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Uncompetitive nanomolar dimeric indenoindole inhibitors of the human breast cancer resistance pump ABCG2

Nathalie Guragossian^{a,b,+}, Billel Belhani^{a,c,+}, Alexis Moreno^{b,+}, Magda Teixeira Nunes^b, Lucia Gonzalez-Lobato^b, Christelle Marminon^{a,d}, Laurent Berthier^e, Amanda Do Rocio Andrade Pires^{b,f}, Csilla Özvegy-Laczka^g, Balázs Sarkadi^g, Raphaël Terreur^e, Zouhair Bouaziz^a, Malika Berredjem^c, Joachim Jose^h, Attilio Di Pietro^b, Pierre Falson^{b,*}, Marc Le Borgne^{a,d,*}

^aEA 4446 Bioactive Molecules and Medicinal Chemistry, Université Claude Bernard Lyon 1, 69373 Lyon, France

^bDrug Resistance & Membrane Proteins group - Molecular Microbiology and Structural Biochemistry Laboratory, CNRS-UCBL1 UMR 5086, IBCP, 69367 Lyon, France

^cLaboratory of Applied Organic Chemistry, Synthesis of Biomolecules and Molecular Modelling Group, Badji Mokhtar - Annaba University, 23000 Annaba, Algeria

^dSmall Molecules for Biological Targets Team, Centre de recherche en cancérologie de Lyon, Centre Léon Bérard, CNRS 5286, INSERM 1052, Université Claude Bernard Lyon 1, Univ Lyon, Lyon, 69373, France

^eLaboratoire de Biologie Tissulaire et Ingénierie, CNRS-UCBL1 UMR 5305, IBCP, 69367 Lyon, France

^fDepartamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba, Brazil

^gInstitute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, 1117 Budapest, Hungary

^hInstitut für Pharmazeutische und Medizinische Chemie, PharmaCampus - Westfälische Wilhelms-Universität Münster, D-48149 Münster, Germany

⁺ Authors contributed equally to this manuscript.

* Corresponding Authors: Pierre Falson: pierre.falson@ibcp.fr; [0000-0002-9760-4577](tel:0000-0002-9760-4577).
Marc Le Borgne: marc.le-borgne@univ-lyon1.fr; [0000-0003-1398-075X](tel:0000-0003-1398-075X)

Abstract: Multidrug resistance membrane pumps reduce the efficacy of chemotherapies by exporting a wide panel of structurally-divergent drugs. Here, to take advantage of the polyspecificity of the human Breast Cancer Resistance Protein (BCRP/ABCG2) and the dimeric nature of this pump, new dimeric indenoindole-based inhibitors from the monomeric α,β -unsaturated ketone **4b** and phenolic derivative **5a** were designed. A library of 18 homo/hetero-dimers was synthesized. Homo-dimerization shifted the inhibition efficacy from sub-micromolar to nanomolar range, correlated with the presence of **5a**, linked by a 2-6 methylene-long linker. Non-toxic, the best dimers displayed a therapeutic ratio as high as 70,000. It has been found that the high potency of the best compound **7b** that displays a K_I of 17 nM is due to an uncompetitive behavior toward mitoxantrone efflux and specific for that drug, compared to Hoechst 33342 efflux. Such property may be useful to target such anticancer drug efflux mediated by ABCG2. Finally, at a molecular level, an uncompetitive mechanism by which substrate promotes inhibitor binding implies that at least 2 ligands should bind simultaneously to the drug-binding pocket of ABCG2.

Keywords: indenoindole, dimers, human breast cancer resistance protein, selectivity, uncompetitive, nanomolar, docking

1. Introduction

The World Health Organization currently reports 9.6 million deaths per year worldwide due to cancer. A major problem facing many cancer patients is related to resistance to chemotherapies, often resulting in cancer relapse or metastasis. This resistance is triggered by the overexpression of drug-efflux pumps belonging to the ATP-Binding Cassette (ABC) superfamily, P-glycoprotein, P-gp/ABCB1 [1] and Breast Cancer Resistance Protein, BCRP/ABCG2 [2], which recognize and translocate across membranes small structurally-unrelated endo- and xenobiotics including drugs [3,4]. X-ray and cryo-EM structures of ABCB1 [5,6] and ABCG2 [7] gave contrasted information about that polyspecificity. Both pumps have a large drug-binding pocket in which that of ABCB1 displays distinct H and R subsites (for Hoechst 33342 and rhodamine) [8], while for ABCG2 the compounds so far structurally located bind in the same region, such as the substrate estrone-3-sulfate or inhibitors MZ29 or MB136 [9,10].

Among the most active compounds inhibiting ABCG2-mediated drug efflux, a first design of inhibitors led to the mycotoxin fumitremorgin C derivative Ko143 (Fig. 1) [11], so far taken as a reference. More recently, the chromone derivative MBL-II-141 (Fig. 1) substantially increased overall survival of tumor-bearing mice treated with irinotecan without noticeable toxicity [12]. Lastly, quinazoline derivatives led to potent inhibitors [13], although with limited therapeutic ratio (TR). Here, we evaluated a strategy for designing new inhibitors by taking advantage of the uncompetition mechanism, a mode of inhibition in which the substrate enhances the affinity for the inhibitor, with the aim to favor the inhibition of the pump for given drugs. We took advantage of the homodimeric topology of ABCG2, a 72-kDa half transporter, which potentially binds 2 copies of a ligand, as recently observed with the Ko143 derivative MZ29 (Fig. 1) [9], or MB136, a derivative of tariquidar (Fig. 1) [10]. More recently, a triazole bridged flavonoid homodimer (Ac22(Az8)₂) showed low toxicity and potent ABCG2-inhibitory activity ($EC_{50} = 1-2$ nM) [14].

However, clinical development has been frustrating, reorienting the clinical relevance of these pumps in a more specific MDR cellular context [15]. Indeed, these pumps are also involved in protection against xenotoxins at barriers such as those present in brain or placenta [16]. ABCG2 is also overexpressed in pluripotent stem cells [17] for which it constitutes a marker. Of note it is equally overexpressed in cancer stem cells that are strongly suspected to be responsible for metastasis. Consequently, ABCG2 may constitute a privileged target for preventing metastasis [15].

To explore this concept of dimeric molecules, we started with recently developed indeno[1,2-*b*]indole compounds inhibiting ABCG2 in the sub-micromolar range [18,19]. The best α,β -unsaturated ketone **4b** [18] with a methyl group in position 7 and the best phenolic derivative **5a** [19] with a methoxy group in *meta* were chosen (Fig. 2). Then a library of 18 homo- and hetero-dimers was synthesized. For each type of dimer, six different linker lengths were introduced. Finally, the efficacy of all dimers to inhibit the ABCG2-mediated mitoxantrone efflux was evaluated. Structure activity relationship, selectivity towards P-gp

and MRP-1, ATPase activity, ABCG2 protein 5D3 monoclonal antibody interaction, and inhibition mechanism were also investigated.

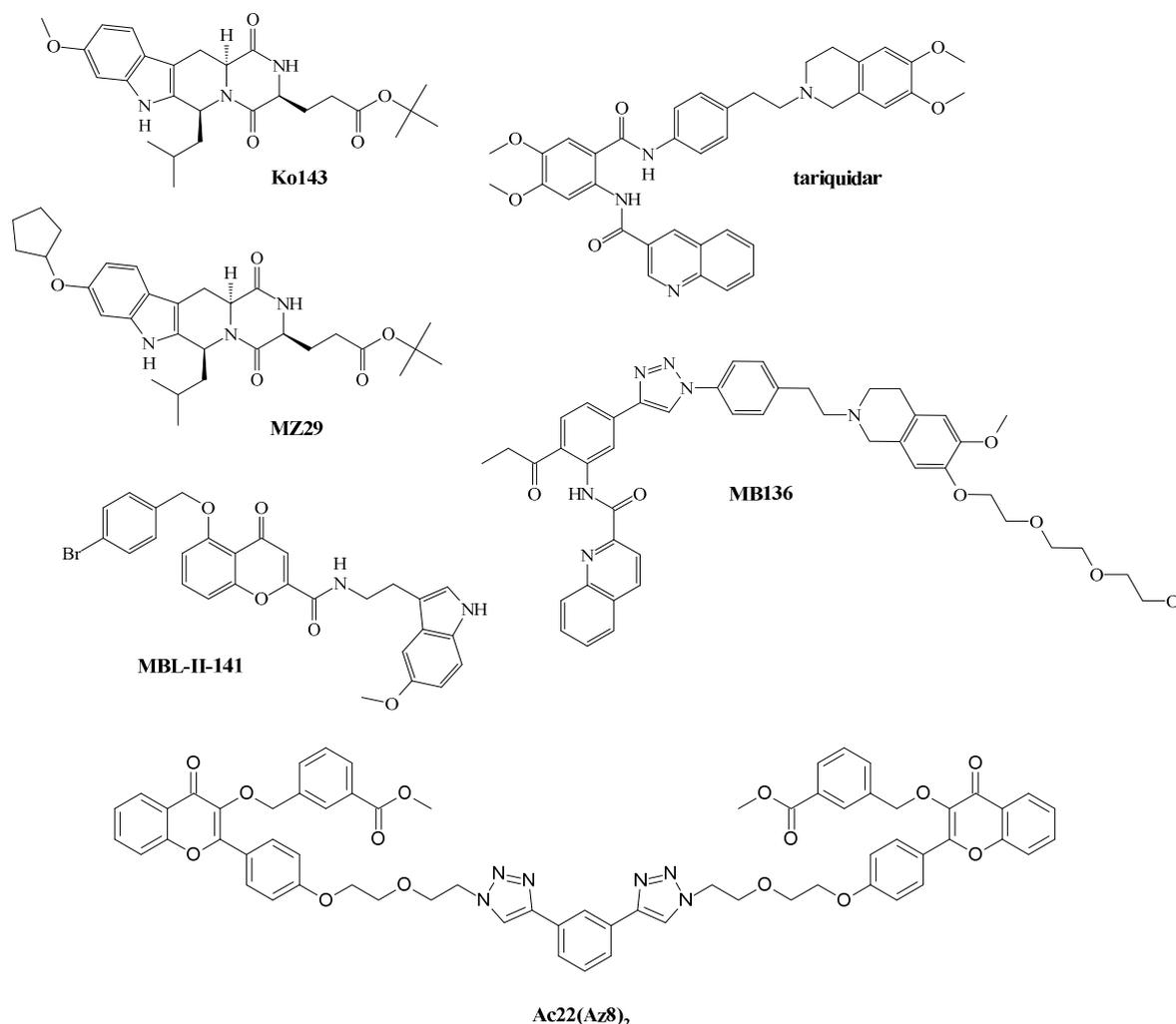


Fig. 1. Chemical structures of Ko143, MZ29, tariquidar, MB136, MBL-II-141 and Ac22(Az8)₂.

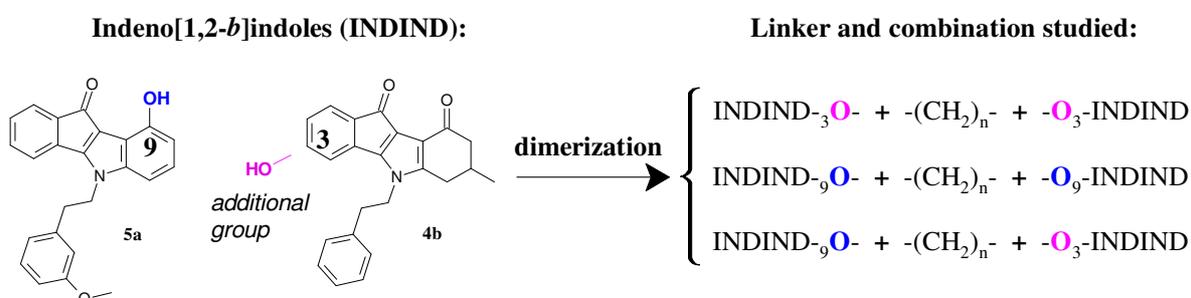


Fig. 2. Chemical structures of monomeric indeno[1,2-*b*]indoles **4b**, **5a** and modulation sites for dimerization (OH groups used for dimerization are shown in blue and magenta, linker length (n) varied between 2 and 12 carbons).

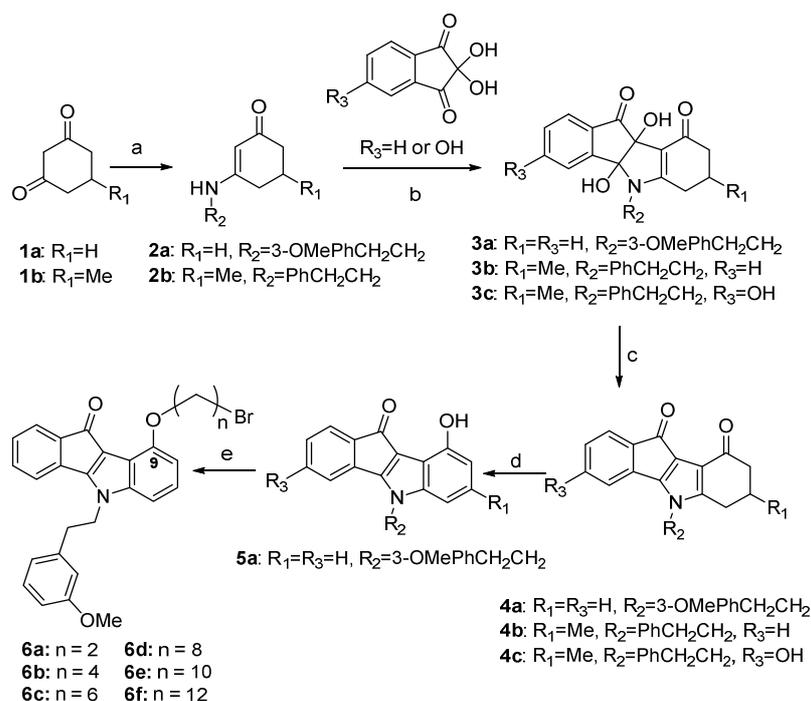
2. Results and discussion

2.1. Chemistry

The tetrahydroindeno[1,2-*b*]indole-9,10-diones **4a,b** and 9-hydroxyindeno[1,2-*b*]indol-10-one **5a** were synthesized as previously reported [18,19]. The new 3-hydroxylated indeno[1,2-*b*]indole **4c** (introduction of a hydroxyl group for the dimerization of **4b**) was obtained in a totally regioselective manner in two steps. Condensation of the enaminone **2b** [20,21] with 5-hydroxyninhydrin [22] led to the trihydroxylated derivative **3c** as a unique regioisomer, which was then deoxygenated using the tetraethylthionylamide (TETA) to afford **4c** (Scheme 1).

We initially considered the synthesis of **5a** homodimers, **7a-f**, linked by two to twelve methylene units (Scheme 2), following two possible methods. The first one started with the synthesis and isolation of the alkylated monomer intermediates **6a-f**. The latter were achieved by *O*-alkylation of the phenolic compound **5a**, by using 1.2 equiv. of the corresponding linker in CH₃CN and 1.5 equiv. of K₂CO₃ under microwave (μ .w.) heating (Scheme 1, condition [e]). The bromo intermediate **6d** was then engaged with **5a** in DMF to produce the targeted homodimer **7d** (according to the condition [g], Scheme 2). After these two steps (conditions [e] + [g]), **7d** was isolated with an overall yield of only 16% (and a total irradiation time of 45 min). In a second approach, the homodimer **7d** was directly obtained by condensation of 1 equiv. of **5a** with 0.6 equiv. of the corresponding linker (Scheme 2, condition [f]). Then **7d** was obtained with a 61% yield after 40 min of μ .w. heating. Therefore, we opted for the one-step pathway (only condition (f)) to synthesize all homodimers **7a-f** and **8a-f**.

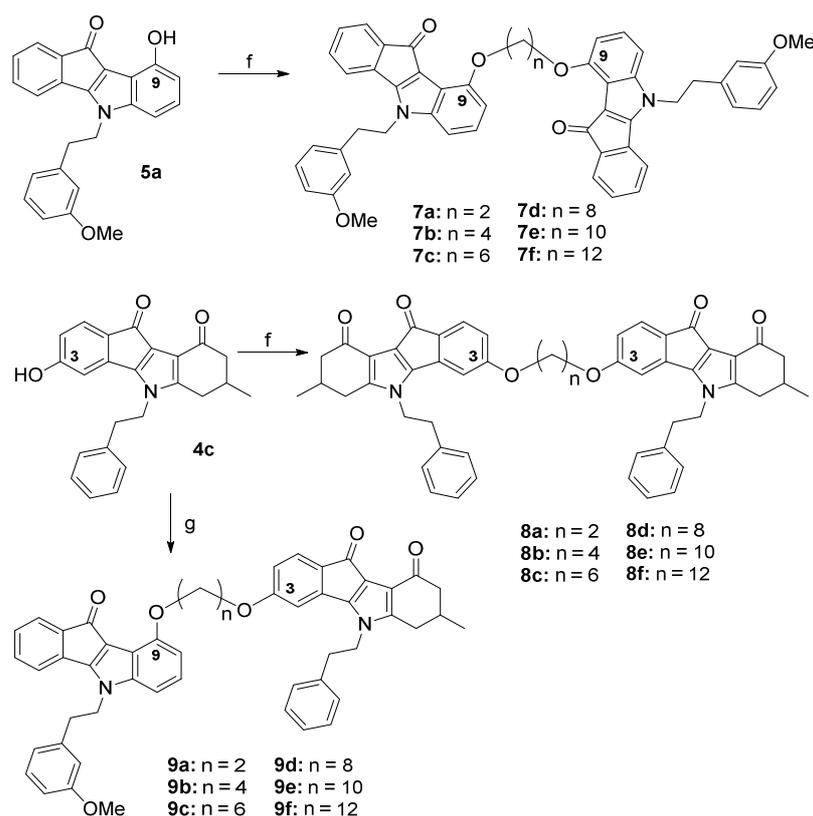
For the synthesis of heterodimers **9a-f**, 3-hydroxylated indenoindole **4c** reacted with each bromo intermediate (**6a-f**) and directly gave **9a-f** with yields between 60 and 72%.



Scheme 1. Reagents and conditions: (a) R_2NH_2 , toluene, reflux, 4-18 h; (b) MeOH, r.t., 20-24 h; (c) $(Et_2N)_2SO$ (TETA), DMF, AcOH, r.t., 22-30 h; (d) 10% Pd-C, Ph_2O , reflux, 6 h; (e) $Br(CH_2)_nBr$, K_2CO_3 , CH_3CN , $\mu.w.$, 130 °C, 20-50 min.

2.2. Biology

We first evaluated the efficacy of all dimers to inhibit the ABCG2-mediated mitoxantrone efflux in ABCG2-transfected human fibroblast embryonic kidney HEK 293 cells (Fig. 3). Homodimerization of **4c** that generated the **8a-f** series did not lead to any improvement of the inhibition potential (Fig. 3b). The half-maximal inhibition concentration, IC_{50} (Table 1), remained in the same micromolar range as the monomer, except for a slight 2-fold improvement obtained with **8a** having an ethyl linker. Dimerization of **5a** led to much more potent products, **7a-f**, with IC_{50} values shifted from micro- to nano-molar ranges (Fig. 3a). Ethyl, butyl and hexyl linkers in **7a-c** generated the most efficient inhibitors, while additional methylene groups in **7d-f** abolished their inhibition potential. Heterodimerization of **5a-4c** leading to **9a-f** gave intermediate results (Fig. 3c). The best compound with IC_{50} in the nanomolar range, **9c** bearing a hexyl linker, was followed by **9a** and **9b** with ethyl and butyl linkers.



Scheme 2. Reagents and conditions: (f) $Br(CH_2)_nBr$, K_2CO_3 , CH_3CN , $\mu.w.$, 130 °C, 35-65 min; (g) **6a-f**, K_2CO_3 , DMF, $\mu.w.$, 160 °C, 10-60 min.

Importantly, the best dimers showed a very limited, if any, toxicity (Fig. S2). This poor intrinsic toxicity observed for **7a**, **7b**, **7c** and **9c** resulted in very high TR values ranging

between 2,000 and 70,000 (Table 1). Low IC_{50} and high TR values make these new molecules as the most potent ABCG2 inhibitors ever conceived. Indeed, the quinazoline-based inhibitors of the nucleus dye Hoechst 33342 ABCG2-mediated efflux recently reported [13] had similar IC_{50} , but their TR values only reached 400 which might limit any practical therapeutic use. We did not observe a marked transport by ABCG2 of the dimers as indicated by the close behavior of WT and G2 strains toward them in terms of cytotoxicity (Fig. S2). We evaluated the specificity of the best dimers toward the other main MDR pumps, P-gp and Multidrug Resistance Protein 1 (MRP1). Compounds **7a**, **7b**, **7c**, **9b**, **9c**, **9d**, **8a** and **8b** displayed no inhibition of calcein efflux mediated by either MRP1 or P-gp, while only **9a** displayed a significant, although poor, capacity to block the rhodamine 123 efflux mediated by P-gp (Fig. S3). ATPase activity of ABCG2 and interaction with the conformational antibody 5D3 with **4b**, **5a**, **7a**, **7b**, **7c** and **9c** were also investigated and the results described in Fig. S4.

Table 1. Inhibition parameters for the ABCG2-mediated mitoxantrone efflux.

Cpd	Linker	IC_{50} , nM ^a	IG_{50} , μ M ^b	TR ^c
5a	-	732 ± 166^d	-	-
7a	(CH ₂) ₂	38.4 ± 4.4^d	WT: 917 ± 645 G2: > 100, no toxicity	≥ 2630
7b	(CH ₂) ₄	24.1 ± 3.4^d	WT: 79.6 ± 16.6 G2: 51.8 ± 14.7	WT: 3302 G2: 2158
7c	(CH ₂) ₆	27.4 ± 3.3^d	≥ 1946	$\geq 70,000$
7d-f	(CH ₂) ₈₋₁₂	$> 10^4$	-	-
4b	-	768 ± 183^f	-	-
8a	(CH ₂) ₂	319 ± 106^f	242 ± 134	758
8b	(CH ₂) ₄	867 ± 199^e	109 ± 34	126
8c-f	(CH ₂) ₆₋₁₂	$> 10^4$	-	-
9a	(CH ₂) ₂	68.0 ± 13.9^d	221 ± 49	3250
9b	(CH ₂) ₄	132.7 ± 40.3^f	295 ± 120	2234
9c	(CH ₂) ₆	32.6 ± 6.4^d	> 100, no toxicity	> 3067
9d	(CH ₂) ₈	388.6 ± 46.2^d	> 20, no toxicity	> 52
9e-f	(CH ₂) ₁₀₋₁₂	$> 10^4$	-	-

^aHalf-maximal inhibition concentrations, IC_{50} , estimated from Fig. S1. ^bConcentrations producing 50% cell growth inhibition, IG_{50} , estimated from Fig. S2. Most could only be roughly estimated, as due to solubility constraints. When no specific effect of the strain line was observed, fits were carried out with both data corresponding to HEK293-pCDNA3 and HEK293-ABCG2 cell lines. ^cTherapeutic Ratio (TR) = IG_{50}/IC_{50} . ^d**** p < 0.0001, ^e*** p < 0.001, ^f** p < 0.01.

Then we explored the inhibition mechanism of the drug efflux mediated by ABCG2 for the best compounds, incubating HEK293 and HEK293-ABCG2 cells with substrates,

mitoxantrone or H \ddot{o} chst 33342, and **7b** or **9c** dimers or their parent monomers (Fig. 4). Resulting drug-efflux data sets were fitted with competitive, non-competitive, uncompetitive and mixed inhibition models [8,23] (Table S1), as detailed in the Supporting Information with mitoxantrone and **7b** (Fig. S5-8). Models were ranked in respect of their corrected Akaike Index Criterion (AICc) [24], the lower the AIC, the better the model (Fig. 4f, Table S2). **5a** displayed two close patterns of non-competitive and mixed inhibitions (Fig. 4a) while **4b** behaved as a mixed inhibitor (Fig. 4b), exhibiting sub-micromolar K_I . In contrast, **7b** displayed an uncompetitive behavior (Fig. 4c) with a K_I of 17 nM, which to our knowledge is the lowest ever reported. **7b** displayed a non-competitive-mixed inhibition toward H \ddot{o} chst 33342 efflux (Fig. 4d) with a 2-fold higher K_{I2} . **9c** showed close mixed and uncompetitive inhibition patterns toward mitoxantrone efflux with K_{I2} of 38-46 nM (Fig. 4e).

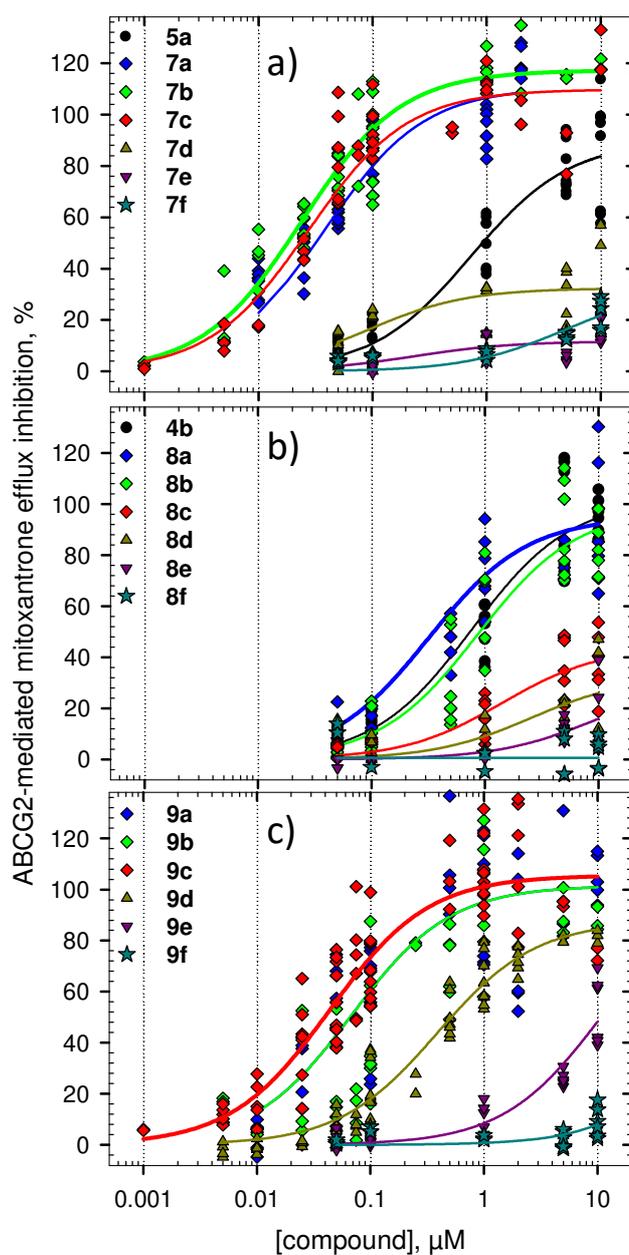


Fig. 3. Evaluation of the inhibitory efficacy of the ABCG2-mediated mitoxantrone efflux by dimers and parent compounds. HEK293-ABCG2 cells were incubated with 5 μ M

mitoxantrone as substrate and 1 to 10^4 nM compound. Data were from 2 (d-f series) to 4 (a-c series) independent experiments done in triplicates (Fig. S1).

The uncompetitive behavior of **7b** toward mitoxantrone assumes that the substrate promotes the binding of **7b**, but how does it operate? To go further, we carried out docking experiments using the recent cryo-EM ABCG2 structure [7] (Fig. 5). We first carried out molecular dynamics in the presence of a lipid bilayer leading to a thermodynamically stable state. We used the latter to carry out docking in two steps, binding first mitoxantrone and then **7b**. Mitoxantrone preferentially bound at the apex of the drug-binding pocket, between the two lipid leaflets (Fig. 5a and 5b), in the same area as cholesterol [7] or estrone-3-sulfate, E₁S [10], previously identified in the corresponding structures. Typically, the quinone B-ring of mitoxantrone stacked between the phenyl rings of the two phenylalanine 439 (Fig. 5c), residues also found to interact with cholesterol or E₁S. In addition, as observed for the latter, the two Asn-436 residues were recruited to generate two hydrogen bonds with the two alkanolamine groups of mitoxantrone. To confirm that interaction, we mutated the Asn-436 residues into alanine, which indeed abolished the transport of mitoxantrone (Fig. 5d), as also previously observed with E₁S [10].

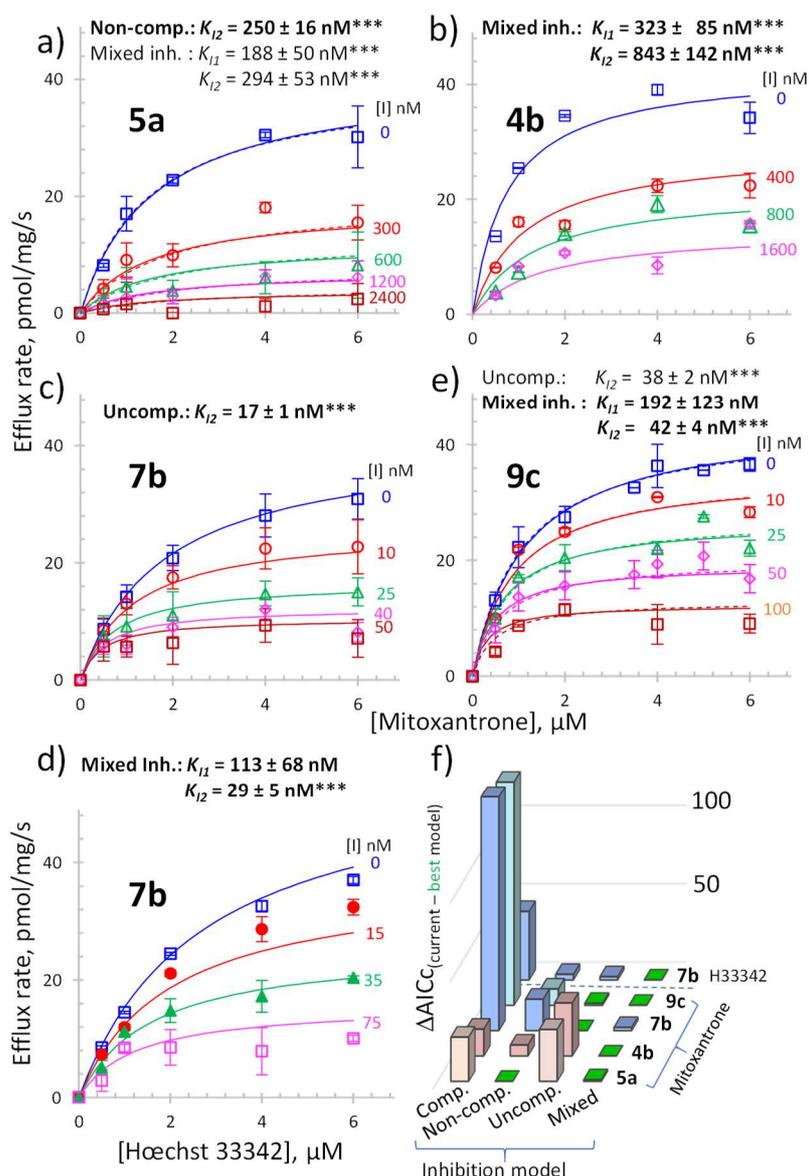


Fig. 4. Inhibition mechanisms by **4b**, **5a**, **7b** and **9c**. a-e) ABCG2-mediated mitoxantrone or Hoechst 33342 efflux rates plotted with respect to substrate and inhibitor concentrations, corresponding to the residual intracellular substrate amounts in HEK293-*ABCG2* vs HEK293. Best and close models are displayed with their inhibitor constant (in bold for the best model). Data set ranging from 72 to 204 points were from 1 (d), 2 (a, b), 3 (e) or 5 (c) experiments done in duplicates (c, e) or triplicates (a, b, d). *** $p < 0.001$. f) Ranking of inhibition models using their AICc. The difference of a given AICc with the lowest found corresponding to the best model is plotted. Best and close models are colored in green (Fig. S5-8).

Subsequent docking of **7b** led to the best pose in close contact with mitoxantrone, taking advantage of the latter to reduce its solvent surface area. We found a binding energy of -82 kcal/mol to which mitoxantrone contributed to 40%, a value obtained by looking at the relative contribution of each residue and mitoxantrone to **7b** binding. This contribution may explain the uncompetitive behavior of **7b**, benefiting of the mitoxantrone vicinity to increase its affinity. As bound, **7b** occupies most of the drug-binding cavity and locks ABCG2 by

steric hindrance in its inward-facing conformation. Such a mechanism is reinforced here by the high affinity of **7b** for the drug-binding pocket, as suggested by its very low K_i .

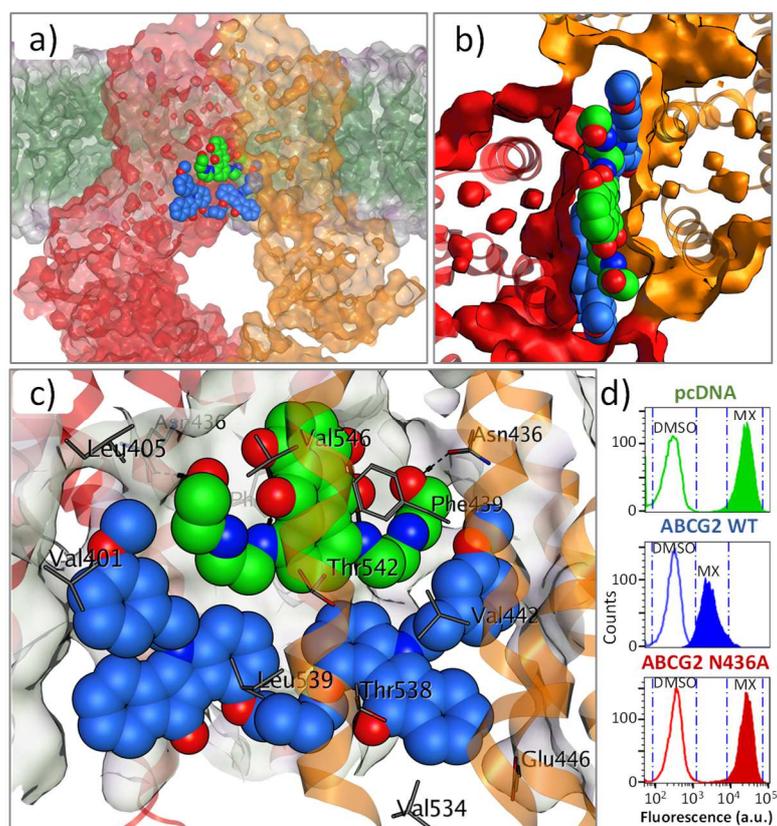


Fig. 5. Molecular docking of **7b** (blue) and mitoxantrone (green) into the cryo-EM structure of ABCG2. a-c) ABCG2 (PDB 5NJ3 [7], red + orange) after dynamic simulation with lipids (pale green). Close residues are labelled. d) Flow cytometry analysis of mitoxantrone (MX) intracellular fluorescence accumulation in control HEK293 cells (pcDNA) or expressing WT or N436A versions of ABCG2, and DMSO as control.

3. Conclusions

In this study, a library of 18 new homo- and hetero-dimers was designed and synthesized as highly potent and selective inhibitors of the human breast cancer resistance pump ABCG2. Dimerization of the monomeric indeno[1,2-*b*]indole **5a** led to the most active compounds **7a-c**, with ethyl, butyl and hexyl linkers, respectively. Extensive investigations have shown that the efficacy of compound **7b** (K_i of 17 nM) is due to an uncompetitive behavior toward mitoxantrone efflux.

Altogether these data lead us to conclude that different compounds can bind simultaneously and in distinct subsites into the large drug-binding pocket of ABCG2, as already observed for P-gp [8] and in contrast to the structural information recently released [9,10]. Lastly and importantly, further inhibitor design should consider this specific feature of ABCG2 to generate new inhibitors. A recent study [25] even confirms the involvement of ABC transporters (e.g. ABCG2) in acquired resistance to antibody-drug conjugates (ADCs)

containing pyrrolobenzodiazepine dimers. In the fight against cancer, a lot of hope is borne by ADCs. It becomes urgent to discover new nontoxic, highly active and selective inhibitors of ABCG2 to counter failures of anticancer drugs belonging to chemotherapy as well as targeted therapy.

4. Experimental

4.1. Chemistry

4.1.1. General

All chemicals and solvents were purchased from Acros, Aldrich or Alfa Aesar and used as such without further purification. Deuterated solvents were obtained from Eurisotop. Petroleum ether 40-60 °C is abbreviated as Pet. ether. Syntheses of compounds **2a**, **2b**, **3a**, **3b**, **4a**, **4b** and **5a** were already described [18,19].

Melting points were determined on an Electrothermal 9200 capillary apparatus. The IR spectra were recorded on a Perkin Elmer Spectrum Two IR Spectrometer.

The ¹H and ¹³C NMR spectra were recorded at 400 MHz on a Brücker DRX 400 spectrometer. Chemical shifts are expressed in ppm (δ) downfield from internal tetramethylsilane and coupling constants *J* are reported in hertz (Hz). The following abbreviations are used: s, singlet; bs, broad singlet; d, doublet; t, triplet; dd, doublet of doublet; dt, doublet of triplet; q, quartet; m, multiplet; Cquat, quaternary carbons.

The mass spectra were performed by direct ionization (EI or CI) on a ThermoFinnigan MAT 95 XL apparatus.

Chromatographic separations were performed on silica gel columns by column chromatography (Kieselgel 300–400 mesh). All reactions were monitored by TLC on GF254 plates that were visualized under a UV lamp (254 nm). Evaporation of solvent was performed in vacuum with rotating evaporator.

The purity of the final compounds (greater than 95%) was determined by uHPLC/MS on an Agilent 1290 system using a Agilent 1290 Infinity ZORBAX Eclipse Plus C18 column (2.1 x 50 mm, 1.8 μm particle size) with a gradient mobile phase of H₂O/CH₃CN (90:10, v/v) with 0.1% of formic acid to H₂O/CH₃CN (10:90, v/v) with 0.1% of formic acid at a flow rate of 0.5 mL/min, with UV monitoring at the wavelength of 254 nm with a runtime of 10 min.

4.1.2. Synthesis of key intermediate **4c**

4.1.2.1. *3,4b,9b-Trihydroxy-7-methyl-5-phenethyl-4b,5,6,7,8,9b-hexahydroindeno[1,2-b]indole-9,10-dione (3c)*

Equimolar amounts of 5-hydroxyninhydrin (2.0 g, 10.3 mmol, 1 equiv.) and enaminone **2b** (2.36 g, 10.3 mmol, 1 equiv.) were dissolved in methanol (20 mL). The reaction mixture was stirred at room temperature for 24 h. The precipitate was then filtered and washed with methanol to get compound **3c** as beige solid (3.46 g, 8.54 mmol, 83%). Mp 231 °C; R_f (acetone/MeOH, 9:1) 0.29. ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.76 (s, 2H, OH-3), 7.56 (dd,

$J = 8.3, 6.0$ Hz, 2H, Harom), 7.36-7.26 (m, 10H, Harom), 7.21 (s, 2H, Harom), 6.96 (dd, $J = 8.3, 1.3$ Hz, 2H, Harom), 6.79 (s, 1H, OH-4a), 6.74 (s, 1H, OH-4a), 5.63 (s, 1H, OH-9b), 5.60 (s, 1H, OH-9b), 3.94-3.64 (m, 4H, 2NCH₂), 3.12-2.86 (m, 4H, 2CH₂Ph), 2.43 (d, $J = 13.6$ Hz, 1H, Ha of CH₂-6), 2.30 (d, $J = 13.2$ Hz, 1H, Ha of CH₂-6), 2.11-2.00 (m, 2H, 2Hb of CH₂-6), 1.94-1.80 (m, 6H, 2CH₂-8 + 2CH-7), 0.94 (d, $J = 5.0$ Hz, 3H, CH₃), 0.89 (d, $J = 5.6$ Hz, 3H, CH₃). ¹³C NMR + DEPT (100 MHz, DMSO-*d*₆): δ 196.04 (C=O), 195.60 (C=O), 188.67 (C=O), 188.37 (C=O), 164.77 (Cquat), 164.73 (Cquat), 164.20 (Cquat), 164.09 (Cquat), 151.47 (Cquat), 151.17 (Cquat), 139.02 (2Cquat), 129.06 (2CH), 129.01 (2CH), 128.53 (5CH), 126.77 (Cquat), 126.52 (Cquat), 126.43 (CH), 125.62 (CH), 125.47 (CH), 118.68 (CH), 118.64 (CH), 109.60 (CH), 109.54 (CH), 105.00 (2Cquat), 95.58 (Cquat), 95.34 (Cquat), 83.66 (Cquat), 83.38 (Cquat), 45.42 (CH₂), 45.31 (CH₂), 43.51 (CH₂), 43.47 (CH₂), 36.89 (CH₂), 36.85 (CH₂), 30.03 (CH₂), 29.95 (CH₂), 29.31 (CH), 28.88 (CH), 20.82 (CH₃), 20.80 (CH₃). IR (ATR) 3302, 1709, 1613, 1588, 1563 cm⁻¹. HRMS m/z calcd for C₂₄H₂₄NO₅[M+H]⁺ 406.1649, found 406.1641.

4.1.2.2. 3-Hydroxy-7-methyl-5-phenethyl-5,6,7,8-tetrahydroindeno[1,2-*b*]indole-9,10-dione (**4c**)

Compound **3c** (0.97 g, 2.40 mmol, 1 equiv.), TETA (1.18g, 6.16 mmol, 2.5 equiv.), and acetic acid (1.13 mL) were dissolved in DMF (6.8 mL) and stirred at room temperature during 24 h. A crushed ice was added and the mixture was stirred for 1 h. The precipitate was filtered and washed with water and then dried. The filtrate was extracted with dichloromethane, dried over Na₂SO₄, and evaporated. Finally, the residue was purified by flash column chromatography on silica gel with CH₂Cl₂/acetone (90:10) as eluent to give an orange solid (561 mg, 1.51 mmol, 63%). Mp 280 °C; R_f (CH₂Cl₂/acetone, 2:1) 0.41. ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.35 (s, 1H, OH), 7.34-7.22 (m, 3H, Harom), 7.18-7.15 (m, 3H, Harom), 6.70 (d, $J = 2.1$ Hz, 1H, H-4), 6.47 (dd, $J = 8.0, 2.1$ Hz, 1H, H-2), 4.37-4.21 (m, 2H, NCH₂), 3.12-2.99 (m, 2H, CH₂Ph), 2.41 (d, $J = 13.3$ Hz, 1H, Ha of CH₂-6), 2.26 (d, $J = 12.8$ Hz, 1H, Hb of CH₂-6), 2.07-1.87 (m, 3H, CH₂-8 and H-7), 0.93 (d, $J = 5.4$ Hz, 3H, CH₃). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 191.99 (C=O), 183.40 (C=O), 161.92 (Cquat), 150.63 (Cquat), 150.41 (Cquat), 137.44 (Cquat), 136.85 (Cquat), 129.11 (2CH), 128.59 (Cquat), 128.48 (2CH), 126.79 (CH), 124.91 (CH), 119.74 (Cquat), 115.90 (Cquat), 111.70 (CH), 107.63 (CH), 46.84 (CH₂), 45.90 (CH₂), 36.01 (CH₂), 30.36 (CH), 28.92 (CH₂), 20.67 (CH₃). IR (ATR) 3283, 1703, 1658, 1598 cm⁻¹. HRMS m/z calcd for C₂₄H₂₂NO₃[M+H]⁺ 372.1594, found 372.1595.

4.1.3. General procedures for the synthesis of compounds **6a-f**

A solution of **5a** (0.54 mmol, 1 equiv.), K₂CO₃ (0.81 mmol, 1.5 equiv.), and the corresponding dibromoalkane (0.65 mmol, 1.2 equiv.) in acetonitrile was heated for 20 to 50 min under microwave irradiation at 130 °C. The mixture was then filtered and concentrated. The residue was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (98:2) as the eluent to give **6**.

4.1.3.1. 9-(2-Bromoethoxy)-5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one (**6a**). The title compound was synthesised according to the general procedure solution employing **5a** (200 mg, 0.54 mmol, 1 equiv.), K₂CO₃ (112 mg, 0.81 mmol, 1.5 equiv.) and 1,2-dibromoethane (56 μ L, 0.65 mmol, 1.2 equiv.), heated for 40 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (98:2) as the eluent to give an orange solid. Yield = 58% (149 mg, 0.131 mmol). Mp 98 °C; R_f (CH₂Cl₂/acetone, 98:2) 0.44. ¹H NMR (400 MHz, CDCl₃): δ 7.39-7.37 (m, 1H, Harom), 7.14-7.11 (m, 3H, Harom), 7.07 (t, *J* = 8.2 Hz, 1H, Harom), 6.85-6.83 (m, 2H, Harom), 6.70-6.65 (m, 3H, Harom), 6.58 (m, 1H, Harom), 4.46 (t, *J* = 6.6 Hz, 2H, OCH₂), 4.41 (t, *J* = 7.1 Hz, 2H, NCH₂), 3.80 (t, *J* = 6.7 Hz, 2H, CH₂Br), 3.67 (s, 3H, OCH₃), 3.10 (t, *J* = 7.2 Hz, 2H, CH₂Ph). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 184.21 (Cquat), 160.20 (Cquat), 158.06 (Cquat), 152.56 (Cquat), 143.89 (Cquat), 140.94 (Cquat), 138.94 (Cquat), 134.65 (Cquat), 132.03 (CH), 130.24 (CH), 129.68 (CH), 124.43 (CH), 123.30 (CH), 121.37 (CH), 118.19 (CH), 115.21 (CH), 115.07 (Cquat), 114.39 (CH), 112.64 (CH), 107.95 (CH), 105.25 (CH), 69.88 (CH₂), 55.52 (CH₃), 47.48 (CH₂), 36.71 (CH₂), 29.47 (CH₂). IR (ATR) 2927, 1697, 1601, 1575, 1261 cm⁻¹. HRMS *m/z* calcd for C₂₆H₂₃BrNO₃[M+H]⁺ 476.0856, found 476.0846.

4.1.3.2. 9-(4-Bromobutoxy)-5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one (**6b**). The title compound was synthesised according to the general procedure solution employing **5a** (200 mg, 0.54 mmol, 1 equiv.), K₂CO₃ (112 mg, 0.81 mmol, 1.5 equiv.) and 1,4-dibromobutane (78 μ L, 0.65 mmol, 1.2 equiv.), heated for 50 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (98:2) as the eluent to give an orange solid. Yield = 64% (174 mg, 0.346 mmol). Mp 121 °C; R_f (CH₂Cl₂/acetone, 98:2) 0.73. ¹H NMR (400 MHz, CDCl₃): δ 7.38-7.36 (m, 1H, Harom), 7.16-7.05 (m, 4H, Harom), 6.84-6.79 (m, 2H, Harom), 6.71-6.66 (m, 2H, Harom), 6.62-6.59 (m, 2H, Harom), 4.40 (t, *J* = 7.2 Hz, 2H, OCH₂), 4.16 (t, *J* = 5.9 Hz, 2H, NCH₂), 3.68 (s, 3H, OCH₃), 3.62 (t, *J* = 6.4 Hz, 2H, CH₂Br), 3.10 (t, *J* = 7.2 Hz, 2H, CH₂Ph), 2.36-2.29 (m, 2H, CH₂), 2.14-2.07 (m, 2H, CH₂). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 183.90 (Cquat), 159.87 (Cquat), 157.41 (Cquat), 153.17 (Cquat), 143.32 (Cquat), 140.67 (Cquat), 138.69 (Cquat), 134.48 (Cquat), 131.62 (CH), 129.89 (CH), 129.20 (CH), 124.25 (CH), 122.87 (CH), 121.05 (CH), 117.69 (CH), 114.74 (CH), 114.47 (Cquat), 114.23 (Cquat), 112.27 (CH), 104.99 (CH), 103.72 (CH), 67.16 (CH₂), 55.18 (CH₃), 47.10 (CH₂), 36.40 (CH₂), 34.49 (CH₂), 29.10 (CH₂), 27.81 (CH₂). IR (ATR) 2916, 1695, 1604, 1578, 1521, 1252 cm⁻¹. HRMS *m/z* calcd for C₂₈H₂₇BrNO₃[M+H]⁺ 504.1169, found 504.1165.

4.1.3.3. 9-((6-Bromohexyl)oxy)-5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one (**6c**). The title compound was synthesised according to the general procedure solution employing **5a** (200 mg, 0.54 mmol, 1 equiv.), K₂CO₃ (112 mg, 0.81 mmol, 1.5 equiv.) and 1,6-dibromohexane (100 μ L, 0.65 mmol, 1.2 equiv.), heated for 20 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (98:2) as the eluent to give an orange solid. Yield = 79% (227 mg, 0.426 mmol). Mp 106 °C; R_f (Pet. ether/EtOAc, 2:1) 0.60. ¹H NMR (400 MHz, CDCl₃): δ 7.41-7.38 (m, 1H, Harom), 7.16-7.06 (m, 4H, Harom), 6.85-6.79 (m, 2H, Harom), 6.71-6.67 (m, 2H, Harom), 6.64-6.60 (m, 2H, Harom), 4.43 (t, *J* = 7.2 Hz, 2H, OCH₂), 4.13 (t, *J* = 6.4 Hz, 2H, NCH₂), 3.68 (s, 3H, OCH₃), 3.45 (t, *J* = 6.8 Hz,

2H, CH₂Br), 3.11 (t, *J* = 7.2 Hz, 2H, CH₂Ph), 2.01-1.91 (m, 4H, 2CH₂), 1.73-1.66 (m, 2H, CH₂), 1.60-1.52 (m, 2H, CH₂). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 184.18 (Cquat), 160.24 (Cquat), 157.67 (Cquat), 153.77 (Cquat), 143.71 (Cquat), 141.10 (Cquat), 139.07 (Cquat), 134.91 (Cquat), 131.92 (CH), 130.23 (CH), 129.52 (CH), 124.60 (CH), 123.27 (CH), 121.41 (CH), 117.97 (CH), 115.09 (CH), 114.96 (Cquat), 114.72 (Cquat), 112.64 (CH), 105.49 (CH), 103.89 (CH), 68.67 (CH₂), 55.53 (CH₃), 46.46 (CH₂), 36.77 (CH₂), 34.45 (CH₂), 33.09 (CH₂), 29.50 (CH₂), 28.26 (CH₂), 25.36 (CH₂). IR (ATR) 2929, 2857, 1714, 1698, 1606, 1578, 1269 cm⁻¹. HRMS *m/z* calcd for C₃₀H₃₁BrNO₃[M+H]⁺ 532.1482, found 532.1488.

4.1.3.4. 9-((8-Bromooctyl)oxy)-5-(3-methoxyphenethyl)indeno[1,2-*b*]indol-10(5*H*)-one (**6d**).

The title compound was synthesised according to the general procedure solution employing **5a** (200 mg, 0.54 mmol, 1 equiv.), K₂CO₃ (112 mg, 0.81 mmol, 1.5 equiv.) and 1,8-dibromooctane (120 μL, 0.65 mmol, 1.2 equiv.), heated for 30 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (98:2) as the eluent to give an orange solid. Yield = 78% (236 mg, 0.421 mmol). Mp 101 °C; R_f (Pet. ether/EtOAc, 1:1) 0.66. ¹H NMR (400 MHz, CDCl₃): δ 7.41-7.39 (m, 1H, Harom), 7.17-7.06 (m, 4H, Harom), 6.85-6.83 (m, 1H, Harom), 6.80 (d, *J* = 8.1 Hz, 1H, Harom), 6.71-6.67 (m, 2H, Harom), 6.63-6.60 (m, 2H, Harom), 4.43 (t, *J* = 7.2 Hz, 2H, OCH₂), 4.12 (t, *J* = 6.5 Hz, 2H, NCH₂), 3.68 (s, 3H, OCH₃), 3.41 (t, *J* = 6.9 Hz, 2H, CH₂Br), 3.11 (t, *J* = 7.1 Hz, 2H, CH₂Ph), 2.00-1.93 (m, 2H, CH₂), 1.90-1.83 (m, 2H, CH₂), 1.67-1.60 (m, 2H, CH₂), 1.50-1.36 (m, 6H, 3CH₂). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 183.87 (Cquat), 159.91 (Cquat), 157.32 (Cquat), 153.52 (Cquat), 143.37 (Cquat), 140.78 (Cquat), 138.76 (Cquat), 134.60 (Cquat), 131.59 (CH), 129.91 (CH), 129.20 (CH), 124.27 (CH), 122.95 (CH), 121.10 (CH), 117.64 (CH), 114.77 (CH), 114.68 (Cquat), 114.39 (Cquat) 112.31 (CH), 105.13 (CH), 103.49 (CH), 68.61 (CH₂), 55.21 (CH₃), 47.14 (CH₂), 36.46 (CH₂), 34.18 (CH₂), 32.87 (CH₂), 29.28 (CH₂), 29.15 (CH₂), 28.68 (CH₂), 28.15 (CH₂), 25.72 (CH₂). IR (ATR) 2931, 2917, 1708, 1696, 1604, 1585, 1274 cm⁻¹. HRMS *m/z* calcd for C₃₂H₃₅BrNO₃[M+H]⁺ 560.1795, found 560.1796.

4.1.3.5. 9-((10-Bromodecyl)oxy)-5-(3-methoxyphenethyl)indeno[1,2-*b*]indol-10(5*H*)-one (**6e**).

The title compound was synthesised according to the general procedure solution employing **5a** (200 mg, 0.54 mmol, 1 equiv.), K₂CO₃ (112 mg, 0.81 mmol, 1.5 equiv.) and 1,10-dibromodecane (146 μL, 0.65 mmol, 1.2 equiv.), heated for 25 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (98:2) as the eluent to give an orange solid. Yield = 50% (159 mg, 0.27 mmol). Mp 47 °C; R_f (Pet. ether/EtOAc, 1:1) 0.41. ¹H NMR (400 MHz, CDCl₃): δ 7.41-7.39 (m, 1H, Harom), 7.16-7.06 (m, 4H, Harom), 6.85-6.83 (m, 1H, Harom), 6.80 (d, *J* = 7.9 Hz, 1H, Harom), 6.71-6.67 (m, 2H, Harom), 6.64-6.60 (m, 2H, Harom), 4.43 (t, *J* = 7.2 Hz, 2H, OCH₂), 4.12 (t, *J* = 6.6 Hz, 2H, NCH₂), 3.68 (s, 3H, OCH₃), 3.39 (t, *J* = 6.9 Hz, 2H, CH₂Br), 3.12 (t, *J* = 7.2 Hz, 2H, CH₂Ph), 2.00-1.93 (m, 2H, CH₂), 1.88-1.80 (m, 2H, CH₂), 1.65-1.57 (m, 2H, CH₂), 1.41-1.31 (m, 10H, 5CH₂). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 184.38 (Cquat), 160.43 (Cquat), 157.83 (Cquat), 154.08 (Cquat), 143.90 (Cquat), 141.32 (Cquat), 139.30 (Cquat), 135.14 (Cquat), 132.12 (CH), 130.44 (CH), 129.72 (CH), 124.81 (CH), 123.47 (CH), 121.63 (CH), 118.16 (CH), 115.29 (CH), 115.21 (Cquat), 114.92 (Cquat), 112.85 (CH), 105.67 (CH), 104.00 (CH), 69.20 (CH₂),

55.75 (CH₃), 47.67 (CH₂), 36.99 (CH₂), 34.69 (CH₂), 33.41 (CH₂), 29.99 (CH₂), 29.93 (CH₂), 29.85 (2CH₂), 29.31 (CH₂), 28.75 (CH₂), 26.34 (CH₂). IR (ATR) 2918, 2847, 1689, 1602, 1585, 1522, 1266 cm⁻¹. HRMS *m/z* calcd for C₃₄H₃₉BrNO₃[M+H]⁺ 588.2108, found 588.2099.

4.1.3.6. *9-((12-Bromododecyl)oxy)-5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one (6f)*. The title compound was synthesised according to the general procedure solution employing **5a** (200 mg, 0.54 mmol, 1 equiv.), K₂CO₃ (112 mg, 0.81 mmol, 1.5 equiv.) and 1,12-dibromododecane (213 mg, 0.65 mmol, 1.2 equiv.), heated for 40 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (98:2) as the eluent to give an orange solid. Yield = 69% (230 mg, 0.373 mmol). Mp 62 °C; R_f (CH₂Cl₂/acetone, 98:2) 0.76. ¹H NMR (400 MHz, CDCl₃): δ 7.41-7.39 (m, 1H, Harom), 7.16-7.11 (m, 3H, Harom), 7.08 (t, *J* = 8.1 Hz, 1H, Harom), 6.86-6.84 (m, 1H, Harom), 6.80 (d, *J* = 9.2 Hz, 1H, Harom), 6.71-6.67 (m, 2H, Harom), 6.64-6.60 (m, 2H, Harom), 4.43 (t, *J* = 7.2 Hz, 2H, OCH₂), 4.12 (t, *J* = 6.7 Hz, 2H, NCH₂), 3.68 (s, 3H, OCH₃), 3.39 (t, *J* = 6.9 Hz, 2H, CH₂Br), 3.12 (t, *J* = 7.2 Hz, 2H, CH₂Ph), 2.00-1.93 (m, 2H, CH₂), 1.88-1.80 (m, 2H, CH₂), 1.62-1.58 (m, 2H, CH₂), 1.42-1.28 (m, 14H, 7CH₂). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 184.49 (Cquat), 160.55 (Cquat), 157.93 (Cquat), 154.21 (Cquat), 144.01 (Cquat), 141.44 (Cquat), 139.40 (Cquat), 135.26 (Cquat), 132.22 (CH), 130.55 (CH), 129.82 (CH), 124.92 (CH), 123.59 (CH), 121.74 (CH), 118.27 (CH), 115.39 (CH), 115.34 (Cquat), 115.04 (Cquat), 112.97 (CH), 105.79 (CH), 104.09 (CH), 69.36 (CH₂), 55.85 (CH₃), 47.79 (CH₂), 37.11 (CH₂), 34.79 (CH₂), 33.51 (CH₂), 30.23 (CH₂), 30.20 (CH₂), 30.19 (CH₂), 30.11 (CH₂), 30.03 (CH₂), 29.98 (CH₂), 29.43 (CH₂), 28.85 (CH₂), 26.48 (CH₂). IR (ATR) 2923, 2852, 1687, 1602, 1579, 1263 cm⁻¹. HRMS *m/z* calcd for C₃₆H₄₃BrNO₃[M+H]⁺ 616.2421, found 616.2408.

4.1.4. General procedures for the synthesis of compounds **7a-f**

Method 1: A solution of **5a** (0.32 mmol, 1 equiv.), K₂CO₃ (0.48 mmol, 1.5 equiv.), and the brominated monomer intermediate **6** (0.32 mmol, 1 equiv.) in DMF was heated for 10 min under microwave irradiation at 160 °C. The mixture was then diluted with H₂O and extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and the solvent was then evaporated under vacuum. The resultant residue was purified by silica gel column chromatography using CH₂Cl₂/acetone (98:2) as the eluent to afford **7**.

Method 2: A solution of **5a** (0.54 mmol, 1 equiv.), K₂CO₃ (0.81 mmol, 1.5 equiv.), and the corresponding dibromoalkane (0.32 mmol, 0.6 equiv.) in acetonitrile was heated for 35 to 65 min under microwave irradiation at 130 °C. The solvent was then evaporated under vacuum and the resultant residue was purified by silica gel column chromatography using CH₂Cl₂/acetone (98:2) as the eluent to afford **7**.

4.1.4.1. *9,9'-(Ethane-1,2-diylbis(oxy))bis(5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one) (7a)*. The title compound was synthesised according to the method 2 of the general procedure employing **5a** (200 mg, 0.542 mmol, 1 equiv.), K₂CO₃ (112 mg, 0.813 mmol, 1.5

equiv.) and 1,2-dibromoethane (23 μ L, 0.27 mmol, 0.5 equiv.) heated for 65 min under microwave irradiation at 130 $^{\circ}$ C. The product was purified by flash chromatography on silica gel using CH_2Cl_2 /acetone (98:2) as the eluent to give a yellow solid. Yield = 75% (311 mg, 0.406 mmol). Mp 192 $^{\circ}$ C; R_f (CH_2Cl_2 /acetone, 98:2) 0.25. ^1H NMR (400 MHz, CDCl_3): δ 7.41-7.38 (m, 2H, Harom), 7.14-7.10 (m, 8H, Harom), 6.94 (d, J = 7.8 Hz, 2H, Harom), 6.86-6.83 (m, 4H, Harom), 6.71-6.67 (m, 4H, Harom), 6.60 (m, 2H, Harom), 4.73 (s, 4H, 2OCH_2), 4.43 (t, J = 7.2 Hz, 4H, 2NCH_2), 3.68 (s, 6H, 2OCH_3), 3.12 (t, J = 7.1 Hz, 4H, $2\text{CH}_2\text{Ph}$). ^{13}C NMR + DEPT (100 MHz, CDCl_3): δ 184.13 (2Cquat), 160.20 (2Cquat), 157.77 (2Cquat), 153.36 (2Cquat), 143.72 (2Cquat), 141.11(2Cquat), 139.09 (2Cquat), 134.91 (2Cquat), 131.90 (2CH), 130.21 (2CH), 129.50 (2CH), 124.83 (2CH), 123.20 (2CH), 121.40 (2CH), 117.99 (2CH), 114.99 (2CH+2Cquat), 114.77 (2Cquat), 112.69 (2CH), 107.34 (2CH), 104.45 (2CH), 68.16 (2CH_2), 55.52 (2CH_3), 47.50 (2CH_2), 36.79 (2CH_2). IR (ATR) 1702, 1602, 1576, 1521, 1271 cm^{-1} . HRMS m/z calcd for $\text{C}_{50}\text{H}_{41}\text{N}_2\text{O}_6[\text{M}+\text{H}]^+$ 765.2959, found 765.2938.

4.1.4.2. *9,9'-(Butane-1,4-diylbis(oxy))bis(5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one)* (**7b**). The title compound was synthesised according to the method 2 of the general procedure employing **5a** (200 mg, 0.542 mmol, 1 equiv.), K_2CO_3 (112 mg, 0.81 mmol, 1.5 equiv.) and 1,4-dibromobutane (32 μ L, 0.27 mmol, 0.5 equiv.) heated for 50 min under microwave irradiation at 130 $^{\circ}$ C. The product was purified by flash chromatography on silica gel using CH_2Cl_2 /acetone (98:2) as the eluent to give an orange solid. Yield = 45% (192 mg, 0.243 mmol). Mp 215 $^{\circ}$ C; R_f (CH_2Cl_2 /acetone, 98:2) 0.60. ^1H NMR (400 MHz, CDCl_3): δ 7.41-7.39 (m, 2H, Harom), 7.17-7.12 (m, 6H, Harom), 7.06 (t, J = 8.1 Hz, 2H, Harom), 6.84-6.82 (m, 2H, Harom), 6.76 (d, J = 8.2 Hz, 2H, Harom), 6.71-6.66 (m, 6H, Harom), 6.61 (m, 2H, Harom), 4.41 (t, J = 7.2 Hz, 4H, 2OCH_2), 4.32 (m, 4H, 2NCH_2), 3.68 (s, 6H, 2OCH_3), 3.11 (t, J = 7.1 Hz, 4H, $2\text{CH}_2\text{Ph}$), 2.41 (m, 4H, 2CH_2). ^{13}C NMR + DEPT (100 MHz, CDCl_3): δ 184.04 (2Cquat), 160.04 (2Cquat), 157.34 (2Cquat), 153.69 (2Cquat), 143.42 (2Cquat), 140.98 (2Cquat), 138.92 (2Cquat), 134.80 (2Cquat), 131.69 (2CH), 130.05 (2CH), 129.26 (2CH), 124.57 (2CH), 123.02 (2CH), 121.24 (2CH), 117.70 (2CH), 114.89 (2Cquat), 114.86 (2CH), 114.35 (2Cquat), 112.48 (2CH), 105.17 (2CH), 103.38 (2CH), 68.16 (2CH_2), 55.35 (2CH_3), 47.26 (2CH_2), 36.59 (2CH_2), 26.06 (2CH_2). IR (ATR) 1699, 1577, 1488, 1272 cm^{-1} . HRMS m/z calcd for $\text{C}_{52}\text{H}_{45}\text{N}_2\text{O}_6[\text{M}+\text{H}]^+$ 793.3272, found 793.3261.

4.1.4.3. *9,9'-(Hexane-1,6-diylbis(oxy))bis(5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one)* (**7c**). The title compound was synthesised according to the method 1 of the general procedure employing **5a** (100 mg, 0.271 mmol, 1 equiv.), K_2CO_3 (56 mg, 0.406 mmol, 1.5 equiv.) and brominated monomer intermediate **6c** (144 mg, 0.271 mmol, 1 equiv.) heated for 10 min under microwave irradiation at 160 $^{\circ}$ C. The product was purified by flash chromatography on silica gel using CH_2Cl_2 /acetone (98:2) as the eluent to give an orange solid. Yield = 64% (142 mg, 0.173 mmol). Mp 217 $^{\circ}$ C; R_f (CH_2Cl_2 /acetone, 98:2) 0.63. ^1H NMR (400 MHz, CDCl_3): δ 7.39-7.37 (m, 2H, Harom), 7.17-7.11 (m, 6H, Harom), 7.04 (t, J = 8.1 Hz, 2H, Harom), 6.85-6.82 (m, 2H, Harom), 6.76 (d, J = 8.2 Hz, 2H, Harom), 6.71-6.61 (m, 8H, Harom), 4.41 (t, J = 7.1 Hz, 4H, 2OCH_2), 4.18 (t, J = 6.5 Hz, 4H, 2NCH_2), 3.68 (s, 6H, 2OCH_3), 3.11 (t, J = 7.1 Hz, 4H, $2\text{CH}_2\text{Ph}$), 2.05-2.02 (m, 4H, 2CH_2), 1.79 (m, 4H, 2CH_2).

^{13}C NMR + DEPT (100 MHz, CDCl_3): δ 183.76 (2Cquat), 159.91 (2Cquat), 157.16 (2Cquat), 153.60 (2Cquat), 143.30 (2Cquat), 140.82 (2Cquat), 138.79 (2Cquat), 134.68 (2Cquat), 131.50 (2CH), 129.91 (2CH), 129.11 (2CH), 124.31 (2CH), 122.94 (2CH), 121.10 (2CH), 117.51 (2CH), 114.77 (4CH), 114.32 (2Cquat), 112.33 (2CH), 105.18 (2CH), 103.27 (2CH), 68.45 (2CH₂), 55.22 (2CH₃), 47.12 (2CH₂), 36.48 (2CH₂), 29.19 (2CH₂), 25.34 (2CH₂). IR (ATR) 1680, 1601, 1580, 1261 cm^{-1} . HRMS m/z calcd for $\text{C}_{54}\text{H}_{49}\text{N}_2\text{O}_6[\text{M}+\text{H}]^+$ 821.3585, found 821.3579.

4.1.4.4. *9,9'-(Octane-1,8-diylbis(oxy))bis(5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one)* (**7d**). The title compound was synthesised according to the method 2 of the general procedure employing **5a** (100 mg, 0.27 mmol, 1 equiv.), K_2CO_3 (56 mg, 0.406 mmol, 1.5 equiv.) and 1,8-dibromooctane (25 μL , 0.135 mmol, 0.5 equiv.) heated for 40 min under microwave irradiation at 130 $^\circ\text{C}$. The product was purified by flash chromatography on silica gel using $\text{CH}_2\text{Cl}_2/\text{acetone}$ (98:2) as the eluent to give an orange solid. Yield = 61% (140 mg, 0.165 mmol). Mp 152 $^\circ\text{C}$; R_f (Pet. ether/EtOAc, 2:1) 0.33. ^1H NMR (400 MHz, CDCl_3): δ 7.40-7.38 (m, 2H, Harom), 7.16-7.05 (m, 8H, Harom), 6.84-6.77 (m, 4H, Harom), 6.70-6.66 (m, 4H, Harom), 6.63-6.60 (m, 4H, Harom), 4.41 (t, $J = 7.2$ Hz, 4H, 2OCH₂), 4.13 (t, $J = 6.6$ Hz, 4H, 2NCH₂), 3.67 (s, 6H, 2OCH₃), 3.11 (t, $J = 7.1$ Hz, 4H, 2CH₂Ph), 2.01-1.94 (m, 4H, 2CH₂), 1.64-1.61 (m, 4H, 2CH₂), 1.48 (m, 4H, 2CH₂). ^{13}C NMR + DEPT (100 MHz, CDCl_3): δ 183.81 (2Cquat), 159.90 (2Cquat), 157.23 (2Cquat), 153.57 (2Cquat), 143.37 (2Cquat), 140.81 (2Cquat), 138.80 (2Cquat), 134.64 (2Cquat), 131.54 (2CH), 129.91 (2CH), 129.15 (2CH), 124.30 (2CH), 122.97 (2CH), 121.11 (2CH), 117.59 (2CH), 114.73 (2CH+2Cquat), 114.41 (2Cquat), 112.34 (2CH), 105.20 (2CH), 103.40 (2CH), 68.74 (2CH₂), 55.22 (2CH₃), 47.13 (2CH₂), 36.46 (2CH₂), 29.30 (4CH₂), 25.78 (2CH₂). IR (ATR) 2938, 1680, 1604, 1580, 1257 cm^{-1} . HRMS m/z calcd for $\text{C}_{56}\text{H}_{53}\text{N}_2\text{O}_6[\text{M}+\text{H}]^+$ 849.3898, found 849.3876.

4.1.4.5. *9,9'-(Decane-1,10-diylbis(oxy))bis(5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one)* (**7e**). The title compound was synthesised according to the method 2 of the general procedure employing **5a** (200 mg, 0.54 mmol, 1 equiv.), K_2CO_3 (112 mg, 0.81 mmol, 1.5 equiv.) and 1,10-dibromodecane (25 μL , 0.27 mmol, 0.5 equiv.) heated for 35 min under microwave irradiation at 130 $^\circ\text{C}$. The product was purified by flash chromatography on silica gel using $\text{CH}_2\text{Cl}_2/\text{acetone}$ (98:2) as the eluent to give an orange solid. Yield = 46% (218 mg, 0.248 mmol). Mp 95 $^\circ\text{C}$; R_f (Pet. ether/EtOAc, 2:1) 0.45. ^1H NMR (400 MHz, CDCl_3): δ 7.40-7.38 (m, 2H, Harom), 7.15-7.05 (m, 8H, Harom), 6.83-6.81 (m, 2H, Harom), 6.78 (d, $J = 8.1$ Hz, 2H, Harom), 6.70-6.67 (m, 4H, Harom), 6.65-6.59 (m, 4H, Harom), 4.41 (t, $J = 7.2$ Hz, 4H, 2NCH₂), 4.11 (t, 4H, $J = 6.8$ Hz, 2OCH₂), 3.67 (s, 6H, 2OCH₃), 3.10 (t, $J = 7.1$ Hz, 4H, 2CH₂Ph), 2.00-1.93 (m, 4H, 2CH₂), 1.65-1.56 (m, 4H, 2CH₂), 1.42-1.38 (m, 8H, 4CH₂). ^{13}C NMR + DEPT (100 MHz, CDCl_3): δ 184.02 (2Cquat), 160.09 (2Cquat), 157.46 (2Cquat), 153.76 (2Cquat), 143.56 (2Cquat), 141.00 (2Cquat), 139.00 (2Cquat), 134.81 (2Cquat), 131.76 (2CH), 130.10 (2CH), 129.35 (2CH), 124.49 (2CH), 123.15 (2CH), 121.30 (2CH), 117.82 (2Cquat), 114.92 (2CH), 114.90 (2CH), 114.60 (2Cquat), 112.54 (2CH), 105.39 (2CH), 103.63 (2CH), 68.94 (2CH₂), 55.42 (2CH₃), 47.32 (2CH₂), 36.66 (2CH₂), 29.81 (2CH₂), 29.60 (2CH₂), 29.53 (2CH₂), 26.06 (2CH₂). IR (ATR) 2921, 2850, 1687, 1603, 1254 cm^{-1} . HRMS m/z calcd for $\text{C}_{58}\text{H}_{57}\text{N}_2\text{O}_6[\text{M}+\text{H}]^+$ 877.4211, found 877.4180.

4.1.4.6. *9,9'-(Dodecane-1,12-diylbis(oxy))bis(5-(3-methoxyphenethyl)indeno[1,2-b]indol-10(5H)-one) (7f)*. The title compound was synthesised according to the method 2 of the general procedure employing **5a** (200 mg, 0.54 mmol, 1 equiv.), K₂CO₃ (112 mg, 0.81 mmol, 1.5 equiv.) and 1,12-dibromododecane (88 mg, 0.27 mmol, 0.5 equiv.) heated for 50 min under microwave irradiation at 130 °C. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (98:2) as the eluent to give an orange solid. Yield = 71% (347 mg, 0.383 mmol). Mp 133 °C; R_f (CH₂Cl₂/acetone, 95:5) 0.48. ¹H NMR (400 MHz, CDCl₃): δ 7.41-7.39 (m, 2H, Harom), 7.16-7.05 (m, 8H, Harom), 6.84-6.82 (m, 2H, Harom), 6.79 (d, *J* = 8.1 Hz, 2H, Harom), 6.70-6.59 (m, 8H, Harom), 4.41 (t, *J* = 7.2 Hz, 4H, 2OCH₂), 4.11 (t, *J* = 6.7 Hz, 4H, 2NCH₂), 3.67 (s, 6H, 2OCH₃), 3.10 (t, *J* = 7.1 Hz, 4H, 2CH₂Ph), 2.00-1.93 (m, 4H, 2CH₂), 1.61-1.55 (m, 4H, 2CH₂), 1.42-1.25 (m, 12H, 6CH₂). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 183.83 (2Cquat), 159.89 (2Cquat), 157.27 (2Cquat), 153.57 (2Cquat), 143.38 (2Cquat), 140.80 (2Cquat), 138.80 (2Cquat), 134.62 (2Cquat), 131.56 (2CH), 129.90 (2CH), 129.17 (2CH), 124.28 (2CH), 122.95 (2CH), 121.11 (2CH), 117.62 (2Cquat), 114.73 (2CH), 114.69 (2CH), 114.41 (2Cquat), 112.34 (2CH), 105.19 (2CH), 103.44 (2CH), 68.76 (2CH₂), 55.22 (2CH₃), 47.12 (2CH₂), 36.46 (2CH₂), 29.70 (2CH₂), 29.66 (2CH₂), 29.43 (2CH₂), 29.33 (2CH₂), 25.86 (2CH₂). IR (ATR) 2917, 1686, 1602, 1260 cm⁻¹. HRMS *m/z* calcd for C₆₀H₆₁N₂O₆[M+H]⁺ 905.4524, found 905.4480.

4.1.5. General procedures for the synthesis of compounds **8a-f**

A solution of **4c** (0.54 mmol, 1 equiv.), K₂CO₃ (0.81 mmol, 1.5 equiv.), and the corresponding dibromoalkane (0.32 mmol, 0.6 equiv.) in acetonitrile was heated for 40 to 50 min under microwave irradiation at 130 °C. The solvent was then evaporated under vacuum and the resultant residue was purified by silica gel column chromatography using CH₂Cl₂/MeOH (95:5, 97:3 or 98:2) as the eluent to afford **8**.

4.1.5.1. *3,3'-(Ethane-1,2-diylbis(oxy))bis(7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione) (8a)*. The title compound was synthesised according to the general procedure employing **4c** (250 mg, 0.67 mmol, 1 equiv.), K₂CO₃ (139 mg, 1.00 mmol, 1.5 equiv.) and 1,2-dibromoethane (35 μL, 0.404 mmol, 0.6 equiv.) for 50 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/MeOH (95:5) as the eluent to give a yellow solid. Yield = 80% (412 mg, 0.536 mmol). Mp 288 °C; R_f (CH₂Cl₂/acetone, 95:5) 0.41. ¹H NMR (500 MHz, DMSO-*d*₆+CDCl₃): δ 7.24-7.00 (m, 12H, Harom), 6.82-6.63 (m, 4H, Harom), 4.43-4.39 (s, 4H, 2OCH₂), 4.34-4.27 (m, 4H, 2NCH₂), 3.05 (t, *J* = 6.6 Hz, 4H, 2CH₂Ph), 2.53 (m, 2H, 2Ha of CH₂-6), 2.31 (d, *J* = 15.3 Hz, 2H, 2Hb of CH₂-6), 2.11-2.01 (m, 6H, 2CH₂-8 + 2H-7), 0.98 (d, *J* = 6.2 Hz, 6H, 2CH₃). ¹³C NMR + DEPT (125 MHz, DMSO-*d*₆+CDCl₃): δ 190.60 (2Cquat), 182.96 (2Cquat), 162.73 (2Cquat), 150.77 (2Cquat), 150.45 (2Cquat), 137.64 (2Cquat), 137.15 (2Cquat), 131.51 (2Cquat), 129.16 (4CH), 128.64 (4CH), 126.94 (2CH), 124.53 (2CH), 120.81 (2Cquat), 116.79 (2Cquat), 111.31 (2CH), 107.74 (2CH), 67.64 (2CH₂), 47.13 (2CH₂), 46.40 (2CH₂), 36.51 (2CH₂), 30.47 (2CH), 29.66 (2CH₂), 20.60 (2CH₃). IR (ATR) 2954, 1698, 1665, 1611, 1503, 1274 cm⁻¹. HRMS *m/z* calcd for C₅₀H₄₅N₂O₆[M+H]⁺ 769.3272, found 769.3267.

4.1.5.2. *3,3'-(Butane-1,4-diylbis(oxy))bis(7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione)* (**8b**). The title compound was synthesised according to the general procedure employing **4c** (200 mg, 0.539 mmol, 1 equiv.), K₂CO₃ (110 mg, 0.808 mmol, 1.5 equiv.) and 1,4-dibromobutane (38 μ L, 0.323 mmol, 0.6 equiv.) for 40 min. The product was washed with acetonitrile to give an orange solid. Yield = 75% (322 mg, 0.404 mmol). Mp 370 °C; R_f (CH₂Cl₂/MeOH, 95:5) 0.32; ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.22-7.09 (m, 12H, Harom), 6.60-6.58 (m, 4H, Harom), 4.27-4.25 (m, 4H, 2NCH₂ or 2OCH₂), 4.15 (m, 4H, 2OCH₂ or 2NCH₂), 3.04 (d, *J* = 5.7 Hz, 4H, 2CH₂Ph), 2.55 (d, *J* = 12.1 Hz, 2H, 2Ha of CH₂-6), 2.29 (d, *J* = 14.1 Hz, 2H, 2Hb of CH₂-6), 2.10-2.08 (m, 6H, 2CH₂-8 + 2H-7), 1.94 (m, 4H, 2CH₂), 0.97 (d, *J* = 7.4 Hz, 6H, 2CH₃). ¹³C NMR + DEPT (125 MHz, DMSO-*d*₆): δ 190.63 (2Cquat), 182.99 (2Cquat), 162.97 (2Cquat), 150.74 (2Cquat), 150.53 (2Cquat), 137.73 (2Cquat), 137.00 (2Cquat), 130.94 (2Cquat), 129.16 (4CH), 128.63 (4CH), 126.93 (2CH), 124.48 (2CH), 120.61 (2Cquat), 116.69 (2Cquat), 110.83 (2CH), 107.68 (2CH), 68.48 (2CH₂), 47.05 (2CH₂), 46.38 (2CH₂), 36.36 (2CH₂), 30.39 (2CH), 29.57 (2CH₂), 25.68 (2CH₂), 20.62 (2CH₃). IR (ATR) 1700, 1663, 1612, 1504, 1216 cm⁻¹. HRMS *m/z* calcd for C₅₂H₄₉N₂O₆[M+H]⁺ 797.3585, found 797.3583.

4.1.5.3. *3,3'-(Hexane-1,6-diylbis(oxy))bis(7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione)* (**8c**). The title compound was synthesised according to the general procedure employing **4c** (300 mg, 0.801 mmol, 1 equiv.), K₂CO₃ (167 mg, 1.21 mmol, 1.5 equiv.) and 1,6-dibromohexane (60 μ L, 0.404 mmol, 0.5 equiv.) for 40 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂-MeOH (97:3) as the eluent to give an orange solid. Yield = 46% (304 mg, 0.368 mmol). Mp 263 °C; R_f (CH₂Cl₂/MeOH, 95:5) 0.36. ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.24-7.17 (m, 8H, Harom), 7.11-7.08 (m, 4H, Harom), 6.61 (d, *J* = 2.1 Hz, 2H, Harom), 6.56 (dd, *J* = 8.0, 1.8 Hz, 2H, Harom), 4.31-4.22 (m, 4H, 2NCH₂), 4.06 (t, *J* = 6.3 Hz, 4H, 2OCH₂), 3.06-3.03 (m, 4H, 2CH₂Ph), 2.30 (dd, *J* = 15.2, 2.9 Hz, 2H, 2Ha of CH₂-6), 2.11-2.00 (m, 6H, 2CH₂-8 + 2Hb of CH₂-6), 1.83-1.80 (m, 4H, 2CH₂), 1.58-1.55 (m, 4H, 2CH₂), 1.27-1.25 (m, 2H, 2H-7), 0.98 (d, 6H, *J* = 6.3 Hz, 2CH₃). ¹³C NMR + DEPT (125 MHz, DMSO-*d*₆): δ 190.71 (2Cquat), 183.12 (2Cquat), 163.16 (2Cquat), 150.81 (2Cquat), 150.42 (2Cquat), 137.71 (2Cquat), 137.11 (2Cquat), 131.02 (2Cquat), 129.21 (4CH), 128.70 (4CH), 127.02 (2CH), 124.63 (2CH), 120.83 (2Cquat), 116.81 (2Cquat), 110.75 (2CH), 107.59 (2CH), 68.65 (2CH₂), 47.19 (2CH₂), 46.46 (2CH₂), 36.57 (2CH₂), 30.56 (2CH), 29.70 (2CH₂), 28.88 (2CH₂), 25.44 (2CH₂), 20.74 (2CH₃). IR (ATR) 1703, 1659, 1610, 1504, 1274 cm⁻¹. HRMS *m/z* calcd for C₅₄H₅₃N₂O₆[M+H]⁺ 825.3898, found 825.3858.

4.1.5.4. *3,3'-(Octane-1,8-diylbis(oxy))bis(7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione)* (**8d**). The title compound was synthesised according to the general procedure employing **4c** (200 mg, 0.539 mmol, 1 equiv.), K₂CO₃ (110 mg, 0.808 mmol, 1.5 equiv.) and 1,8-dibromooctane (59 μ L, 0.323 mmol, 0.6 equiv.) for 40 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/MeOH (98:2) as the eluent to give an orange solid. Yield = 71% (326 mg, 0.382 mmol). Mp 248 °C; R_f (CH₂Cl₂/MeOH, 95:5) 0.36. ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.25-7.17 (m, 8H, Harom),

7.12 (d, $J = 7.6$ Hz, 4H, Harom), 6.66 (d, $J = 2.1$ Hz, 2H, Harom), 6.57 (dd, 2H, $J = 8.0, 2.1$ Hz, Harom), 4.37-4.26 (m, 4H, 2NCH₂), 4.05 (t, 4H, $J = 6.4$ Hz, 2OCH₂), 3.06 (t, $J = 6.6$ Hz, 4H, 2CH₂Ph), 2.57 (d, $J = 13.7$ Hz, 2H, 2Ha of CH₂-6), 2.31 (d, $J = 15.7$ Hz, 2H, 2Hb of CH₂-6), 2.12-2.03 (m, 6H, 2CH₂-8 + 2H-7), 1.79-1.75 (m, 4H, 2CH₂), 1.51-1.43 (m, 8H, 4CH₂), 1.00 (d, $J = 3.8$ Hz, 6H, 2CH₃). ¹³C NMR + DEPT (125 MHz, DMSO-*d*₆): δ 190.57 (2Cquat), 183.01 (2Cquat), 163.13 (2Cquat), 150.79 (2Cquat), 150.44 (2Cquat), 137.76 (2Cquat), 137.07 (2Cquat), 130.90 (2Cquat), 129.17 (4CH), 128.62 (4CH), 126.93 (2CH), 124.54 (2CH), 120.65 (2Cquat), 116.73 (2Cquat), 110.99 (2CH), 107.54 (2CH), 68.80 (2CH₂), 47.07 (2CH₂), 46.39 (2CH₂), 36.39 (2CH₂), 30.43 (2CH), 29.59 (2CH₂), 28.85 (2CH₂), 28.75 (2CH₂), 25.57 (2CH₂), 20.61 (2CH₃). IR (ATR) 2939, 1703, 1660, 1609, 1504, 1274 cm⁻¹. HRMS *m/z* calcd for C₅₆H₅₇N₂O₆[M+H]⁺ 853.4211, found 853.4230.

4.1.5.5. *3,3'-(Decane-1,10-diylbis(oxy))bis(7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione) (8e)*. The title compound was synthesised according to the general procedure employing **4c** (200 mg, 0.539 mmol, 1 equiv.), K₂CO₃ (110 mg, 0.808 mmol, 1.5 equiv.) and 1,10-dibromodecane (72 μL, 0.323 mmol, 0.6 equiv.) for 40 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/MeOH (98:2) as the eluent to give an orange solid. Yield = 69% (327 mg, 0.372 mmol). Mp 244 °C; R_f (CH₂Cl₂/MeOH, 95:5) 0.4. ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.26-7.13 (m, 12H, Harom), 6.67-6.57 (m, 4H, Harom), 4.38-4.32 (m, 4H, 2NCH₂), 4.05 (t, $J = 6.3$ Hz, 4H, 2OCH₂), 3.08 (d, $J = 5.4$ Hz, 4H, 2CH₂Ph), 2.59 (d, $J = 12.3$ Hz, 2H, 2Ha of CH₂-6), 2.31 (d, $J = 15.3$ Hz, 2H, 2Hb of CH₂-6), 2.13-2.04 (m, 6H, 2CH₂-8 + 2H-7), 1.77-1.74 (m, 4H, 2CH₂), 1.44-1.28 (m, 12H, 6CH₂), 1.00 (d, $J = 5.8$ Hz, 6H, 2CH₃). ¹³C NMR + DEPT (125 MHz, DMSO-*d*₆): δ 190.57 (2Cquat), 183.01 (2Cquat), 163.13 (2Cquat), 150.82 (2Cquat), 150.44 (2Cquat), 137.77 (2Cquat), 137.07 (2Cquat), 130.90 (2Cquat), 129.18 (4CH), 128.62 (4CH), 126.92 (2CH), 124.54 (2CH), 120.64 (2Cquat), 116.73 (2Cquat), 111.01 (2CH), 107.54 (2CH), 68.80 (2CH₂), 47.08 (2CH₂), 46.39 (2CH₂), 36.39 (2CH₂), 30.45 (2CH), 29.60 (2CH₂), 28.98 (2CH₂), 28.87 (2CH₂), 28.82 (2CH₂), 25.64 (2CH₂), 20.61 (2CH₃). IR (ATR) 2922, 2850, 1703, 1660, 1610, 1504, 1274 cm⁻¹. HRMS *m/z* calcd for C₅₈H₆₁N₂O₆[M+H]⁺ 881.4524, found 881.4531.

4.1.5.6. *3,3'-(Dodecane-1,12-diylbis(oxy))bis(7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione) (8f)*. The title compound was synthesised according to the general procedure employing **4c** (200 mg, 0.539 mmol, 1 equiv.), K₂CO₃ (110 mg, 0.808 mmol, 1.5 equiv.) and 1,12-dibromododecane (100 mg, 0.323 mmol, 0.6 equiv.) for 50 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/MeOH (95:5) as the eluent to give an orange solid. Yield = 74% (362 mg, 0.399 mmol). Mp 221 °C; R_f (CH₂Cl₂/acetone, 9:1) 0.41. ¹H NMR (400 MHz, CDCl₃): δ 7.36 (d, $J = 7.8$ Hz, 2H, Harom), 7.23-7.21 (m, 6H, Harom), 6.97-6.94 (m, 4H, Harom), 6.45-6.43 (m, 4H, Harom), 4.17-4.04 (m, 4H, 2NCH₂), 3.94 (t, $J = 6.6$ Hz, 4H, 2OCH₂), 3.08-3.01 (m, 4H, 2CH₂Ph), 2.36 (d, $J = 12.7$ Hz, 2H, 2Ha of CH₂-6), 2.01-1.93 (m, 6H, 2CH₂-8 + 2Hb of CH₂-6), 1.84-1.74 (m, 4H, 2CH₂), 1.63-1.57 (m, 2H, 2H-7), 1.47-1.31 (m, 16H, 8CH₂), 0.88 (d, $J = 6.1$ Hz, 6H, 2CH₃). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 192.47 (2Cquat), 184.26 (2Cquat), 163.17 (2Cquat), 150.59 (4Cquat), 137.14 (2Cquat), 137.12 (2Cquat), 131.30 (2Cquat), 129.36 (4CH), 129.23

(4CH), 127.63 (2CH), 125.66 (2CH), 121.84 (2Cquat), 117.09 (2Cquat), 109.09 (2CH), 107.81 (2CH), 68.82 (2CH₂), 47.65 (2CH₂), 46.39 (2CH₂), 37.23 (2CH₂), 31.14 (2CH), 29.93 (2CH₂), 29.76 (4CH₂), 29.60 (2CH₂), 29.39 (2CH₂), 26.24 (2CH₂), 21.33 (2CH₃). IR (ATR) 2919, 1701, 1662, 1609, 1503, 1274 cm⁻¹. HRMS *m/z* calcd for C₆₀H₆₅N₂O₆[M+H]⁺ 909.4837, found 909.4836.

4.1.6. General procedures for the synthesis of compounds **9a-f**

A solution of **4c** (0.32 mmol, 1 equiv.), K₂CO₃ (0.48 mmol, 1.5 equiv.), and the brominated monomer intermediate **6** (0.32 mmol, 1 equiv.) in DMF was heated for 10 to 60 min under microwave irradiation at 160 °C. The mixture was then diluted with H₂O and extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and the solvent was then evaporated under vacuum. The resultant residue was purified by silica gel column chromatography using CH₂Cl₂/acetone (90:10, 95:5 or 98:2) as the eluent to afford **9**.

4.1.6.1. *3-(2-((5-(3-Methoxyphenethyl)-10-oxo-5,10-dihydroindeno[1,2-b]indol-9-yl)oxy)ethoxy)-7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione* (**9a**). The title compound was synthesised according to the general procedure employing **4c** (100 mg, 0.273 mmol, 1 equiv.), K₂CO₃ (56 mg, 0.409 mmol, 1.5 equiv.) and brominated monomer intermediate **6a** (130 mg, 0.273 mmol, 1 equiv.) for 40 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (95:5) as the eluent to give an orange solid. Yield = 62% (130 mg, 0.169 mmol). Mp 204 °C; R_f (CH₂Cl₂-MeOH, 95:5) 0.47. ¹H NMR (400 MHz, CDCl₃): δ 7.34-7.29 (m, 2H, Harom), 7.20-7.18 (m, 3H, Harom), 7.13- 7.05 (m, 4H, Harom), 6.94-6.91 (m, 2H, Harom), 6.85-6.81 (m, 2H, Harom), 6.72 (d, *J* = 7.8 Hz, 1H, Harom), 6.69-6.58 (m, 5H, Harom), 4.51 (s, 4H, 2OCH₂), 4.40 (t, *J* = 7.1 Hz, 2H, NCH₂), 4.15-4.03 (m, 2H, NCH₂), 3.67 (s, 3H, OCH₃), 3.09 (t, *J* = 7.1 Hz, 2H, CH₂Ph), 3.02 (t, *J* = 5.7 Hz, 2H, CH₂Ph), 2.35 (d, *J* = 12.7 Hz, 1H, Ha of CH₂-6), 1.96-1.90 (m, 3H, CH₂-8 + Hb of CH₂-6), 1.58-1.52 (m, 1H, H-7), 0.86 (d, 3H, *J* = 5.8 Hz, CH₃). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 192.73 (Cquat), 184.58 (Cquat), 184.49 (Cquat), 163.16 (Cquat), 160.49 (Cquat), 158.30 (Cquat), 153.26 (Cquat), 151.06 (Cquat), 150.87 (Cquat), 144.19 (Cquat), 141.23 (Cquat), 139.26 (Cquat), 137.56 (Cquat), 137.30 (Cquat), 134.96 (Cquat), 132.38 (CH), 132.05 (Cquat), 130.52 (CH), 129.92 (CH), 129.71 (2CH), 129.50 (2CH), 127.85 (CH), 125.89 (CH), 124.80 (CH), 123.44 (CH), 122.05 (Cquat), 121.69 (CH), 118.54 (CH), 117.33 (Cquat), 115.47 (Cquat), 115.40 (CH), 114.77 (Cquat), 112.90 (CH), 110.45 (CH), 108.70 (CH), 107.93 (CH), 105.43 (CH), 68.57 (CH₂), 68.13 (CH₂), 55.82 (CH₃), 47.93 (CH₂), 47.77 (CH₂), 46.71 (CH₂), 37.55 (CH₂), 36.98 (CH₂), 31.44 (CH), 30.22 (CH₂), 21.61 (CH₃). IR (ATR) 2951, 1708, 1675, 1598, 1578, 1523, 1501, 1268 cm⁻¹. HRMS *m/z* calcd for C₅₀H₄₃N₂O₆[M+H]⁺ 767.3116, found 767.3118.

4.1.6.2. *3-(4-((5-(3-Methoxyphenethyl)-10-oxo-5,10-dihydroindeno[1,2-b]indol-9-yl)oxy)butoxy)-7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione* (**9b**). The title compound was synthesised according to the general procedure employing **4c** (147 mg, 0.396 mmol, 1 equiv.), K₂CO₃ (82 mg, 0.59 mmol, 1.5 equiv.) and brominated

monomer intermediate **6b** (200 mg, 0.396 mmol, 1 equiv.) for 40 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂-acetone (98:2) as the eluent to give an orange solid. Yield = 60% (189 mg, 0.238 mmol). Mp 140 °C; R_f (CH₂Cl₂/acetone, 98:2) 0.5. ¹H NMR (400 MHz, CDCl₃): δ 7.33-7.31 (m, 1H, Harom), 7.22-7.20 (m, 3H, Harom), 7.13-7.03 (m, 5H, Harom), 6.94-6.92 (m, 2H, Harom), 6.75-6.73 (m, 2H, Harom), 6.67-6.55 (m, 4H, Harom), 6.39 (d, *J* = 2.0 Hz, 1H, Harom), 6.30 (dd, *J* = 8.1, 2.1 Hz, 1H, Harom), 4.33 (t, *J* = 7.1 Hz, 2H, OCH₂), 4.25-4.20 (m, 4H, OCH₂ + NCH₂), 4.11-3.97 (m, 2H, NCH₂), 3.65 (s, 3H, OCH₃), 3.08-2.99 (m, 4H, 2CH₂Ph), 2.36 (d, *J* = 12.3 Hz, 1H, Ha of CH₂-6), 2.27-2.20 (m, 2H, CH₂), 2.17-2.14 (m, 2H, CH₂), 1.97-1.91 (m, 3H, CH₂-8 + Hb of CH₂-6), 1.56-1.50 (m, 1H, H-7), 0.88 (d, *J* = 6.0 Hz, 3H, CH₃). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 191.68 (Cquat), 183.68 (2Cquat), 162.19 (Cquat), 159.49 (Cquat), 157.08 (Cquat), 152.79 (Cquat), 150.11 (Cquat), 149.46 (Cquat), 143.02 (Cquat), 140.33 (Cquat), 138.37 (Cquat), 136.49 (Cquat), 136.09 (Cquat), 134.09 (Cquat), 131.35 (CH), 130.09 (Cquat), 129.48 (CH), 128.67 (2CH), 128.60 (CH), 128.53 (2CH), 126.93 (CH), 124.65 (CH), 123.81 (CH), 122.14 (CH), 120.90 (Cquat), 120.73 (CH), 117.49 (CH), 116.38 (Cquat), 114.31 (CH), 114.12 (Cquat), 113.85 (Cquat), 111.99 (CH), 109.92 (CH), 106.22 (CH), 104.36 (CH), 103.24 (CH), 68.00 (CH₂), 67.42 (CH₂), 54.84 (CH₃), 46.92 (CH₂), 46.67 (CH₂), 45.75 (CH₂), 36.52 (CH₂), 35.93 (CH₂), 30.59 (CH), 29.23 (CH₂), 25.76 (CH₂), 25.37 (CH₂), 20.66 (CH₃). IR (ATR) 2922, 1696, 1670, 1601, 1525, 1503, 1263 cm⁻¹. HRMS *m/z* calcd for C₅₂H₄₇N₂O₆[M+H]⁺ 795.3429, found 795.3419.

4.1.6.3. 3-((6-((5-(3-Methoxyphenethyl)-10-oxo-5,10-dihydroindeno[1,2-*b*]indol-9-yl)oxy)hexyl)oxy)-7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-dione (**9c**). The title compound was synthesised according to the general procedure employing **4c** (34 mg, 0.094 mmol, 1 equiv.), K₂CO₃ (19 mg, 0.14 mmol, 1.5 equiv.) and brominated monomer intermediate **6c** (50 mg, 0.094 mmol, 1 equiv.) for 10 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (90:10) as the eluent to give an orange solid. Yield = 60% (46 mg, 0.056 mmol). Mp 107 °C; R_f (9:1 CH₂Cl₂-acetone) 0.48. ¹H NMR (400 MHz, CDCl₃): δ 7.33-7.31 (m, 1H, Harom), 7.22-7.17 (m, 4H, Harom), 7.10-7.04 (m, 4H, Harom), 6.94-6.91 (m, 2H, Harom), 6.78-6.76 (m, 1H, Harom), 6.73 (d, *J* = 8.2 Hz, 1H, Harom), 6.66 (dd, *J* = 7.9, 2.1 Hz, 1H, Harom), 6.62-6.58 (m, 2H, Harom), 6.56-6.55 (m, 1H, Harom), 6.40-6.36 (m, 2H, Harom), 4.34 (t, *J* = 7.1 Hz, 2H, OCH₂), 4.13 (t, *J* = 6.1 Hz, 2H, OCH₂), 4.04-3.97 (m, 4H, 2NCH₂), 3.65 (s, 3H, OCH₃), 3.07-2.99 (m, 4H, 2CH₂Ph), 2.38 (d, *J* = 12.7 Hz, 1H, Ha of CH₂-6), 1.99-1.96 (m, 3H, CH₂-8 + Hb of CH₂-6), 1.93-1.80 (m, 6H, 3CH₂), 1.66-1.52 (m, 3H, CH₂ + H-7), 0.88 (d, *J* = 5.9 Hz, 3H, CH₃). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 192.16 (Cquat), 184.08 (Cquat), 183.92 (Cquat), 162.97 (Cquat), 159.87 (Cquat), 157.42 (Cquat), 153.42 (Cquat), 150.41 (Cquat), 150.03 (Cquat), 143.33 (Cquat), 140.69 (Cquat), 138.74 (Cquat), 136.87 (Cquat), 136.50 (Cquat), 134.49 (Cquat), 131.70 (CH), 130.66 (Cquat), 129.87 (CH), 129.08 (CH), 129.06 (2CH), 128.90 (2CH), 127.31 (CH), 125.30 (CH), 124.19 (CH), 122.69 (CH), 121.43 (Cquat), 121.10 (CH), 117.79 (CH), 116.73 (Cquat), 114.68 (CH), 114.53 (Cquat), 114.23 (Cquat), 112.35 (CH), 109.25 (CH), 107.25 (CH), 104.72 (CH), 103.51 (CH), 67.99 (CH₂), 67.66 (CH₂), 55.22 (CH₃), 47.31 (CH₂), 47.07 (CH₂), 46.12 (CH₂), 36.92 (CH₂), 36.38 (CH₂), 30.88 (CH), 29.64 (CH₂), 29.08 (CH₂), 28.80 (CH₂), 24.96 (CH₂), 24.70 (CH₂), 21.03 (CH₃). IR (ATR) 2921,

1702, 1603, 1486, 1264 cm^{-1} . HRMS m/z calcd for $\text{C}_{54}\text{H}_{51}\text{N}_2\text{O}_6[\text{M}+\text{H}]^+$ 823.3742, found 823.3723.

4.1.6.4. *3-((8-((5-(3-Methoxyphenethyl)-10-oxo-5,10-dihydroindeno[1,2-b]indol-9-yl)oxy)octyl)oxy)-7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione (9d)*. The title compound was synthesised according to the general procedure employing **4c** (66 mg, 0.178 mmol, 1 equiv.), K_2CO_3 (37 mg, 0.268 mmol, 1.5 equiv.) and brominated monomer intermediate **6d** (100 mg, 0.178 mmol, 1 equiv.) for 50 min. The product was purified by flash chromatography on silica gel using $\text{CH}_2\text{Cl}_2/\text{acetone}$ (90:10) as the eluent to give an orange solid. Yield = 72% (109 mg, 0.128 mmol). Mp 163 $^\circ\text{C}$; R_f ($\text{CH}_2\text{Cl}_2/\text{acetone}$, 98:2) 0.69. ^1H NMR (400 MHz, CDCl_3): δ 7.35-7.32 (m, 2H, Harom), 7.23-7.21 (m, 3H, Harom), 7.14-7.04 (m, 4H, Harom), 6.96-6.93 (m, 2H, Harom), 6.82-6.76 (m, 2H, Harom), 6.69-6.58 (m, 4H, Harom), 6.47 (d, $J = 1.9$ Hz, 1H, Harom), 6.43 (dd, $J = 8.1, 1.9$ Hz, 1H, Harom), 4.40 (t, $J = 7.1$ Hz, 2H, OCH_2), 4.13-4.06 (m, 4H, $\text{OCH}_2 + \text{NCH}_2$), 3.95 (t, $J = 6.5$ Hz, 2H, NCH_2), 3.67 (s, 3H, OCH_3), 3.10-3.04 (m, 4H, 2 CH_2Ph), 2.37 (d, $J = 12.5$ Hz, 1H, Ha of CH_2 -6), 2.00-1.92 (m, 5H, $\text{CH}_2 + \text{CH}_2$ -8 + Hb of CH_2 -6), 1.82-1.78 (m, 2H, CH_2), 1.69-1.65 (m, 2H, CH_2), 1.60-1.67 (m, 7H, 3 $\text{CH}_2 + \text{H}$ -7), 0.88 (d, $J = 5.8$ Hz, 3H, CH_3). ^{13}C NMR + DEPT (100 MHz, CDCl_3): δ 192.18 (Cquat), 184.05 (Cquat), 183.86 (Cquat), 162.92 (Cquat), 159.89 (Cquat), 153.47 (Cquat), 150.38 (Cquat), 157.36 (Cquat), 150.16 (Cquat), 143.38 (Cquat), 140.73 (Cquat), 138.74 (Cquat), 136.88 (Cquat), 136.73 (Cquat), 134.54 (Cquat), 131.67 (CH), 130.92 (Cquat), 129.89 (CH), 129.17 (2CH), 129.07 (2CH), 128.91 (CH), 127.32 (CH), 125.45 (CH), 124.27 (CH), 122.84 (CH), 121.53 (Cquat), 121.11 (CH), 117.73 (CH), 116.75 (Cquat), 114.74 (CH), 114.59 (Cquat), 114.32 (Cquat), 112.34 (CH), 108.87 (CH), 107.61 (CH), 105.04 (CH), 103.57 (CH), 68.61 (CH_2), 68.52 (CH_2), 55.22 (CH_3), 47.35 (CH_2), 47.13 (CH_2), 46.10 (CH_2), 36.94 (CH_2), 36.42 (CH_2), 30.86 (CH), 29.63 (CH_2), 29.32 (CH_2), 29.16 (2 CH_2), 29.06 (CH_2), 25.83 (CH_2), 25.65 (CH_2), 21.02 (CH_3). IR (ATR) 2916, 1702, 1605, 1451, 1266 cm^{-1} . HRMS m/z calcd for $\text{C}_{56}\text{H}_{55}\text{N}_2\text{O}_6[\text{M}+\text{H}]^+$ 851.4055, found 851.4032.

4.1.6.5. *3-((10-((5-(3-Methoxyphenethyl)-10-oxo-5,10-dihydroindeno[1,2-b]indol-9-yl)oxy)decyl)oxy)-7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-b]indole-9,10(5H,6H)-dione (9e)*. The title compound was synthesised according to the general procedure employing **4c** (63 mg, 0.17 mmol, 1 equiv.), K_2CO_3 (35 mg, 0.255 mmol, 1.5 equiv.) and brominated monomer intermediate **6e** (100 mg, 0.17 mmol, 1 equiv.) for 15 min. The product was purified by flash chromatography on silica gel using $\text{CH}_2\text{Cl}_2/\text{acetone}$ (98:2) as the eluent to give an orange solid. Yield = 61% (91 mg, 0.104 mmol). Mp 96 $^\circ\text{C}$; R_f ($\text{CH}_2\text{Cl}_2/\text{acetone}$, 99:1) 0.45. ^1H NMR (400 MHz, CDCl_3): δ 7.38-7.36 (m, 2H, Harom), 7.23-7.22 (m, 3H, Harom), 7.15-7.04 (m, 4H, Harom), 6.97-6.95 (m, 2H, Harom), 6.84-6.82 (m, 1H, Harom), 6.78 (d, $J = 8.1$ Hz, 1H, Harom), 6.70-6.65 (m, 2H, Harom), 6.62-6.59 (m, 2H, Harom), 6.50 (d, 1H, $J = 2.0$ Hz, Harom), 6.45 (dd, $J = 8.1, 2.1$ Hz, 1H, Harom), 4.42 (t, $J = 7.2$ Hz, 2H, OCH_2), 4.18-4.07 (m, 4H, $\text{OCH}_2 + \text{NCH}_2$), 3.95 (t, $J = 6.5$ Hz, 2H, NCH_2), 3.67 (s, 3H, OCH_3), 3.12-3.05 (m, 4H, 2 CH_2Ph), 2.38 (d, $J = 12.5$ Hz, 1H, Ha of CH_2 -6), 2.01-1.93 (m, 5H, $\text{CH}_2 + \text{CH}_2$ -8 + Hb of CH_2 -6), 1.83-1.76 (m, 2H, CH_2), 1.61-1.55 (m, 3H, $\text{CH}_2 + \text{H}$ -7), 1.47-1.38 (m, 10H, 5 CH_2), 0.89 (d, $J = 6.0$ Hz, 3H, CH_3). ^{13}C NMR + DEPT (100 MHz,

CDCl₃): δ 192.77 (Cquat), 184.64 (Cquat), 184.46 (Cquat), 163.53 (Cquat), 160.53 (Cquat), 157.96 (Cquat), 154.12 (Cquat), 150.99 (Cquat), 150.75 (Cquat), 144.01 (Cquat), 141.38 (Cquat), 139.37 (Cquat), 137.49 (Cquat), 137.38 (Cquat), 135.19 (Cquat), 132.26 (CH), 131.58 (Cquat), 130.52 (CH), 129.77 (CH), 129.68 (2CH), 129.54 (2CH), 127.94 (CH), 126.07 (CH), 124.89 (CH), 123.47 (Cquat), 122.16 (CH), 121.72 (CH), 118.32 (CH), 117.39 (Cquat), 115.36 (Cquat), 115.23 (CH), 114.99 (Cquat), 112.97 (CH), 109.52 (CH), 108.18 (CH), 105.75 (CH), 104.17 (CH), 69.28 (CH₂), 69.20 (CH₂), 55.83 (CH₃), 47.98 (CH₂), 47.75 (CH₂), 46.72 (CH₂), 37.57 (CH₂), 36.04 (CH₂), 31.46 (CH), 30.25 (CH₂), 30.03 (CH₂), 30.03 (CH₂), 29.96 (CH₂), 29.93 (CH₂), 29.89 (CH₂), 29.68 (CH₂), 26.59 (CH₂), 26.43 (CH₂), 21.62 (CH₃). IR (ATR) 2920, 2848, 1698, 1667, 1602, 1501, 1264 cm⁻¹. HRMS *m/z* calcd for C₅₈H₅₉N₂O₆[M+H]⁺ 879.4368, found 879.4349.

4.1.6.6. 3-((12-((5-(3-Methoxyphenethyl)-10-oxo-5,10-dihydroindeno[1,2-*b*]indol-9-yl)oxy)dodecyl)oxy)-7-methyl-5-phenethyl-7,8-dihydroindeno[1,2-*b*]indole-9,10(5*H*,6*H*)-dione (**9f**). The title compound was synthesised according to the general procedure employing **4c** (90 mg, 0.24 mmol, 1 equiv.), K₂CO₃ (50 mg, 0.365 mmol, 1.5 equiv.) and brominated monomer intermediate **6f** (150 mg, 0.24 mmol, 1 equiv.) for 40 min. The product was purified by flash chromatography on silica gel using CH₂Cl₂/acetone (98:2) as the eluent to give an orange solid. Yield = 63% (137 mg, 0.151 mmol). Mp 91 °C; R_f (CH₂Cl₂/acetone, 98:2) 0.33. ¹H NMR (400 MHz, CDCl₃): δ 7.37-7.34 (m, 2H, Harom), 7.23-7.21 (m, 3H, Harom), 7.12-7.03 (m, 4H, Harom), 6.96-6.94 (m, 2H, Harom), 6.82-6.80 (m, 1H, Harom), 6.78 (d, 1H, *J* = 8.1 Hz, Harom), 6.69-6.61 (m, 2H, Harom), 6.59-6.58 (m, 2H, Harom), 6.46-6.42 (m, 2H, Harom), 4.40 (t, *J* = 7.2 Hz, 2H, OCH₂), 4.13-4.07 (m, 4H, OCH₂ + NCH₂), 3.94 (t, *J* = 6.5 Hz, 2H, NCH₂), 3.67 (s, 3H, OCH₃), 3.11-3.02 (m, 4H, 2 CH₂Ph), 2.35 (d, *J* = 12.8 Hz, 1H, Ha of CH₂-6), 1.97-1.90 (m, 5H, CH₂-8 + CH₂ + Hb of CH₂-6), 1.62-1.56 (m, 3H, CH₂ and H-7), 1.46-1.32 (m, 16H, 8CH₂), 0.87 (d, *J* = 6.4 Hz, 3H, CH₃). ¹³C NMR + DEPT (100 MHz, CDCl₃): δ 192.44 (Cquat), 184.29 (Cquat), 184.13 (Cquat), 163.16 (Cquat), 160.16 (Cquat), 157.62 (Cquat), 153.76 (Cquat), 150.63 (Cquat), 150.47 (Cquat), 143.66 (Cquat), 141.04 (Cquat), 139.05 (Cquat), 137.15 (Cquat), 137.04 (Cquat), 134.85 (Cquat), 131.93 (CH), 131.24 (Cquat), 130.18 (CH), 129.43 (CH), 129.35 (2CH), 129.21 (2CH), 127.61 (CH), 125.72 (CH), 124.55 (CH), 123.12 (CH), 121.81 (Cquat), 121.40 (CH), 117.98 (CH), 117.02 (Cquat), 115.00 (CH), 114.87 (Cquat), 114.64 (Cquat), 112.64 (CH), 109.12 (CH), 107.84 (CH), 105.40 (CH), 103.85 (CH), 68.98 (CH₂), 68.85 (CH₂), 55.50 (CH₃), 47.64 (CH₂), 47.41 (CH₂), 46.37 (CH₂), 37.22 (CH₂), 36.70 (CH₂), 31.12 (CH), 29.89 (CH₂), 29.85 (CH₂), 29.82 (CH₂), 29.79 (2CH₂), 29.68 (CH₂), 29.65 (CH₂), 29.60 (CH₂), 29.39 (CH₂), 26.28 (CH₂), 26.14 (CH₂), 21.29 (CH₃). IR (ATR) 2919, 1698, 1603, 1484, 1266 cm⁻¹. HRMS *m/z* calcd for C₆₀H₆₃N₂O₆[M+H]⁺ 907.4681, found 907.4668.

4.2. Biology

4.2.1. Reagents

Mitoxantrone, doxorubicin, Hoechst 33342 and Ko143 were purchased from Sigma Aldrich (Saint Quentin-Fallavier, France), dissolved in 100% dimethylsulfoxide at 20 mM stock-concentration and stored at -20 °C.

4.2.2. Cell cultures

Cell cultures were carried out as previously described [18,26,27]. The human fibroblast embryonic kidney 293, HEK293, cell line was transfected with the pcDNA3.1(+) plasmid, either empty (HEK293-*pcDNA3*) or coding for the human ABCG2 (HEK293-*ABCG2*) and selected with 750 µg/mL geneticin (Sigma Aldrich). HEK293-Flp-In™ cells were cotransfected with the pOG44 Flp recombinant expression vector and with the pCDNA5/FRT plasmid, either empty (HEK293-*pcDNA5*) or coding for the human ABCC1 (HEK293-*ABCC1*) and selected with 200 µg/mL hygromycin B (ThermoFisher Scientific). The murine embryonic fibroblast NIH3T3 parental cell line and NIH3T3/ABCB1 drug resistant cell line transfected with human MDR1/A-G185 [28], were from American Type Culture Collection (Manassas, VA. The NIH3T3/ABCB1 growth medium was also supplemented with 60 ng/mL colchicine. Cells were grown in cell growth medium containing Gibco® DMEM (Dulbecco/Vogt modified Eagle's minimal essential medium) High glucose with GlutaMAX™ (Fisher Scientific), 10% fetal bovine serum (Hyclone, GE Healthcare) and 1% penicillin/streptomycin (Sigma Aldrich) and maintained at 37 °C, 5% CO₂ under controlled humidity.

4.2.3. ABCG2-mediated mitoxantrone efflux and inhibition

Assays were carried out as routinely established in the laboratory [19]. Cells were seeded at a density of 1.0×10^5 cells/well into 24-well culture plates. After 48 hours incubation, the cells were exposed to 5 µM mitoxantrone for 30 min at 37 °C, in the presence or absence of each compound, and then washed with phosphate buffered saline (PBS) and trypsinized. The intracellular fluorescence was monitored with a FACS Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a 633-nm red laser, using the FL4 channel and at least 5000 events were collected. The percentage of inhibition was calculated by using the following equation: % inhibition = $(C-M)/(C_{ev}-M) \times 100$, where C is the intracellular fluorescence of ABCG2-expressing cells in the presence of compounds and mitoxantrone, M is the intracellular fluorescence of the same cells with only 5 µM mitoxantrone, and C_{ev} was estimated by measuring the intracellular fluorescence of ABCG2-expressing cells in the presence of 5 µM mitoxantrone and 1 µM of reference inhibitor Ko143 [11].

4.2.4. Mutagenesis

Human *ABCG2* WT and *ABCG2* N436A, coding genes were optimized and synthesized by GeneArt (Thermo Fisher Scientific) in the pcDNA3.1 plasmid. HEK293 cells were transfected by electroporation with the plasmid either empty or carrying *ABCG2* WT or mutant gene. Transfected cells were cultured as above. ABCG2-expressing monoclonal cell lines were then generated by FACS using the phycoerythrin-coupled 5D3 antibody (Santa Cruz Biotech) as native expression reporter. Cells were grown in a humidified atmosphere at 37 °C in 5% CO₂. Mitoxantrone efflux profile of ABCG2 N436A was determined as above and compared to HEK293-*pcDNA3* and HEK293-*ABCG2*, with a MacsQUANT VRB

Analyzer flow cytometer (Miltenyi Biotec) using a 635 nm red laser and recording fluorescence emission in a 655–730 nm R1 channel.

4.2.5. Compounds cytotoxicity

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay previously described [26]. HEK293-*pcDNA3* cells were seeded at a density of 1×10^4 cells/well into 96-well culture plates. After overnight incubation, the cells were treated with 0–100 μM compounds for 72 h. Cells were then exposed to 0.5 g/L MTT and incubated for 4 h at 37 °C. The culture medium was discarded, and 100 μL of a DMSO/ethanol (1:1) solution was added into each well and mixed by gently shaking for 10 min. Absorbance was measured at 570 nm and subtracted from the background at 690 nm using a microplate reader. Data are the mean \pm SD of at least three independent experiments.

4.2.6. Compounds specificity

Specificity of compounds towards ABCG2 was evaluated in triplicate within cells expressing the Multidrug Resistance Protein 1, MRP1, and the P-glycoprotein, P-gp, as previously described [19]. NIH3T3-*ABCB1* expressing the P-gp were seeded at a density of 6×10^4 cells/well into 24-well culture plates and incubated for 48 h at 37 °C, whereas HEK293 cells transfected with *ABCC1* expressing MRP1, were seeded at 2.5×10^5 cells/well for 48 h. The cells were respectively incubated with 0.5 μM rhodamine 123 or 0.2 μM calcein-AM for 30 min at 37 °C, with or without compounds. Cells were then washed with PBS, trypsinized and the intracellular fluorescence was monitored with a FACS Calibur cytometer (Becton Dickinson) using the FL1 channel and at least 5,000 events were collected. The percentage of inhibition was calculated relatively to 5 μM GF120918 for P-gp or 35 μM verapamil for MRP1, using similar equation as above for ABCG2 inhibition.

4.2.7. ATPase activity

Basal vanadate-sensitive ATPase activity of ABCG2 was measured by a colorimetric assay between ascorbic acid and free inorganic phosphate produced by ATP hydrolysis of ABCG2 expressed in *Sf9* membranes and loaded with cholesterol as previously described [29]. *Sf9* insect cell membranes at 1 mg/mL were incubated in a 50 mM Tris-HCl, pH 8.0, 50 mM NaCl and 3.3 mM sodium azide, in the absence or in the presence of 2 μM tested compounds. The reaction was started by the addition of 3.9 mM ATP-Mg, with or without 0.33 mM sodium orthovanadate to distinguish the ABCG2 vanadate-sensitive ATPase activity from endogenous ones. Plates were incubated for 30 min at 37 °C. The reaction was stopped with 10% sodium dodecylsulfate and revealed with a mixture of 7 mM ammonium molybdate reagent and 10% ascorbic acid. The absorbance was measured after 15 min incubation at 880 nm using a plate reader. Data are the mean \pm SD of at least three to five independent experiments done in triplicate.

4.2.8. 5D3 Shift Assay

HEK293-*ABCG2* transfected cells (0.5×10^5 cells) were centrifuged in aliquots at $200 \times g$ for 5 min at 37°C . The pellet was washed and suspended in PBS containing 0.5% BSA. Aliquots of the suspension were incubated with $2 \mu\text{M}$ tested compounds or 1% DMSO with the 5D3-PE primary antibody (purified antihuman *ABCG2*, 5D3 clone) or $10 \mu\text{g/mL}$ of the isotype control for 45 min at 37°C . After centrifugation at $200 \times g$ and washing, the cells were suspended in PBS containing 0.5% BSA and 5D3 binding was determined by flow cytometry using the FL2 channel collecting 10,000 events. The relative level of 5D3 binding was calculated in comparison with the fluorescence measured in the presence of Ko143, which was taken as 100% of 5D3 binding [30]. Data are the mean \pm SD of three independent experiments.

4.2.9. Inhibition mechanism of *ABCG2*-mediated substrate efflux

Assays and mathematical treatment of the experimental data were carried out as previously described [27] and above, in the “*ABCG2*-mediated mitoxantrone efflux and inhibition” §, using 0 to $6 \mu\text{M}$ mitoxantrone or Hoechst 33342 as substrates incubated with HEK293 and HEK293-*ABCG2* cells for 30 min at 37°C , in the presence of various concentrations of compounds in a range centered on their respective IC_{50} , to cover approximately 0, 25, 50, 75 and 100% expected inhibition. The intracellular fluorescence of each accumulated substrate was monitored, as above for mitoxantrone, and using a FACS LSR II (Becton Dickinson Biosciences, Franklin Lakes, New Jersey, USA) for Hoechst 33342, exciting the substrate with a 355 nm laser; and recovering the emission with a 450/50 nm band pass filter. All trials were standardized with mid-range FL1 fluorescence beads (Becton Dickinson Biosciences). Data were collected with CELLQUEST Pro 4.0 software or FACSDIVA 6.1.3 software (Becton Dickinson Biosciences) and then exported to FlowJo (TreeStar, Ashland, Oregon, USA) for analysis. Hoechst 33324 fluorescence was converted to apparent intracellular drug concentrations (pmol.mg^{-1}) by quantifying spectrophotometrically the intracellular amounts as detailed previously [27]. The same procedure was used for mitoxantrone, integrating the peak between 630 and 680 nm (Fig. S5). Regression analyses were carried out using R v3.5.1 with Tinn-R 5 and the set of equations detailed in Table S1 covering a panel of typical inhibition mechanisms based on the following general Scheme S1.

4.2.10. Statistical analyses

Data were plotted and fitted with SigmaPlot V12.5, GraphPad v8.0 or Excel365 as indicated. All data are represented as mean \pm SD. Statistical analyses were performed using Student's t-test. P values are indicated following the convention: ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

4.3. In silico experiments

The cryo-electron microscopy structure of ABCG2 [7] (PDB code 5NJ3) was used to carry out docking and molecular dynamic experiments after having removed the information related to the bound 5D3 antibody and cholesterol.

Molecular docking was carried out with MOE 2016.08 within the drug-binding pocket, as determined by site finder using the triangle matcher method with the first score function set to london dG. Sixty poses were generated from which the first thirty were refined using induced fit algorithm and scored again by the GBVI/WSA dG (Generalized-Born Volume Integral/Weighted Surface area) function [31].

Molecular dynamics simulations were carried out in a lipid environment, forming a DOPC lipid bilayer around the membrane region of ABCG2. Simulations were done with Amber v16, using Amberff14SB as force-field for proteins and General AMBER Force Field (gaff) for ligands and DOPC [32]. Ligand charges were determined using the AM1-BCC method [33]. All simulations were performed in solution using five-site transferable interaction potential (TIP3P) as water model and Cl^- and Na^+ ions for the protein-ligand system were placed in the model to neutralize the positive or negative charges around the complex at pH 7. Before MD simulation, the system was minimized through 3,000 steps of steepest descent minimization followed by 3,000 steps of conjugate gradient minimization. For bio-simulations, the system was thermalized during the first 100 ps with a temperature starting at 100 K that reached 303 K with lipid restraints under an NVT ensemble. Succeeding isothermal isobaric ensemble (NPT) of MD was carried out for equilibration during 1 ns with Langevin dynamics for temperature control. Equilibration run was followed by 40 ns-long MD simulation without position restraints under periodic boundary conditions using an NPT ensemble at 303 K. The Particle Mesh Ewald (PME) scheme was used to handle the long-range electrostatics and the cutoff of 12 Å was used for the Van der Waals interaction. The time step of molecular simulations was chosen to be 2 fs with 303 K as final temperature and 1 atm as pressure [34]. The SHAKE algorithm was used to constrain bond lengths on hydrogen atoms at their equilibrium values. Coordinates were saved for analyses every 50 ps. AmberTools16 was used to examine the time-dependence of the root mean squared deviation (RMSD).

Calculation of free binding energies was carried out using the MMGBSA approach provided in the Amber16 suite. Two hundred snapshots were chosen at time intervals of 20 ps from the last 4 ns of MD simulation using a salt concentration of 0.1 M and the Generalized Born (GB) implicit solvent model [35].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ORCID

Billel Belhani – orcid.org/0000-0002-5951-6322

Christelle Marminon – orcid.org/0000-0002-4043-3949

Raphaël Terreux – orcid.org/0000-0003-1243-7413

Zouhair Bouaziz – orcid.org/0000-0002-9948-0272

Malika Berredjem – orcid.org/0000-0002-4312-8771

Joachim Jose – orcid.org/0000-0002-0666-2676

Pierre Falson – orcid.org/0000-0002-9760-4577

Marc Le Borgne – orcid.org/0000-0003-1398-075X

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