m.p. 131–134 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.91 (s, 1H), 8.42 (d, J = 1.8 Hz, 1H), 7.62 (s, 1H), 7.40 (dd, J = 8.7, 1.9 Hz, 1H), 7.18 (d, J = 8.7 Hz, 1H), 3.83 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 184.3 (CH), 139.8 (CH), 136.7 (C), 127.2 (C), 126.9 (CH), 124.9 (CH), 117.7 (C), 116.9 (C), 111.5 (CH), 34.1 (CH_3); LRMS (ESI+) *m/z* (%) 240 (100) $[M(^{81}Br)+H]^+$, 238 $[M(^{79}Br)+H]^+$ (95); HRMS (ESI+) *m/z* calc. for $C_{10}H_9BrNO$ 237.9868 $[M+H]^+$, found 237.9862.

*4.1.1.5. N-Ethyl-5-bromoindole-3-carboxaldehyde (**2q**).* The crude product was prepared according to general procedure A starting from 5-bromoindole-3-carboxaldehyde (100 mg, 0.45 mmol) and bromoethane (97 mg, 0.89 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 7:3), the pure product (96 mg, 0.38 mmol, 85%) was obtained as a yellowish

powder. R_f = 0.13 (7:3 cyclohexane/EtOAc); m.p. 113–115 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.84 (s, 1H, CH=O), 8.37 (d, J = 1.9 Hz, 1H, H4), 7.64 (s, 1H, H2), 7.32 (dd, J = 8.7, 2.0 Hz, 1H, H6), 7.15 (d, J = 8.7 Hz, 1H, H7), 4.13 (q, J = 7.3 Hz, 2H, CH_2), 1.49 (t, J = 7.3 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 184.3 (CH=O), 138.2 (C2), 135.7 (C8), 126.9 (C5), 126.9 (C6), 124.7 (C4), 117.5 (C3), 116.5 (C9), 111.6 (C7), 42.2 (CH_2), 15.0 (CH_3); LRMS (ESI+) *m/z* (%) 254 (95) [M(^{81}Br)+H]$^+$, 252 (100) [M(^{79}Br)+H]$^+$; HRMS (ESI+) *m/z* calc. for $C_{11}H_{11}BrNO$ 252.0024 [M+H]$^+$, found 252.0018.

*4.1.1.6. N-Butyl-5-bromoindole-3-carboxaldehyde (**2r**).* The crude product was prepared according to general procedure A starting from 5-bromoindole-3-carboxaldehyde (307 mg, 1.37 mmol) and bromobutane (377 mg, 2.75 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 7:3), the pure product (358 mg, 1.28 mmol, 93%) was obtained as a yellow oil. R_f = 0.21 (7:3 cyclohexane/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.84 (s, 1H), 8.37 (d, J = 1.9 Hz, 1H), 7.61 (s, 1H), 7.31 (dd, J = 8.7, 1.9 Hz, 1H), 7.15 (d, J = 8.7 Hz, 1H), 4.07 (t, J = 7.2 Hz, 2H), 1.74–1.84 (m, 2H), 1.21–1.38 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 184.3 (CH), 139.0 (CH), 135.9 (C), 126.9 (C), 126.9 (CH), 124.7 (CH), 117.4 (C), 116.4 (C), 111.7 (CH), 47.3 (CH_2), 31.8 (CH_2), 20.1 (CH_2), 13.7 (CH_3); LRMS (ESI+) *m/z* (%) 304 (10), 302 (10), 282 (100) [M(^{81}Br)+H]$^+$, 280 (95) [M(^{79}Br)+H]$^+$; HRMS (ESI+) *m/z* calc. for $C_{13}H_{15}BrNO$ 280.0337 [M+H]$^+$, found 280.0338.

*4.1.1.7. N-(Cyclohexylmethyl)-5-bromoindole-3-carboxaldehyde (**2s**).* The crude product was prepared according to general procedure A starting from 5-bromoindole-3-carboxaldehyde (200 mg, 0.90 mmol) and bromomethylcyclohexane (316 mg, 1.80 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 9:1 to 8:2), the pure product (186 mg, 0.58 mmol, 65%) was obtained as a yellowish crystal. R_f = 0.37 (7:3 cyclohexane/EtOAc); m.p. 89–92 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.90 (s, 1H), 8.41 (d, J = 2.0 Hz, 1H), 7.60 (s, 1H), 7.35 (dd, J = 8.7, 2.0 Hz, 1H), 7.18 (d, J = 8.7 Hz, 1H), 3.92 (d, J = 7.3 Hz, 2H), 1.76–1.89 (m, 1H), 1.53–1.76 (m, 5H), 1.05–1.23 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 184.4 (CH), 139.6 (CH), 136.3 (C), 127.0 (CH), 126.9 (C), 124.8 (CH), 117.3 (C), 116.5 (C), 112.0 (CH), 54.0 (CH_2), 38.5 (CH), 31.0 (2xCH_2), 26.2 (CH_2), 25.7 (2xCH_2); LRMS (ESI+) *m/z* (%) 344 (20), 322 (95) [M(^{81}Br)+H]$^+$, 320 (100) [M(^{79}Br)+H]$^+$; HRMS (ESI+) *m/z* calc. for $C_{16}H_{19}BrNO$ 320.0650 [M+H]$^+$, found 320.0663.

*4.1.2. General procedure B for the synthesis of compounds (**3c**, m, o–s)*

To a solution of 4,6-dihydroxybenzofuran-3(2*H*)-one **1** in ethanol (3 mL/mmol) were added an aqueous solution of potassium hydroxide (50%, 5 mL/mmol) and a benzaldehyde derivative (1.0–3.5 equiv.). The solution was refluxed until TLC showed complete disappearance of the starting material (2–5 h). After cooling, ethanol was removed under reduced pressure, then the residue was diluted into distilled water (50 mL/mmol) and an aqueous solution of hydrochloric acid (10%) was added to adjust the pH to 2–3. The mixture was then extracted with ethyl acetate or dichloromethane. The combined organic layers were washed with water and brine, dried over $MgSO_4$, filtered off and concentrated under reduced pressure to afford the corresponding crude (*Z*)-2-benzylidenebenzofuran-3(2*H*)-one derivative.

*4.1.2.1. (Z)-2-(N-Butyl-3-methylindol-2-ylmethylene)-4,6-dihydroxybenzofuran-3(2H)-one (**3c**).* The crude product was prepared according to general procedure B starting from **1** (60 mg, 0.36 mmol) and aldehyde **2c** (100 mg, 0.46 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 5:5) and recrystallization in acetonitrile, the pure product (61 mg, 0.17 mmol, 47%) was obtained as red powder. R_f = 0.18 (96:4 CH_2Cl_2/MeOH); m.p. 197–199 °C; ^{1}H NMR (400 MHz, Acetone-d_6) δ ppm 9.88 (bs, 1H), 9.26 (bs, 1H), 7.60 (dd, J = 8.0, 0.7 Hz, 1H), 7.44 (dd, J = 8.3, 1.0 Hz, 1H), 7.23 (ddd, J = 8.0, 7.1, 1.0 Hz, 1H), 6.83 (s, 1H), 7.07 (ddd, J = 8.3, 7.1, 0.7 Hz, 1H), 6.30 (d, J = 1.5 Hz, 1H), 6.17 (d, J = 1.5 Hz, 1H), 4.31 (t, J = 7.3 Hz), 2.42 (s, 3H), 1.69–1.78 (m, 2H), 1.26–1.38 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, Acetone-d_6) δ ppm 180.7 (C), 168.6 (C), 168.4 (C), 159.0 (C), 148.4 (C), 139.0 (C), 129.5 (C), 129.4 (C), 124.1 (CH), 120.1 (CH), 120.1 (CH), 115.5 (C), 110.6 (CH), 104.4 (C), 100.1 (CH), 98.7 (CH), 92.1 (CH), 44.4 (CH_2), 33.3 (CH_3), 20.8 (CH_2), 14.1 (CH_2), 10.7 (CH_3); LRMS (ESI+) *m/z* (%) 364 (100) [M+H]$^+$; HRMS (ESI+) *m/z* calc. for $C_{22}H_{22}NO_4$ 364.1549 [M+H]$^+$, found 364.1537.

*4.1.2.2. (Z)-2-[N-(Cyclohexylmethyl)-5-methoxyindol-3-ylmethylene]-4,6-dihydroxybenzofuran-3(2H)-one (**3m**).* The crude product was prepared according to general procedure B starting from **1** (60 mg, 0.36 mmol) and aldehyde **2m** (125 mg, 0.46 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 4:6) and recrystallization in acetonitrile, the pure product (36 mg, 0.09 mmol, 24%) was obtained as red powder. m.p. 188–191 °C; ^{1}H NMR (400 MHz, Acetone-d_6) δ ppm 9.70 (bs, 1H), 8.88 (bs, 1H), 8.07 (s, 1H), 7.59 (d, J = 2.4 Hz, 1H), 7.45 (d, J = 8.9 Hz, 1H), 7.09 (s, 1H), 6.90 (dd, J = 8.9, 2.4 Hz, 1H), 6.34 (d, J = 1.7 Hz, 1H), 6.12 (d, J = 1.7 Hz, 1H), 4.14 (d, J = 7.3 Hz, 2H), 3.92 (s, 3H), 1.87–2.02 (m, 1H), 1.58–1.76 (m, 5H), 1.16–1.26 (m, 3H), 1.02–1.16 (m, 2H); ^{13}C NMR (100 MHz, Acetone-d_6) δ ppm 181.2 (C), 167.7 (C), 167.2 (C), 158.5 (C), 156.5 (C), 146.0 (C), 134.9 (CH), 132.8 (C), 129.4 (C), 113.9 (CH), 112.4 (CH), 108.6 (C), 105.2 (C), 105.0 (CH), 101.6 (CH), 98.1 (CH), 91.9 (CH), 56.0 (CH_3), 53.8 (CH_2), 39.5 (CH), 31.4 (2xCH_2), 27.0 (CH_2), 26.4 (2xCH_2); LRMS (ESI+) *m/z* (%) 420 (100) [M+H]$^+$; HRMS (ESI+) *m/z* calc. for $C_{25}H_{26}NO_5$ 420.1811 [M+H]$^+$, found 420.1803.

*4.1.2.3. (Z)-2-[N-(3-Bromobenzyl)-5-methoxyindol-3-ylmethylene]-4,6-dihydroxybenzofuran-3(2H)-one (**3o**).* The crude product was prepared according to general procedure B starting from **1** (25 mg, 0.15 mmol) and aldehyde **2o** (104 mg, 0.30 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 5:5) and recrystallization in acetonitrile, the pure product (24 mg, 0.05 mmol, 32%) was obtained as orange powder. R_f = 0.16 (96:4 CH_2Cl_2/MeOH); m.p. > 270 °C (decomposition); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.72 (bs, 1H), 10.72 (bs, 1H), 8.24 (s, 1H), 7.56 (d, J = 2.3 Hz, 1H), 7.45–7.50 (m, 2H), 7.39 (d, J = 8.9 Hz, 1H), 7.29 (dd, J = 8.1, 8.1 Hz, 1H), 7.17–7.23 (m, 1H), 7.02 (s, 1H), 6.84 (dd, J = 8.9, 2.4 Hz, 1H), 6.21 (d, J = 1.7 Hz, 1H), 6.06 (d, J = 1.7 Hz, 1H), 5.54 (s, 2H), 3.83 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 178.3 (C), 166.9 (C), 166.4 (C), 157.9 (C), 154.9 (CH), 145.2 (C), 140.4 (C), 133.4 (CH), 130.9 (CH), 130.8 (CH), 130.4 (CH), 129.7 (C), 128.1 (C), 126.0 (CH), 121.8 (C), 112.9 (CH), 111.7 (CH), 107.9 (C), 103.7 (C), 102.4 (CH), 101.1 (CH), 97.5 (CH), 90.3 (CH), 55.4 (CH_3), 48.9 (CH_2); LRMS (ESI-) *m/z* (%) 492 (80) [M(^{81}Br)-H]$^-$, 490 (70) [M(^{79}Br)-H]$^-$, 255 (85), 239 (70), 157 (100); HRMS (ESI-) *m/z* calc. for $C_{25}H_{17}BrNO_5$ 490.0290 [M-H]$^-$, found 490.0294.

*4.1.2.4. (Z)-2-(N-Methyl-5-bromoindol-3-ylmethylene)-4,6-dihydroxybenzofuran-3(2H)-one (**3p**).* The crude product was prepared according to general procedure B starting from **1** (37 mg, 0.22 mmol) and aldehyde **2p** (105 mg, 0.44 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 5:5) and recrystallization in acetonitrile, the pure product (35 mg, 0.09 mmol, 41%) was obtained as orange powder. R_f = 0.16 (96:4 CH_2Cl_2/MeOH); m.p. > 300 °C (decomposition); ^{1}H NMR (400 MHz, Acetone-d_6) δ ppm 9.72 (bs, 1H), 8.92 (bs, 1H), 8.24 (d,

J = 1.8 Hz, 1H), 8.14 (s, 1H), 7.49 (d, J = 8.7 Hz, 1H), 7.41 (dd, J = 8.7, 1.8 Hz, 1H), 7.04 (s, 1H, CH), 6.34 (d, J = 1.7 Hz, 1H), 6.13 (d, J = 1.7 Hz, 1H), 3.99 (s, 3H); ^{13}C NMR (100 MHz, Acetone-d_6) δ ppm 181.1 (C), 167.4 (C), 167.4 (C), 158.6 (C), 146.7 (C), 136.9 (C), 136.1 (CH), 130.2 (C), 126.2 (CH), 122.7 (CH), 114.9 (C), 113.0 (CH), 108.5 (C), 105.1 (C), 103.6 (CH), 98.3 (CH), 91.9 (CH), 33.8 (CH_3); LRMS (ESI-) m/z (%) 386 (95) [M(^{81}Br)-H]$^-$, 384 (100) [M(^{79}Br)-H]$^-$; HRMS (ESI-) m/z calc. for $C_{18}H_{11}BrNO_4$ 383.9871 [M-H]$^-$, found 383.9870.

*4.1.2.5. (Z)-2-(N-Ethyl-5-bromoindol-3-ylmethylene)-4,6-dihydroxybenzofuran-3(2H)-one (**3q**).* The crude product was prepared according to general procedure B starting from **1** (27 mg, 0.16 mmol) and aldehyde **2q** (83 mg, 0.33 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 5:5) and recrystallization in acetonitrile, the pure product (21 mg, 0.05 mmol, 33%) was obtained as orange powder. R_f = 0.15 (96:4 CH_2Cl_2/MeOH); m.p. > 270 °C (decomposition); ^{1}H NMR (400 MHz, Acetone-d_6) δ ppm 9.74 (bs, 1H), 8.94 (bs, 1H), 8.25 (d, J = 1.8 Hz, 1H), 8.20 (s, 1H), 7.54 (d, J = 8.7 Hz, 1H), 7.40 (dd, J = 8.7, 1.9 Hz, 1H), 7.04 (s, 1H), 6.35 (d, J = 1.7 Hz, 1H), 6.13 (d, J = 1.7 Hz, 1H), 4.40 (q, J = 7.3 Hz, 2H), 1.52 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, Acetone-d_6) δ ppm 181.1 (C), 167.9 (C), 167.4 (C), 158.6 (C), 146.7 (C), 135.8 (C), 134.6 (CH), 130.4 (C), 126.2 (CH), 122.9 (CH), 114.8 (C), 113.1 (CH), 108.7 (C), 105.1 (C), 103.6 (CH), 98.3 (CH), 92.0 (CH), 42.5 (CH_2), 15.7 (CH_3); LRMS (ESI-) m/z (%) 400 (100) [M(^{81}Br)-H]$^-$, 398 (90) [M(^{79}Br)-H]$^-$, 255 (40), 157 (60); HRMS (ESI-) m/z calc. for $C_{19}H_{13}BrNO_4$ 398.0028 [M-H]$^-$, found 398.0028.

*4.1.2.6. (Z)-2-(N-Butyl-5-bromoindol-3-ylmethylene)-4,6-dihydroxybenzofuran-3(2H)-one (**3r**).* The crude product was prepared according to general procedure B starting from **1** (60 mg, 0.36 mmol) and aldehyde **2r** (129 mg, 0.46 mmol). After purification by recrystallization in acetonitrile, the pure product (148 mg, 0.35 mmol, 96%) was obtained as red powder. R_f = 0.16 (96:4 CH_2Cl_2/MeOH); m.p. > 260 °C (decomposition); ^{1}H NMR (400 MHz, Acetone-d_6) δ ppm 9.76 (bs, 1H), 8.93 (bs, 1H), 8.25 (d, J = 1.8 Hz, 1H), 8.19 (s, 1H), 7.55 (d, J = 8.7 Hz, 1H), 7.39 (dd, J = 8.7, 1.8 Hz, 1H), 7.04 (s, 1H), 6.34 (d, J = 1.3 Hz, 1H), 6.14 (d, J = 1.3 Hz, 1H), 4.36 (t, J = 7.2 Hz, 2H), 1.85–1.94 (m, 2H), 1.33–1.43 (m, 2H), 0.95 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, Acetone-d_6) δ ppm 181.1 (C), 167.4 5 (C), 167.4 (C), 158.6 (C), 146.6 (C), 136.1 (C), 135.1 (CH), 130.3 (C), 126.1 (CH), 122.8 (CH), 114.8 (C), 113.2 (CH), 108.6 (C), 105.1 (C), 103.6 (CH), 98.2 (CH), 91.9 (CH), 47.4 (CH_2), 32.9 (CH_2), 20.6 (CH_2), 13.9 (CH_3); LRMS (ESI+) m/z (%) 430 (50) [M(^{81}Br)+H]$^+$, 428 (60) [M(^{79}Br)+H]$^+$, 381 (100), 353 (60), 227 (57), 210 (57); HRMS (ESI+) m/z calc. for $C_{21}H_{19}BrNO_4$ 428.0497 [M+H]$^+$, found 428.0495.

*4.1.2.7. (Z)-2-[N-(Cyclohexylmethyl)-5-bromoindol-3-ylmethylene]-4,6-dihydroxybenzofuran-3(2H)-one (**3s**).* The crude product was prepared according to general procedure starting from **1** (63 mg, 0.38 mmol) and aldehyde **2s** (241 mg, 0.75 mmol). After purification by column chromatography on silica gel (cyclohexane/EtOAc 8:2 to 5:5) and recrystallization in acetonitrile, the pure product (100 mg, 0.21 mmol, 56%) was obtained as orange powder. R_f = 0.20 (96:4 CH_2Cl_2/MeOH); m.p. > 270 °C (decomposition); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.75 (s, 1H), 10.73 (s, 1H), 8.25 (d, J = 1.9 Hz, 1H), 8.10 (s, 1H), 7.58 (d, J = 8.7 Hz, 1H), 7.35 (dd, J = 8.7, 1.9 Hz, 1H), 6.97 (s, 1H), 6.23 (d, J = 1.8 Hz, 1H), 6.06 (d, J = 1.8 Hz, 1H), 4.14 (d, J = 7.3 Hz, 2H), 1.75–1.90 (m, 1H), 1.43–1.72 (m, 5H, 5H), 0.93–1.24 (m, 5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 178.2 (C), 166.9 (C), 166.5 (C), 157.9 (C), 145.5 (C), 135.2 (C), 134.4 (CH), 128.7 (C), 124.9 (CH), 121.8 (CH), 113.4 (C), 113.0 (CH), 107.1 (C), 103.5 (C), 101.7 (CH), 97.5 (CH), 90.4 (CH), 52.1 (CH_2), 38.2 (CH), 30.0 (2xCH_2), 25.8 (CH_2), 25.1 (2xCH_2); LRMS (ESI+) m/z (%) 470 (90) [M(^{81}Br)+H]$^+$, 468 (100) [M(^{79}Br)+H]$^+$, 381 (65), 353 (70), 331 (33), 210 (50); HRMS (ESI+) m/z calc. for $C_{24}H_{23}BrNO_4$ 468.0810 [M+H]$^+$, found 468.0809.

4.2. Biology

4.2.1. Reagents

Calcein-AM, elacridar (GF120918), MK-571 and cyclosporine A were obtained from Sigma-Aldrich (France). All other reagents were purchased from other companies (such as Euromedex France) at the highest available purity grade (>95%).

4.2.2. Cell lines

The MDCKII WT and MDCKII ABCC2 cell lines were kindly provided by Prof. Dr. Piet Borst, (The Netherlands Cancer Institute Amsterdam, Netherlands). These are epithelial kidney cells and the MDCKII-ABCC2 cell line is obtained by transfection with a pCMV-cMOAT retrovirus (clone 17; MDCKII-MOAT17) [39].

To evaluate the selectivity of our compounds on other ABC transporters, we used the NIH3T3 parental cell line and NIH3T3/ABCB1 drug resistant cell line transfected with human MDR1/A-G185 [49], purchased from American Type Culture Collection (Manassas, VA) and used as described previously [23]. The BHK21 (Baby Hamster Kidney-21) cells and BHK21-ABCC1 stably transfected with wild-type ABCC1 cells were used to evaluate the effect on ABCC1 [50]. Flp-In™-293 cells were transfected by electroporation using Neon® Transfection System (ThermoFisher scientific) with pcDNA5-FRT-*ABCC2* plasmid and pOG44 Flp-recombinant expression vector. After selection by hygromycine B (ThermoFisher scientific), the Flp-In system allows a stable integration and expression of *ABCC2* to deliver single copy isogenic cell lines. Flp-In-293-*ABCC2* cells were grown at 37 °C in 5% CO_2.

4.2.3. Cell culture

All cell lines were grown at 37 °C in 5% CO2. MDCKII and NIH3T3 cells were cultured in Dulbecco's modified Eagles's medium (DMEM high glucose) (PAA) supplemented with 10% fetal bovine serum (FBS, PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France), 1% penicillin/streptomycin (PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France), without selection for the ABCC2-transfected cell line, while for the NIH3T3-ABCB1 cell line we used 60 ng/mL of colchicine (Sigma- Aldrich company, Saint Quentin Fallavier, France). BHK-21 cells were cultured in DMEM-F12, GlutaMAX Supplement culture medium (Gibco-Life Technologies, Saint Aubin, France) supplemented with 15 mM HEPES, 1% penicillin/streptomycin (PAA) and 5% of heat-inactivated fetal bovine serum (PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France), in the presence of 200 mM hygromycin for transfected cells.

4.2.4. ABCC2-mediated calcein transport

4.2.4.1. High-throughput flow cytometry analysis for ABCC2 inhibitors screening. After a thorough understanding of the transport of calcein in the MDCKII cells, we chose the ideal conditions to set up a high-throughput screening protocol. For this purpose, we used the Macsquant VYB (Miltenyi Biotec) cytometer integrated with a microplate reader and an autosampler. Calcein-AM was used as a substrate at 25 nM, in the presence of 1 μM GF120918 and 25 μM MK-571 to inhibit endogenous ABCB1 and ABCC1. Our reference for ABCC2 inhibition was 25 μM cyclosporine. Using 96-well plates, the control cell line and the ABCC2-transfected cell line, we could screen up to 20 compounds/h. Cells were seeded at a density of 3 × 103 cells/well for 48 h at 37 °C in 5% CO_2, then treated for 30 min with 5 μM of the inhibitor candidate, washed with a phosphate buffered saline (PBS) solution and detached using Accumax or Accutase enzyme solutions. Calcein was excited using

488 nm laser; the intracellular fluorescence was monitored using the B1 (525/50 nm) channel, and at least 5000 events were collected. The percentage of inhibition was calculated by using the following equation: **% inhibition** = **(ABC_{S+I} − ABC_S)/($Control_{S+I}$ − ABC_S)*100**, where ABC_{S+I} is the intracellular fluorescence of the transfected cells in the presence of substrates + inhibitors, ABC_S is the intracellular fluorescence of the transfected cells with only substrate, and $Control_{S+I}$ corresponds to the intracellular fluorescence of control cells in the presence of substrates and inhibitors. In the initial screening that led to the identification of the class of compounds optimized as ABCC2 inhibitors, compounds were tested only once.

In order to calculate the IC_{50} of calcein transport in MDCKII WT and ABCC2 cells, we tested several concentrations of the inhibitor and plotted the inhibition curves, then extracted the concentration for which 50% of calcein transport is inhibited. An additional confirmatory assay was carried on in a similar manner in Flp-In™293 and Flp-In™293 cells transfected with ABCC2. The inhibitors were tested in flow cytometry at 5 μM, the cells were loaded with 25 nM calcein-AM, and used 25 μM cyclosporine as reference.

4.2.4.2. Selectivity of inhibitor candidates towards ABCB1 and ABCC1. NIH-3T3 cells transfected with ABCB1 were seeded at a density of 3.5 × 103 cells/well into 96-wells culture plates and incubated 48 h at 37 °C in 5% CO_2, whereas BHK21 cells transfected with ABCC1 were seeded at 10^4 cells/well for 48 h. The transport mediated by the ABC transporters was evaluated by exposing the cells to calcein-AM (25 nM) in the presence or absence of the tested compounds at 5 μM, then washed with PBS solution and detached using Accumax or Accutase enzyme solutions. We used cyclosporine A as a positive reference in our tests at 2.5 μM for ABCB1 and 25 μM for ABCC1.

4.2.5. Cytotoxicity

Cell survival was studied using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. We tested the cytotoxicity of compounds on MDCKII WT and MDCKII ABCC2 cell lines. Cells were seeded in 96-well plates at the right density so that they don't reach 100% confluence after 72 h of incubation at 37 °C. After 24 h, we cultivated cells in the presence or absence of the tested compounds (0–50 μM). After 72 h, we added 22 μL of MTT solution (5 mg/mL) to each well and the plates were incubated at 37 °C for 3 h. The medium was discarded, and replaced with 100 μL solution of DMSO/isopropanol (1:1). After 10–20 min of gentle shaking, absorbance was measured in a microplate reader at 570 nm, and this was corrected with the absorbance measured at 690 nm. Data shown represents the mean ± SD of at least three independent experiments.

4.3. Statistical analysis

All experiments were run in duplicates or triplicates, with positive and negative controls, and then analyzed statistically using specific software and analysis tools. We used FlowJo V10 for the analysis of flow-cytometry data, and Excel 2013 package for the interpretation and graphical representation of data. Statistical differences between group means were determined by student's two-tailed *t*-test for unpaired samples.

Acknowledgements

We are grateful to Dr. Laurent Ettouati, Mrs Christine Ranquet and Mrs Elodie Monniot for their assistance in the stock management of the small molecule library of EA 4446 B2MC and HPLC/MS analyses of compounds. We would like to thank chemists involved in chemical syntheses of this library, in particular Drs. Zouhair Bouaziz, Christelle Marminon, Pascal Nebois, Laurent Ettouati, Sylvie Radix, Thierry Lomberget, Nadia Walchshofer, Roland Barret and Marie-Emmanuelle Million. We also thank ChemAxon for providing us a license to their cheminformatics software Instant JChem. EB was financially supported by the Erasmus + PhD mobility program accessed from the University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca, Romania, and a fellowship received from the French National Research Agency, ANR-13-BSV5-0001-01 (to PF and AB).

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