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Structure–activity relationship of natural and synthetic coumarins inhibiting the multidrug transporter P-glycoprotein

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Abstract—A set of 32 natural and synthetic coumarins were tested in order to evaluate their activity on human leukemic cells (K562/R7) overexpressing P-glycoprotein (P-gp). Their ability to reduce the P-gp-mediated drug efflux of daunorubicin out of cells was evaluated at 10 μM. Four natural compounds, previously isolated from *Calophyllum dispar* (Clusiaceae) and substituted by a common α-(hydroxyisopropyl)dihydrofuran moiety, exhibited a significant inhibitory effect on P-gp when compared to the positive control cyclosporin A. A 3D-quantitative structure–activity relationship (3D-QSAR) analysis of the coumarins was performed using the biological results obtained by comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) of P-gp. Results showed a favorable electrostatic and steric volume, like the α-(hydroxyisopropyl)dihydrofuran moiety, beside C_5–C_6 or C_7–C_8 positions. In addition, the analysis revealed an important hydrophobic, neutral charge group, like phenyl, in position C_4 on the coumarinic ring.

1. Introduction

Multidrug resistance (MDR) is a major problem in the treatment of cancer. One main mechanism of this resistance is the over-expression, in cancer cells, of P-glycoprotein (P-gp), an ATP-dependent transmembrane efflux pump. P-gp recognizes a wide class of apolar compounds including anticancer agents and, functioning as efflux pump, extrudes them outside the cell.[1–3] Structurally, P-gp is a 1280 amino acid integral membrane phosphoglycoprotein with two homologous halves connected by a short stretch of linker region.[4] Each half consists of six putative transmembrane (TM) domains followed by a consensus nucleotide binding domain (NBD). The two halves of P-gp are essential for activity of the transporter as measured by the ability to confer drug resistance or drug-stimulated ATPase activity.[4] Different possibilities could be considered to limit the efflux of xenobiotics by P-gp, such as the blockage of specific substrate recognition, ATP hydrolysis, ATP binding, coupling of ATP hydrolysis to translocation of the substrate, or regulation of P-gp function by affecting posttranslational modifications. A wide panel of P-gp modulators have been studied; some, like cyclosporin A, verapamil, and progesterone, have also been evaluated in clinical trials.[5–9] Clinical trials using first- or second-generation modulators failed because of intrinsic toxicity of inhibitors or pharmacokinetic interactions. Over the last years, third-generation modulators have been developed which are especially selected for their ability to specifically inhibit P-gp in the absence of other pharmacological activity.[8]

In the research of P-gp inhibitors, some natural substances have been described as potential chemosensitizers.

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Flavonoids have been described as modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites.[10] A series of jatrophane polyesters isolated from the Mediterranean spurge *Euphorbia dendroides* L. served as models for the establishment of structure–activity relationships within this class of P-gp inhibitors.[11] Recently, another botanic family, the Clusiaceae, has been considered with interest for the presence of inhibitors of P-gp, as in the case of the phenolics isolated from different *Hypericum* species.[12] However, no data have been reported on coumarins, another class of chemical compounds largely present in the Clusiaceae family though some furanocoumarins, such as bergamottin and 6′,7′-dihydroxybergamottin, extracted from the grapefruit juice (Rutaceae), have already shown some inhibitory activity on P-gp.[12,13] In the course of our studies on the biologic interest of coumarins isolated from the Clusiaceae family,[14] we have evaluated the activity of 19 natural coumarins isolated from the Malaysian *Calophyllum dispar* on human cells overexpressing P-gp. Some synthetic coumarins with simple variations of the heterocyclic nucleus have also been tested. The present paper reports the results of this biological evaluation.

Finally, a 3D-quantitative structure–activity relationship analysis of these coumarins has been realized using the biological results obtained by comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) of P-gp. To our knowledge, only a few 3D-QSAR analyses of potent modulators of P-gp have been reported to date;[15,16] this is the first report of a study of coumarinic structures.

2. Results

2.1. Natural coumarins

Natural coumarins isolated from the *Calophyllum* genus belong, from a biogenetic point of view, to a homogeneous group of naturally occurring oxygen heterocycles with a biosynthetic scheme related to that of neoflavonoids. Except for some species of *Calophyllum*, the basic structure of these compounds consists in a 5,7-dioxygenated coumarinic nucleus, substituted by an alkyl or an aryl group at position 4. The other common elements are, at positions 6 and 8, a prenyl group and an α-unsaturated acyl moiety; both elements generally undergo cyclization with *ortho*-phenol groups, thus generating additional rings in the molecule, such as a pyran or a chromanone ring. Previous studies of *Calophyllum* coumarins reported two major types of biological activities. Tetracyclic coumarins such as calanolides are non-nucleoside inhibitors of HIV-1 reverse transcriptase,[17,18] whereas tricyclic coumarins exhibit interesting activities as anti-tumor promoters.[19,20]

Two *Calophyllum* species, *C. dispar* and *C. brasiliense*,[14,21] elaborate coumarins that are uncommon in the genus, substituted by saturated acyl chains and by prenyl variations typically associated with mammea-type coumarins, a class of compounds mainly present in the genus Mammea (Clusiaceae). In previous papers, we reported nineteen coumarins, mostly bicyclic, as novel natural compounds isolated from the stem bark of *C. dispar*.[22] In the course of our search for bioactive compounds from higher plants, we evaluated the activity of this homogeneous set of natural coumarins on P-gp.

2.2. Synthetic coumarins

Based on the first evaluations of the P-gp-inhibiting activity of some natural coumarins isolated from *C. dispar*, we synthesized coumarin structures to evaluate the impact of different sub-structures on biological activity. Some variations of the coumarinic nucleus were introduced, including an additional fused ring to elaborate benzocoumarins. Synthesis by one-step palladium-catalyzed addition between naphthol derivatives and propiolate reagents led to the elaboration of 5,6-benzocoumarin **1**, 7,8-benzocoumarin **2**, 4-phenyl-5,6-benzocoumarin **5**, and pyranocoumarin **10** (Fig. 1).[23]

As expected, palladium catalysis was not adapted to the synthesis of 4-phenylcoumarins.[23] The reaction procedure was then changed for the classical Pechmann condensation (Fig. 2),[24] catalyzed with trifluoroacetic acid or indium III chloride. This reaction presented some efficacy for the synthesis of 4-arylcoumarins, and led to the elaboration of 5,7-dihydroxy-4-picolinylcoumarin **28**, 8-hydroxy-4-phenyl-7,8-benzocoumarin **6**, 7-hydroxy-4-phenylcoumarin **7**, and 5,7-dihydroxy-4-phenylcoumarin **8**.

Finally, some prenylation reactions were carried out to evaluate the P-gp activity of coumarinic derivatives carrying hydrophobic prenyl group substitutions (**4**, **11**, and **14**).

2.3. Evaluation of P-glycoprotein inhibition

The activity of natural and synthetic coumarins was tested on a human erythroleukemic cell line overexpressing P-gp, K562/R7. Their ability to inhibit P-gp-mediated drug efflux was quantified by comparing the intracellular fluorescence of daunorubicin (DNR) in the presence

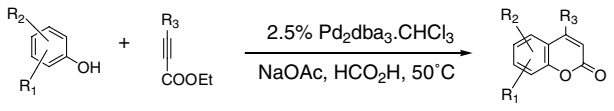


Figure 1. Palladium-catalyzed addition (R_1 and R_2 = H, OH, acyl, prenyl, and R_3 = H, alkyl, phenyl).

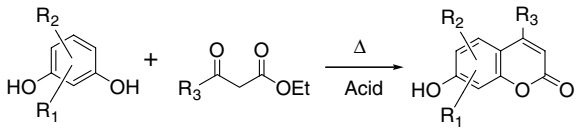


Figure 2. Pechmann condensation in the presence of trifluoroacetic acid or Indium III chloride (R_1 and R_2 = H, OH, acyl, prenyl, and R_3 = H, alkyl, phenyl, picolinyl).

or absence of inhibitor, and was expressed in comparison with values recorded with the positive control cyclosporin A (CsA): (Table 1)

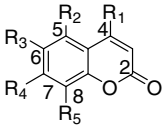
$$\text{Activity} = \frac{F_{\text{product}} - F_{\text{DNR}}}{F_{(\text{DNR+CsA})} - F_{\text{DNR}}} \times 100$$

where F_{product} stands for induced shift in fluorescence of DNR with the presence of coumarin, F_{DNR} for induced shift in fluorescence of DNR alone in the absence of inhibitor (negative control), and $F_{(\text{DNR+CsA})}$ for induced shift in fluorescence of DNR with the presence of CsA (positive control). Pure compounds at a concentration of 10 μM were used to limit the number of active compounds and also to reduce potential cytotoxic effects. Considering the stability of the cell population (size and granulosity), as shown by the analysis of the biparametric histogram of FACS (FSC–SSC), no cytotoxic effect of coumarins was observed on the K562/R7 cell line at this concentration. At this low concentration, coumarins with activity values, compared to cyclosporin A, of 20% and above were considered as potential inhibitors of P-gp (Table 2).

2.4. QSAR

A series of 32 coumarin molecules was modeled. For each model a geometric optimization was performed using the Sybyl molecular modeling package with the Merck molecular force field (MMFF94). During the optimization process, the dielectric constant was set to 80.0 and the electrostatic cutoff was set to 20 Å. After energy minimization, the conformation of each structure was checked to confirm the accurate energy state of the conformer (i.e., the lowest energy state). Each

Table 1. Structure of natural and synthetic coumarins



Compound	Origin[a]	R_1	R_2	R_3[b]	R_4	R_5[b]	(R_2–R_3)[c]	(R_4–R_5)[c]
1	S	H	—	—	H	H	**g**	—
2	S	H	H	H	—	—	—	**h**
3	S	H	H	H	OH	H	—	—
4	S	H	H	H	*O*-Prenyl	H	—	—
5	S	Phenyl	—	—	H	H	**g**	—
6	S	Phenyl	H	H	—	—	—	**i**
7	S	Phenyl	H	H	OH	H	—	—
8	S	Phenyl	OH	H	OH	H	—	—
9	S	Phenyl	H	H	OH	OH	—	—
10	S	Phenyl	H	OH	—	—	**f**	—
11	S	Phenyl	H	H	*O*-Prenyl	H	—	—
12	N	Phenyl	OH	Prenyl	OH	1-Oxobutyl	—	—
13	N	Phenyl	OH	3-MOB	OH	Prenyl	—	—
14	S	Phenyl	*O*-Prenyl	H	*O*-Prenyl	Prenyl	—	—
15	N	Phenyl	OH	1-Oxobutyl	OH	HMBE	—	—
16	N	Phenyl	OH	HMBE	OH	1-Oxobutyl	—	—
17	N	Phenyl	OH	2-MOB	OH	HMBE	—	—
18	N	Phenyl	OH	HMBE	OH	2-MOB	—	—
19	N	Phenyl	OH	3-MOB	OH	HMBE	—	—
20	N	Phenyl	OH	HMBE	OH	3-MOB	—	—
21	N	Phenyl	OH	1-Oxobutyl	—	—	—	**a**
22	N	Phenyl	OH	2-MOB	—	—	—	**a**
23	N	Phenyl	OH	3-MOB	—	—	—	**a**
24	N	Phenyl	OH	2-MOB	—	—	—	**c**
25	N	Phenyl	OH	3-MOB	—	—	—	**d**
26	N	Phenyl	—	—	OH	3-MOB	**b**	—
27	N	Phenyl	—	—	OH	3-MOB	**e**	—
28	S	Picolinyl	OH	H	OH	H	—	—
29	N	Propyl	OH	1-Oxobutyl	—	—	—	**a**
30	N	Propyl	OH	2-MOB	—	—	—	**a**
31	N	Propyl	OH	2-MOB	—	—	—	**c**
32	N	Propyl	OH	2-MOB	OH	HMBE	—	—

[a] S, synthetic coumarin and N, natural coumarin.
[b] HMBE, 2-hydroxy-3-methylbut-3-enyl, 2-MOB, 2-methyl-1-oxobutyl and 3-MOB for 3-methyl-1-oxobutyl.
[c]

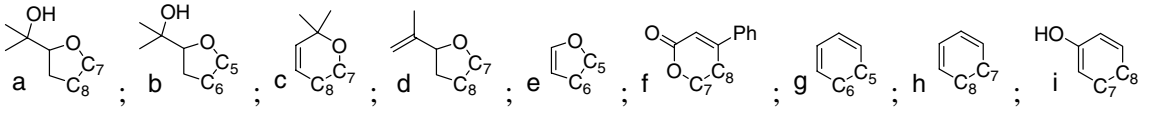


Table 2. Experimental, CoMFA, and CoMSIA values for P-gp-mediated drug efflux activities of coumarinic derivatives

Compound	Obsd[a]	CoMFA, $r^2 = 0.633$		CoMSIA, $r^2 = 0.705$	
		Calcd[b]	Dev[c]	Calcd[b]	Dev[c]
1	−10.5	−7.29	−3.24	−13.04	2.51
2	−2.6	−8.90	6.32	−9.99	7.42
3	−7.0	−10.98	3.98	−16.19	9.19
4	−8.8	4.40	−13.16	6.91	−15.67
5	0.7	1.41	−0.75	6.88	−6.22
6	9.5	14.44	−4.96	11.44	−1.96
7	1.7	7.19	−5.52	10.19	−8.52
8	3.4	1.01	2.43	3.93	−0.49
9	3.2	1.35	1.84	2.04	1.15
10	1.2	−1.16	2.37	2.75	−1.53
11	16.4	−0.72	17.12	0.44	15.95
12	9.1	16.41	−7.35	13.55	−4.49
13	3.3	1.26	2.02	2.07	1.21
14	4.1	17.45	−13.30	16.50	−12.35
15	3.9	7.88	−4.00	6.72	−2.84
16	6.9	10.70	−3.83	8.47	−1.60
17	8.4	8.94	−0.58	4.03	4.33
18	10.5	7.64	2.84	11.49	−1.01
19	2.6	6.59	−4.02	8.79	−6.22
20	8.3	6.27	2.01	6.12	2.16
21	26.5	35.87	−9.37	36.41	−9.91
22	35.9	24.80	11.07	25.95	9.93
23	34.9	21.17	13.75	28.76	6.16
24	3.9	16.15	−12.23	17.52	−13.60
25	4.4	9.63	−5.27	6.07	−1.71
26	32.5	12.91	19.59	12.64	19.86
27	16.5	17.12	−0.66	14.75	1.71
28	1.2	6.00	−4.78	−2.39	3.61
29	7.3	8.92	−1.58	8.39	−1.05
30	6.4	5.75	0.62	5.89	0.48
31	0.6	0.78	−0.15	−3.88	4.51
32	2.0	−8.86	10.90	−2.43	4.47

[a] Obsd, observed percentage values for the P-gp-mediated drug efflux activity of coumarin derivatives.
[b] Calcd, calculated values by CoMFA or CoMSIA.
[c] Dev, deviation values (obsd − calcd) per head CoMFA or CoMSIA analyses.

conformation was then added to a chemical structural database.

Despite a certain molecular diversity, all tested compounds have common structural cycles which are easily superimposable. The database was aligned by rigid alignment using atoms of the lactone ring, allowing to have an excellent superposition of the coumarinic nucleus of all molecules. In a second step, lateral chains of molecules were also aligned by rotation of the free torsion angle to the common conformation. For each molecule whose conformation had been modified, a second energy minimization was performed and the internal energy was checked; a difference lower than 20 kJ mol^{-1} was necessary to confirm the reliability of the conformation.

The CoMFA and CoMSIA methods are 3D QSAR techniques which determine activity relationships using molecular fields computed on the 3D model stored in the database. This method is particularly well adapted to treat a set of molecules with a common core. Starting from the structural database, all aligned structures were arranged in a cartesian grid extended by 4 Å in all space directions from the larger molecules. A C3-type carbon atom with a partial charge of +2 was used as a probe to describe the molecular field in the CoMFA study. The electrostatic and the steric interaction energy descriptors were used to describe molecular fields. A partial charge of +2 rather than +1 was used to give a predominant weight to electrostatic description, thus allowing a better representation of polarity and potential hydrogen bonding sites in the final structure–activity relationship equation. The ratio between steric and electrostatic descriptors was checked after statistical analysis; an electrostatic involvement below 65% was necessary to achieve an equilibrated system. The different values of steric and electrostatic descriptors were filtered with a value of 0.5 kcal mol^{-1} to avoid statistical artifacts. The partial least square (PLS) method was chosen with an optimal number of components set to 4. To improve the relationship the 'leave one out' validation strategy was chosen and the activity of all the molecules was predicted. Predicted and experimental values are given in Table 2. The coefficient of correlation in the calibration phase was approximately $r^2 = 0.984$ and $q^2 = 0.633$ for cross-predicted values.

A CoMSIA study was performed using the same molecular databases and alignment. Steric, electrostatic, hydrogen bond acceptor and donor, and hydrophobic descriptors were used as inputs to the spreadsheet. The PLS method was chosen to build a structure–activity relationship between descriptor and activity. This relation was tested by the leave-one-out scheme. The correlation coefficient was approximately $q^2 = 0.705$ and $r^2 = 0.910$ for fitting. The optimal number of

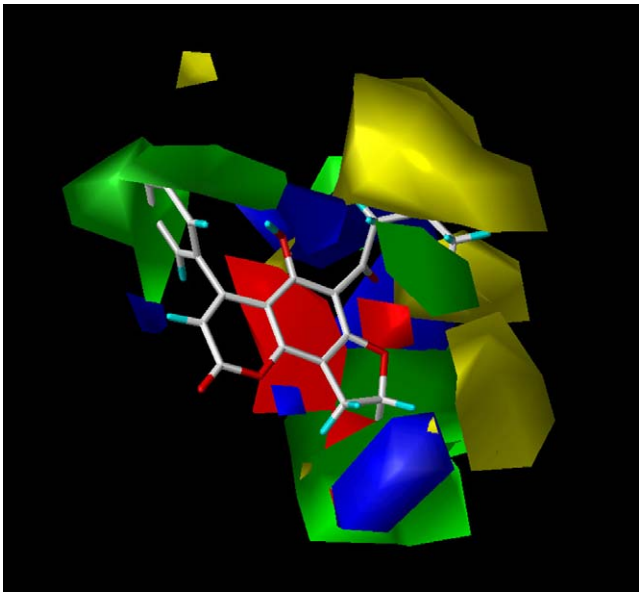


Figure 3. CoMFA with four types of volume, two for steric field and two for electrostatic field: (i) green volume means sterically favorable for activity but yellow volume is unfavorable; (ii) blue volume means electrostatically favorable for activity but red volume is unfavorable.

components for the PLS algorithm was 3. All types of descriptors were filtered with a 0.5 kcal mol^{-1} to select descriptors with an acceptable variation.

3. Discussion

An analysis of the distribution of significant probes in the space around the molecule gave some clue about the shape of the interaction site and the nature of the amino acid at its surface. This analysis was performed using CoMFA and CoMSIA methods. The coefficient of correlation for cross-validated activity gives better prediction for CoMSIA ($q^2 = 0.705$) method than for CoMFA ($q^2 = 0.633$). CoMSIA allowed the analysis of electrostatic, steric, hydrogen bond, and hydrophobic fields, whereas CoMFA allowed the analysis of electrostatic (Coulomb term) and steric (Lennard-Jones term) fields.

Volumes were calculated using the QSAR module of Sybyl 7.0 molecular modeling package. For CoMFA analysis (Fig. 3) the software provided four types of volumes: two for steric fields and two for electrostatic fields. marked in green represent steric interactions favorable for activity, which suggests the existence of a free space on the receptor in the volume. Volumes marked in yellow are unfavorable for activity, suggesting that this space is occupied by an amino acid of the receptor. Volumes marked in blue and red are correlated with the electrostatic field. For CoMSIA (Fig. 4) the same types of volumes were calculated for steric and electrostatic fields, but hydrogen bond and hydrophobic fields could also be generated. An analysis of all these volumes revealed common features and provided several clues about the shape of the interaction site, allowing to design an optimized molecule.

As shown in Table 2, the inhibition activity was largely influenced by slight modifications of the substitution pattern on the coumarinic nucleus. In Figure 4B, the yellow contour around the 4-position of the coumarinic core shows that the hydrophobic bulk was favorable there. Additional information for this region was provided by observation of the favorable green area above the 4-position in Figure 3 (steric map). As a consequence, the substitution of this position by a phenyl

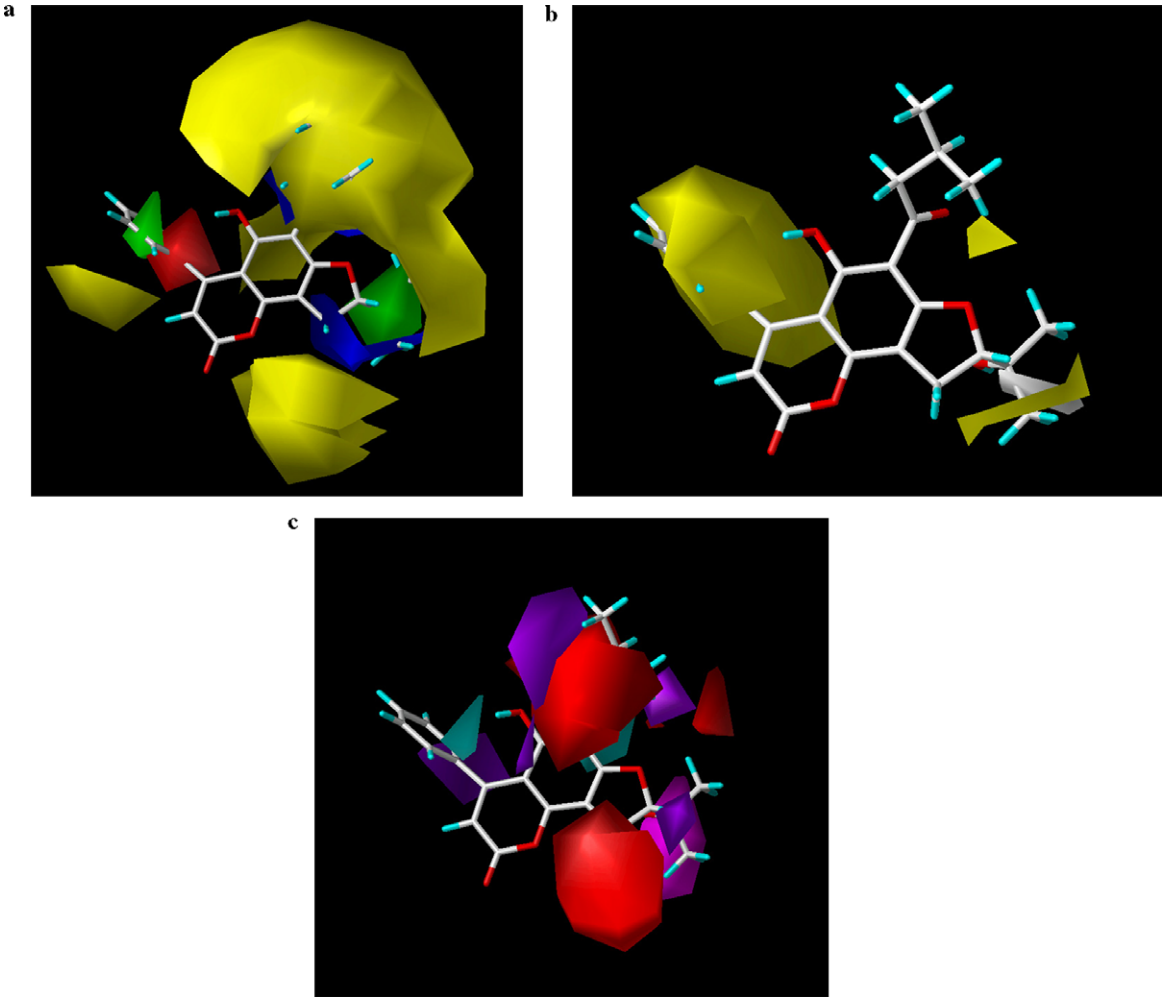


Figure 4. CoMSIA (a) with four types of volume, two for steric field (green for favorable but yellow for unfavorable) and two electrostatic field (blue for favorable but red for unfavorable); (b) with two types of hydrophobic volume (yellow for favorable but white for unfavorable); (c) with four types of volume, two for hydrogen bond donor field (cyan for favorable but violet for unfavorable) and two for hydrogen bond acceptor field (magenta for favorable but red for unfavorable).

moiety increased the activity. This can also be seen in 4-phenylcoumarins, **17**, **21**, **22**, and **24**, for which greater activities were recorded when compared to their 4-propyl analogues, **32**, **29**, **30**, and **31** respectively. Furthermore, no 4-hydrogenated coumarins (**1**–**4**) exhibited any positive activity. This confirms the interest for a large and hydrophobic substituent at the 4-position.

The regioisomeric relationships between different compounds provide useful information on the nature of substitutions on C_5–C_6 and C_7–C_8. Acyclic substitutions between 6- or 8-prenylcoumarins (**12** and **13**) and 6- or 8-(2-hydroxy-3-methylbut-3-enyl)coumarins (**15**–**20**) induced only slight variations in the inhibitory activity of P-gp. The fact that these compounds were also substituted at the 8- or 6-positions by a 4- or 5-carbon acyl moiety (1-oxobutyl, 2-MOB or 3-MOB) seems to emphasize the lack of selectivity of such acyclic chains (i.e., acyl, prenyl, and HMBE chains) at these positions.

In the case of coumarins with additional rings, coumarins with a [α-(hydroxyisopropyl)dihydrofuran] group in position C_7–C_8 (**21**–**23**) presented the higher activities of all studied coumarins. The substitution of the hydroxyisopropyl moiety by an isopropenyl group implied a dramatic loss of activity for dihydrofuranocoumarins (e.g., compound **23** compared to **25**). The observation of the favorable blue volume close to position C_7–C_8 in Figure 4A (electrostatic map) seems to indicate that the difference of activity recorded for the two compounds may be dependent on an electrostatic effect, present in **23** (with a hydroxyl group) and absent in **25**. The involvement of this electrostatic effect could also explain the greater activities recorded for coumarins with a [α-(hydroxyisopropyl)dihydrofuran] group (**22** and **30**) compared to their α,α-dimethylpyran analogues (**24** and **31**, respectively). In the case of additional rings at positions C_5–C_6, the substitution by the hydroxyisopropyl moiety also increased the activity of furanocoumarins (e.g., **26** vs **27**). It should be noted that an over-evaluation of unfavorable steric effect at positions C_5–C_6 was observed in QSAR analyses of compound **26**.

Finally, the prenyl group, known in xanthones and flavonoids as a favorable substituent for the inhibition of P-gp-mediated drug efflux because of its lipophilicity,[25] failed to appear in our experiments as an active moiety. No significant variation in activity was recorded by comparing the P-gp-mediated drug efflux of coumarins and of their prenylated analogues (**3**, **8**, **7** vs **4**, **14**, **11**, respectively) and by comparing prenylated coumarins and their related derivatives with 2-hydroxy-3-methylbut-3-enyl substituent (**12**, **13** vs **16**, **19**, respectively).

4. Conclusion

Out of 32 coumarins evaluated for their inhibitory activity on P-gp, four were considered active. A 3D-QSAR study was realized using the CoMFA and the CoMSIA for refining our research and orienting subsequent syntheses. It proved the importance of some substituents present on the basic coumarinic structure, such as the phenyl group at position C_4. The [α-(hydroxyisopropyl)dihydrofuran] group, specially at positions C_7–C_8, also showed some interest for activity compared to other additional groups. These results confirm that such dihydrofuranic moieties are of major importance for coumarinic compounds, as was previously reported for cnidiadin, a furanocoumarin with a [α,β-di(hydroxyisopropyl)dihydrofuran] group at positions C_7–C_8, which exhibited an anti-MDR activity on MDCK-MDR1 cell line.[26] From the results of the QSAR analyses, we can extrapolate the design of new molecules and new compounds will be shortly synthesized.

5. Experimental

5.1. Chemistry

5.1.1. Drugs and reagents. Cyclosporin A (CsA) was obtained from Novartis (Basel, Switzerland), Daunorubicin (DNR) from Aventis (Vitry, France), 7,8-dihydroxy-4-phenylcoumarin (**9**) from Sigma–Aldrich (Saint Quentin Fallavier, France), and Umbelliferon (**3**) from Carl Roth (Karlsruhe, Deutschland).

5.1.2. General. NMR spectra were recorded on DRX 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) and on DRX 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) instruments. Solvents were used as internal references ($CDCl_3$, DMSO-d_6 and acetone-d_6). Mass spectra (EI) were recorded with a GC/MS Nermag R10-10 spectrometer. TLC was carried out using Merck silica gel Si 60 F_{254} 20×20 cm plastic sheets and RP-18 F_{254S} 20×20 cm aluminum sheets and TLC plates on DIOL-F_{254}S 10×10 cm plates. Analytical HPLC was carried out on a Thermo Separation Products system equipped with a P-4000 quaternary gradient pump, a UV-6000LP photodiode array detector using analytical 125–4 mm columns packed with Merck Lichrospher 100 RP-18 (5 μm), and Macherey-Nagel Nucleosil 100-5 C_6H_6 endcapped. HPLC purifications performed with solvent gradient system (water/acetonitrile) and on a flow rate (1 ml/min.) yielded products with chemical purity greater than 93%. MPLC were carried out using Merck silica gel 60 (40–63 μm), Lichroprep 100DIOL (40–63 μm), or Lichroprep 60 RP-18 (40–63 μm) with UV detection at 254 and 366 nm.

5.1.3. Synthesis of 5,6-benzocoumarin (1). Ethylpropiolate (0.1 ml, 0.98 mmol) was added to a solution of 2-naphthol (70 mg, 0.49 mmol), sodium acetate (2.24 mg, 0.027 mmol), and tris(dibenzylideneacetone)di-palladium(0)chloroform($(dba)_3Pd_2{\cdot}CHCl_3$) (12.68 mg, 0.012 mmol) in 2.5 ml of formic acid. The mixture was stirred under nitrogen for 22 h. After dilution with water, the product was extracted with CH_2Cl_2, washed with an aqueous solution of sodium bicarbonate (5%) and a saturated solution of NaCl, filtered, and evaporated until dryness under vacuo. A MPLC with silica gel 60 (40–63 μm) using a gradient of hexane/

AcOEt gave 44.4 mg of compound **1** (46% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.53 (1H, d, J = 9.8 Hz, H-1), 8.26 (1H, d, J = 8.5 Hz, H-10), 8.02 (1H, d, J = 9.1 Hz, H-6), 7.95 (1H, d, J = 7.9 Hz, H-7), 7.72 (1H, td, J = 8.5 Hz and J = 1.0 Hz, H-9), 7.61 (1H, td, J = 7.9 Hz and J = 1.0 Hz, H-8), 7.50 (1H, d, J = 9.1 Hz), 6.62 (1H, d, J = 9.8 Hz, H-2). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 161.4 (C-3), 154.3 (C-4a), 139.6 (C-1), 133.6 (C-6), 130.7 (C-6a), 129.5 (C-7/10a), 128.7 (C-9), 126.5 (C-8), 121.8 (C-10), 117.5 (C-5), 116.1 (C-2), 113.4 (C-10b). MS (EI) *m/e*: 196 $[M]^+$. HRMS ($C_{13}H_8O_2$) calcd 196.0524, found 196.0529.

5.1.4. Synthesis of 7,8-benzocoumarin (2). Compound **2** was prepared, by a procedure similar to that described for compound **1**, from ethylpropiolate and 1-naphthol (8% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.56 (1H, m, H-10), 7.88 (1H, m, H-7), 7.84 (1H, d, J = 9.5 Hz, H-4), 7.69 (1H, d, J = 8.5 Hz, H-6), 7.65 (2H, m, H-8/9), 7.47 (1H, d, J = 8.5 Hz, H-5), 6.53 (1H, d, J = 9.5 Hz, H-3). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 161.4 (C-2), 151.8 (C-10b), 144.7 (C-4), 135.3 (C-6a), 129.2 (C-8), 128.2 (C-7), 127.6 (C-9), 124.9 (C-6), 124.0 (C-5), 123.5 (C-10a), 122.8 (C-10), 116.4 (C-2), 114.7 (C-4a). MS (EI) *m/e*: 196 $[M]^+$. HRMS ($C_{13}H_8O_2$) calcd 196.0524, found 196.0528.

5.1.5. Synthesis of 7-*O*-prenyl-coumarin (4). Tetrabutylammoniumhydroxide, 30 hydrate (1.17 g, 1.47 mmol) was added to a cold solution of umbelliferon **3** (119 mg, 0.73 mmol) and 20 ml CH_2Cl_2, followed in 15 min by the addition of 1-bromo-3-methyl-2-butene (127 µl, 1.1 mmol). The mixture was stirred at room temperature for 2 h. After dilution with water and acidification with HCl (1 N), the product was extracted with CH_2Cl_2, filtered, and evaporated until dryness under vacuo. A MPLC with silica gel 60 (40–63 µm) using a gradient of hexane/AcOEt gave 113.1 mg of compound **4** (67% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.70 (3H, s, H-4′), 1.74 (3H, s, H-5′), 4.50 (2H, d, J = 6.4 Hz, H-1′), 5.40 (1H, t, J = 13.2 Hz and 6.4 Hz, H-2′), 6.17 (1H, d, J = 10.0 Hz, H-3), 6.77 (2H, m, H-4/8), 7.29 (1H, d, J = 8.5 Hz, H-5), 7.55 (1H, d, J = 8.5 Hz, H-6). MS (EI) *m/e*: 230 $[M]^+$. HRMS ($C_{14}H_{14}O_3$) calcd 230.0943, found 230.0947.

5.1.6. Synthesis of 4-phenyl-5,6-benzocoumarin (5). Compound **5** was prepared, by a procedure similar to that described for compound **1**, from ethylphenylpropiolate and 2-naphthol (8% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.02 (1H, d, J = 9.1 Hz, H-6), 7.85 (1H, d, J = 7.9 Hz, H-7), 7.52 (3H, m, H-3′/4′/5′), 7.51 (1H, d, J = 9.1 Hz, H-5), 7.41 (1H, ddd, J = 7.0 Hz and 1.3 Hz, H-8), 7.37 (2H, m, H-2′/6′), 7.25 (1H, d, J = 8.8 Hz, H-10), 7.16 (1H, ddd, J = 7.0 Hz and 1.3 Hz, H-9), 6.39 (1H, s, H-2). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 160.8 (C-3), 156.9 (C-1), 155.2 (C-4a), 140.0 (C-1′), 134.4 (C-6), 131.7 (C-6a), 129.8 (C-10a), 129.7 (C-4′), 129.6(C-3′/5′), 129.4 (C-7), 127.9 (C-2′/6′), 127.1 (C-9), 126.4 (C-10), 125.8 (C-8), 117.9 (C-5), 117.2 (C-2), 113.5 (C-10b). MS (EI) *m/e*: 272 $[M]^+$. HRMS ($C_{19}H_{12}O_2$) calcd 272.0837, found 272.0840.

5.1.7. Synthesis of 8-hydroxy-4-phenyl-7,8-benzocoumarin (6). A solution of 1,6-dihydroxynaphthalen (927 mg, 5.8 mmol), ethylbenzoylacetate (1 ml, 5.8 mmol), and Indium III chloride (128 mg, 0.58 mmol) was refluxed under nitrogen for 2 h. After dilution with water, the product was extracted with CH_2Cl_2, filtered, and evaporated until dryness under vacuo. A MPLC with silica gel 60 (40–63 µm) using a gradient of hexane/AcOEt gave 44.4 mg of compound **6** (18% yield). ^{1}H NMR (acetone-d_6, 500 MHz): δ 6.32 (1H, s, H-3), 7.30 (1H, d, J = 2.2 Hz, H-7), 7.35 (1H, dd, J = 8.8 Hz and 2.2 Hz, H-6), 7.38 (1H, d, J = 8.8 Hz, H-5), 7.55 (1H, d, J = 9.1 Hz, H-9), 7.59 (5H, m, Ar), 8.41 (1H, d, J = 9.1 Hz, H-10), 9.24 (1H, s, 8-OH). ^{13}C NMR (acetone-d_6, 500 MHz): δ 110.0 (C-7), 112.3 (C-4a), 113.1 (C-3), 117.9 (C-10a), 119.7 (C-6), 122.8 (C-9), 123.3 (C-5), 124.6 (C-10), 128.9 (C-3′/5′), 129.2 (C-4′), 129.8 (C-2′/6′), 136.4 (C-1′), 137.6 (C-6a), 152.2 (C-10b), 156.9 (C-4), 158.6 (C-8), 160.0 (C-2). MS (EI) *m/e*: 288 $[M]^+$. HRMS ($C_{19}H_{12}O_3$) calcd 288.0786, found 288.0787.

5.1.8. Synthesis of 7-hydroxy-4-phenylcoumarin (7). Compound **7** was prepared, by a procedure similar to that described for compound **6**, from ethylbenzoylacetate and resorcinol (8% yield). Spectral and HPLC data were compared with those of the commercial product (Sigma–Aldrich). ^{1}H NMR (acetone-d_6, 300 MHz): δ 6.12 (1H, s, H-3), 6.84 (2H, m, H-6/8), 7.35 (1H, d, J = 9.4 Hz, H-5), 7.57 (5H, m, Ar).

5.1.9. Synthesis of 5,7-hydroxy-4-phenylcoumarin (8). Compound **8** was prepared, by a procedure similar to that described for compound **6**, from ethylbenzoylacetate and phloroglucinol (11% yield). Spectral and HPLC data were compared with those of the commercial product (Sigma–Aldrich). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 5.74 (1H, s, H-3), 6.17 (1H, d, J = 2.0 Hz, H-8), 6.27 (1H, d, J = 2.0 Hz, H-6), 7.38 (5H, m, Ar), 10.18 (2H, s, 2 OH).

5.1.10. Synthesis of 5-hydroxy-4,10-diphenyl-2H,8H-benzo[1,2-b; 3,4-b′]dipyran-2,8-dione (10). Compound **10** was prepared, by a procedure similar to that described for compound **1**, from ethylphenylpropiolate and phloroglucinol (7% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.51 (6H, m, H-3″/4″/5″/3′/4′/5′), 7.40 (4H, m, H-2″/6″/2′/6′), 6.84 (1H, *s*, H-6), 6.39(1H, s, 5-OH), 6.21 (1H, s, H-9), 6.04 (1H, s, H-3). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 160.2 (C-8), 158.1 (C-6a), 157.7 (C-5), 157.6 (C-2), 155.3 (C-4), 153.4 (C-10), 153.1 (C-10b), 138.5 (C-1′), 136.5(C-1′), 130.8 (C-4′), 130.0 (C-3′), 129.4 (C-5′), 128.6 (C-3″/4″/5″), 127.8 (2′/6′), 127.4 (C-2′/6′), 115.4 (C-3), 115.0 (C-9), 105.1 (C-4a), 103.0 (C-10a), 101.5 (C-6). MS (EI) *m/e*: 382 $[M]^+$. HRMS ($C_{24}H_{14}O_5$) calcd 382.0841, found 382.0842.

5.1.11. Synthesis of 7-*O*-prenyl-4-phenyl-coumarin (11). Compound **11** was prepared, by a procedure similar to that described for compound **4**, from 7-hydroxy-4-phenylcoumarin **7** (95% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.78 (3H, s, H-4″), 1.81 (3H, s, H-5″), 4.59 (2H, d, J = 6.2 Hz, H-1″), 5.47 (1H, t, J = 12.5 Hz and 6.2 Hz, H-2″), 6.21 (1H, s, H-3), 6.79 (1H, dd,

J = 2.3 Hz and 8.5 Hz, H-6), 6.89 (1H, d, J = 2.3 Hz, H-8), 7.37 (1H, d, J = 8.5 Hz, H-5), 7.46 (5H, m, Ar). MS (EI) *m/e*: 306 $[M]^+$. HRMS ($C_{20}H_{18}O_3$) calcd 306.1256, found 306.1261.

5.1.12. Synthesis of 5,7-di-*O*-prenyl-8-prenyl-4-phenyl-coumarin (14). Compound **14** was prepared, by a procedure similar to that described for compound **4**, from 5,7-dihydroxy-4-phenyl-coumarin **8** (17% yield). ^1H ($CDCl_3$, 500 MHz): δ 1.53 (3H, s, H-4″), 1.62 (3H, s, H-5″), 1.70 (3H, s, H-13), 1.77 (3H, s, H-4‴), 1.82 (3H, s, H-5‴), 1.86 (3H, s, H-12), 3.52 (2H, d, J = 7.3 Hz, H-9), 4.15 (2H, d, J = 6.6 Hz, H-1″), 4.61 (2H, d, J = 6.6 Hz, H-1‴), 4.62 (1H, t, J = 7.0 Hz, H-2″), 5.29 (1H, t, J = 7.2 Hz, H-10), 5.49 (1H, t, J = 6.6 Hz, H-2‴), 5.98 (1H, s, H-3), 6.27 (1H, s, H-6), 7.25 (2H, m, H-2′/6′), 7.35 (3H, m, H-3′/4′/5′). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 18.3 (C-12), 18.4 (C-4″), 18.7 (C-5‴), 22.3 (C-9), 26.0 (C-5″), 26.2 (C-4‴), 26.3 (C-13), 65.8 (C-1″), 65.9 (C-1‴), 94.1 (C-6), 104.0 (C-4a), 111.3 (C-8), 112.8 (C-3), 119.1 (C-2″), 119.8 (C-2‴), 122.4 (C-10), 127.5 (C-2′/6′), 127.7 (C-4′), 127.8 (C-3′/5′), 132.3 (C-11), 137.4 (C-3″), 138.6 (C-3‴), 140.9 (C-1′), 154.2 (C-8a), 155.9 (C-5), 156.3 (C-4), 160.3 (C-7), 161.5 (C-2). MS (EI) *m/e*: 458 $[M]^+$. HRMS ($C_{30}H_{34}O_5$) calcd 458.2457, found 458.2473.

5.1.13. Synthesis of 5,7-dihydroxy-4-picolinylcoumarin (28). A solution of phloroglucinol (128 mg, 1 mmol) and ethylpicolinoylacetate (196 mg, 1 mmol) in 10 ml of trifluoroacetic acid was refluxed for 2 h. A saturated solution of sodium bicarbonate was added until precipitation, then filtered. A VLC with silica gel 60 (40–63 μm) using a gradient of hexane/AcOEt gave 157 mg of compound **28** (61% yield). ^1H NMR (DMSO-d_6, 500 MHz): δ 10.30 (1H, s, 5-OH), 10.25 (1H, s, 7-OH), 8.57 (1H, d, J = 4.4 Hz, H-3′), 7.83 (1H, ddd, J = 1.6 Hz, 1.9 Hz and 7.6 Hz, H-5′), 7.45 (1H, d, J = 7.9 Hz, H-6′), 7.40 (1H, m, H-4′), 6.28 (1H, d, J = 2.2 Hz, H-8), 6.15 (1H, d, J = 2.2 Hz, H-6), 5.87 (1H, s, H-3). ^{13}C NMR (DMSO, 500 MHz): δ 162.8 (C-7), 160.9 (C-2), 158.0 (C-1′), 157.7 (C-5), 157.6 (C-8a), 155.1 (C-4), 149.0 (C-3′), 136.8 (C-5′), 123.8 (C-6′/4′), 110.9 (C-3), 101.3 (C-4a), 100.0 (C-6), 95.5 (C-8). MS (EI) *m/e*: 255 $[M]^+$. HRMS ($C_{14}H_9NO_4$) calcd 255.0532, found 255.0537.

5.2. Biological activity—materials and instruments

5.2.1. Cell culture. The human erythroleukemic cell line K562 was purchased from the American Type Culture Collection. The K562/R7 MDR cell line was obtained by prolonged exposure of K562 cells to doxorubicin.[27] Cell lines were cultured in RPMI 1640 supplemented with 10% newborn-calf serum, 2 mM glutamine, 200 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2 atmosphere.

5.2.2. Drug accumulation assay. One million K562/R7 human leukemic cells expressing high levels of P-glycoprotein were incubated for 1 h at 37 °C in 1 ml RPMI 1640 medium containing a final concentration of 10 μM daunorubicin, in the presence or absence of inhibitor. The cells were then washed twice with ice-cold phosphate-buffered saline (PBSt), and kept on ice until analysis by flow cytometry on a FACS-II (Centre Léon Bérard, Lyon, France). Assays were performed in duplicate, with at least three separate experiments. CsA, an inhibitor of P-glycoprotein, was used as a positive control at 2 μM final concentration. Compounds were tested at 10 μM concentration.

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