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Analogues of the Lignan Pinoresinol as Novel Lead Compounds for P-glycoprotein (P-gp) Inhibitors

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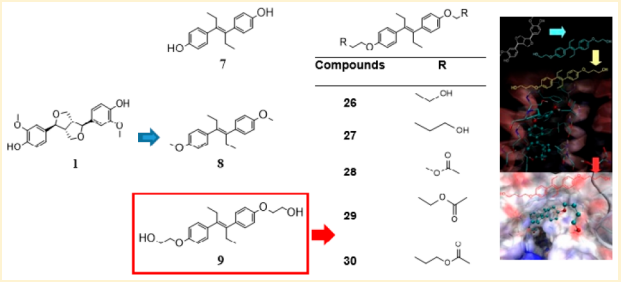
Supporting Information

ABSTRACT: To find novel P-gp-inhibitors, a library of pregnane X receptor (PXR) ligands and the ZINC DrugsNow library were superimposed on the P-gp inhibitor (+)-pinoresinol (**1**) used as a query for a three-dimensional similarity search. After determining the TanimotoCombo index of similarity with **1**, eight compounds from the PXR library and two ZINC compounds were selected for biological evaluation. The P-gp inhibition study showed that compounds **7**, **8**, and **9** successfully increased intracellular doxorubicin (DOX) accumulation in the P-gp overexpressed Lucena 1 cells from 25, 12.5, and 6.25 μM, respectively. Among a series of analogues of **9**, compounds **26**−**30** were shown to be active, with **26** and **27** causing a significant increase in DOX accumulation from 1.56 μM and rendering Lucena 1 sensitive to DOX from 1.56 and 0.78 μM, respectively. Molecular modeling studies showed that both compounds bind to the P-gp at transmembrane helices (TMH) 4, 5, and 6, with **27** also showing contacts with TMH 3.



KEYWORDS: *MDR reversing agents, P-glycoprotein, pinoresinol, diethylstilbestrol derivatives, structure−activity relationship, molecular docking*

The development of drug resistance in cancer cells is a major barrier to successful chemotherapy. Among the various mechanisms behind multidrug resistance (MDR),[1] the increased energy-dependent efflux of a broad set of structurally unrelated cytostatic drugs is one of the most commonly encountered.[2] This phenomenon is mediated by transport proteins,[3] the best characterized of which is P-glycoprotein (P-gp) encoded by the *mdr-1* gene, one of the 49 identified ATP-binding cassette (ABC) transporters.[4] P-gp contains two transmembrane domains, each consisting of six transmembrane α-helices (TMHs) and a nucleotide-binding domain (NBD).[5] The latter hydrolyzes ATP to drive the transport of the binding substrate. The importance of P-gp is that it is expressed in almost half of human tumors,[2] being observed in many types of leukemia,[2] including chronic myelogenous leukemia.[6]

Despite some encouraging results,[7] most of the P-gp inhibitors submitted to clinical trials were disappointing, mainly due to their side effects, interactions with coadministered drugs, or defects in the experimental design.[8,9] The development of drugs able to counteract the resistance mediated by P-gp is thus imperative. Natural compounds have attracted great attention and are considered fourth generation P-gp inhibitors.[9] A preceding paper reported that the lignan (±)-pinoresinol, isolated from *Melia azedarach*,[10] showed inhibitory activity on the transport of doxorubicin (DOX) out of P-gp overexpressed chronic myelogenous leukemia cells, Lucena 1 from 56 μM, as demonstrated by an accumulation assay.[2] By multidrug resistance reversal assay, (±)-pinoresinol was able to sensitize resistant cells to DOX from 7 μM.[2] Further studies revealed that (±)-pinoresinol bonds to P-gp through residues of the TMH 4, 5, and 6, some of which are also involved in the binding of the reference P-gp inhibitors, verapamil and tariquidar.[2]

This scenario and the success of plant-derived products as starting points for drug development[11] led us to search for suitable candidates with improved activity compared to (±)-pinoresinol to specifically counteract the mechanism of drug resistance in cells with enhanced P-gp expression. Since the chemical structure of (+)-pinoresinol (**1**) (Figure 1) includes two hydroxyl groups at a distance of 1.3 nm bound to a central scaffold, we virtually screened our own previously synthesized

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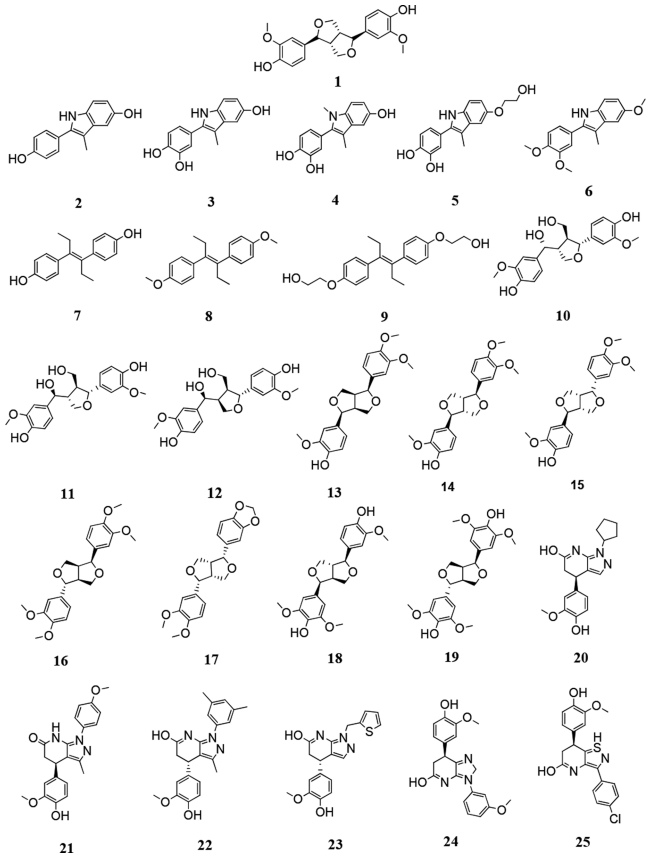

Figure 1. Chemical structures of (+)-pinoresinol (**1**), the PXR ligands **2**−**9**, and the ZINC compounds **10**−**25** showing high similarity with **1** by ROCS analysis.

pregnane X receptor (PXR) library (Figure 1; compounds **2**−**9**), which has a similar structural feature,[12,13] for similarity with **1** using ROCS (ROCS 3.2.2.2: OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com).[14] TanimotoCombo similarity index of shape (shape Tanimoto index) and pharmacophore features (color Tanimoto index) of the query and of compounds **2**−**9** were calculated. Additionally, the ZINC DrugsNow library, containing more than 10 million compounds, was also screened for three-dimensional similarity to **1** using the TanimotoCombo similarity index (sum of shape and color Tanimoto index).

ROCS demonstrated that the identified ZINC molecules **10**−**25** (Figure 1) overlay better with **1** (TanimotoCombo between 1.413 and 1.606) than the bazedoxifene scaffold-based PXR antagonists **2**−**6** (Figure 1, TanimotoCombo between 0.717 and 0.909) and the diethylstilbestrol scaffold-based PXR ligands **7**−**9** (Figure 1, TanimotoCombo between 0.823 and 0.851) (see Supporting Information for representative overlays, Figure S1). On the basis of these results, the optimized structures of these compounds were studied *in silico* by molecular docking, using the human P-gp model previously built based on the homologous P-gp from mouse (*Mus musculus*) as template.[15] In relevant cases, both stereoisomers (±) were investigated, and both OH and NH tautomers (*keto* and *enol* forms) for lactam compounds **20**−**25**. As observed in Table S1, the compounds evaluated showed binding energies ranging from −7.07 to −9.23 kcal/mol. To have a first estimation of the ability to reverse the P-gp-resistant phenotype of cancer cells, compounds **2**−**9**, the most strongly binding nontetrahydrofuran based ZINC compound **23** (binding energy of NH tautomer, −9.23 kcal/mol) and its close structural analogue **21** (binding energy of OH tautomer, −8.56 kcal/mol) were evaluated by a DOX accumulation assay using flow cytometry[2] (see Supporting Information). For this purpose, the chronic myelogenous leukemia cells, Lucena 1,[16] with 58% of the cells expressing P-gp at the surface, were used as a model. These cells derived from the sensitive K562 cell line with less P-gp in the outer membrane (2% of cells). The retention of the cytotoxic clinical drug DOX, a known P-gp fluorescent substrate, inside the cells is commonly used to measure the capacity of a substance to inhibit P-gp-mediated outward transport. Efflux inhibition positively correlates with the intracellular concentration of the probe.[17] The same assay was carried out in the K562 cell line with the aim of discarding any other synergism between the compounds and the fluorescent drug different from the effect on P-gp.

Notably, diethylstilbestrol-based PXR ligands **7**, **8**, and **9** effectively increased intracellular DOX ($p < 0.05$) in Lucena 1 cells with fluorescence intensity ratio (FIR) values corresponding to 1.06, 1.19, and 1.16, respectively, at 25 μM, while the bazedoxifene PXR antagonists **2**−**6** and the ZINC compounds **21** and **23** did not enhance DOX-associated fluorescence ($p > 0.05$) (Table 1). The activity of **7**−**9** was not significantly different with respect to the activity of 25 μM of the well-known reference P-gp modulators verapamil, tamoxifen, and cyclosporine A (CsA) ($p > 0.05$). It is worth noting that **7**−**9** did not cause a significant increase in DOX accumulation in the K562 cell line ($p > 0.05$) (Table S2) indicating that these compounds specifically inhibited P-gp transport. Taken together, the better reversal activity displayed by **7**−**9** compared to that of **2**−**6** and **21** and **23** would indicate that the diethylstilbestrol scaffold is more beneficial for achieving P-gp inhibitory activity than the bazedoxifene- and 1,4,5,7-tetrahydro-6*H*-pyrazolo[3,4-*b*]-pyridin-6-one scaffold.

Following primary screening, and with the aim of determining the minimum effective concentrations (MEC), **7**−**9** were further tested at serial dilutions. As seen in Table 1, compound **7** did not restore DOX accumulation in Lucena 1 cells at concentrations below 25 μM ($p > 0.05$), while **8** and **9** turned out to be effective from 12.5 ($p < 0.01$) and 6.25 μM ($p < 0.001$), respectively, with no differences in activity with respect to verapamil, tamoxifen, and CsA ($p > 0.05$). A dose-dependent response was observed for **8** ($b = 0.0097$; $p = 0.0076$; CI 95% = 0.0043 to 0.0151) and **9** ($b = 0.0090$; $p = 0.0496$; CI 95% = 0.00002 to 0.01797). To the best of our knowledge, this is the first report of the inhibition of diethylstilbestrol **7** on P-gp-mediated transport, although inhibitory properties have been previously reported against the breast cancer resistance protein (BCRP) at 30 μM.[18] In another work, **7** showed a weak activation (45%) of P-gp ATPase activity at 10 μM.[19] Although mild stimulation of ATP hydrolysis may be associated with modulatory activity on P-gp function, as observed for the compound (±)-pinoresinol[2] or cyclosporine A,[20] no studies have previously been performed about the inhibitory effect of **7** on the P-gp-mediated efflux of drugs. Such investigations must be carried out based on the assumption that some molecules able to activate P-gp-ATPase, for instance, the powerful modulator tariquidar,[21] behave as inhibitors, while others, such as DOX, vinblastine, progesterone, or propranolol, act only as substrates.[20,22]

The limited clinical efficacy of multiple drugs as a result of resistance is a complex mechanism not only attributed to the efflux mediated by an ABC transporter.[1] It is therefore highly

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Table 1. Effect of PXR and Selected ZINC Compounds on P-gp Function by Doxorubicin Accumulation and Resistance Reversal Assays in Lucena 1 Cell Line[a]

	FIR/FR										
	concentration (μM)										
compound	25	12.50	6.25	3.12	1.56	0.78	0.39	0.19	0.098	0.048	0.024
2	0.80 ± 0.02										
3	0.72 ± 0.03										
4	0.75 ± 0.08										
5	1.01 ± 0.03										
6	0.90 ± 0.15										
7	1.06 ± 0.01*	1.10 ± 0.08	1.04 ± 0.03								
8	1.19 ± 0.03*	1.09 ± 0.02**	1.09 ± 0.07								
9	1.16 ± 0.08*	1.20 ± 0.04*	1.08 ± 0.007***	1.01 ± 0.06							
21	1.11 ± 0.06										
23	0.96 ± 0.01										
26	1.23 ± 0.08*	1.06 ± 0.02*	1.09 ± 0.01*	1.04 ± 0.009*/ 1.55 ± 0.09**	1.05 ± 0.003*/ 1.30 ± 0.12*	0.98 ± 0.02/ 1.15 ± 0.11					
27	1.32 ± 0.12*	1.18 ± 0.07*	1.08 ± 0.02*	1.06 ± 0.01*/ 1.61 ± 0.14*	1.10 ± 0.02*/ 1.34 ± 0.07*	0.99 ± 0.02/ 1.16 ± 0.08*	1.00 ± 0.02/ 1.03 ± 0.03				
28	1.21 ± 0.07*	1.15 ± 0.05*	1.12 ± 0.02**	1.07 ± 0.009**	1.03 ± 0.03						
29	1.21 ± 0.03***	1.10 ± 0.02*	1.06 ± 0.02*	1.04 ± 0.01*	1.02 ± 0.01						
30	1.18 ± 0.07*	1.10 ± 0.02*	1.09 ± 0.03**	1.08 ± 0.02*	1.01 ± 0.02						
31	0.99 ± 0.04										
32	1.04 ± 0.05										
33	1.03 ± 0.02										
34	0.95 ± 0.06										
Ver	1.35 ± 0.009***	1.26 ± 0.03**	1.20 ± 0.06*	1.16 ± 0.03*	1.07 ± 0.03*	1.07 ± 0.07					
Tam	1.13 ± 1.03*	1.07 ± 0.02*	1.05 ± 0.01*	1.01 ± 0.02							
CsA	1.40 ± 0.01*	1.47 ± 0.12*	1.44 ± 0.06**	1.47 ± 0.07**	1.46 ± 0.12*	1.43 ± 0.09*	1.30 ± 0.04**	1.25 ± 0.06*	1.16 ± 0.04*	1.15 ± 0.01**	1.01 ± 0.04

[a]Ver: verapamil. Tam: tamoxifen. CsA: cyclosporine A. Fluorescence intensity ratio (FIR); analysis of differences between the intracellular accumulations of DOX in cells treated with the tested compounds compared to control cells were performed by one-tailed paired *t*-test. Fold reversal (FR) values were calculated as IC_{50} of DOX alone/IC_{50} of DOX in the presence of the tested compounds. Statistical comparisons between IC_{50} of DOX alone or with compound in each treatment were performed by one-tailed paired *t*-test. Results represent the mean ± SE. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

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beneficial to develop multitargeted agents for overcoming the resistance to pharmacotherapies.[1] As well as P-gp, PXR also plays a key role in the MDR of cancer cells, acting as a xenobiotic sensor that regulates the transcription of genes that ultimately increase the activity of transporters and drug-metabolizing enzymes.[12,13,23] The dual activity of the PXR antagonists **7** and **9**, negatively modulating PXR activity[13] and, at the same time, the outward transport mediated by P-gp, is encouraging for these antagonists to be agents for overcoming MDR.

As previously reported, compound **7** was able to decrease P-gp expression levels in MCF-7/MDR cells.[24] Therefore, the effectiveness of this compound could be a sum of effects, possibly inhibiting both P-gp transport and expression.

Given that compounds **7**−**9** displayed higher P-gp modulating activity than (±)-pinoresinol,[2] and aiming to optimize the P-gp-mediated MDR reversal activity of the identified compounds, an additional series of *O*-functionalized diethylstilbestrol derivatives **26**−**34**, originally prepared as potential PXR modulators (Figure 2), were assayed for P-gp inhibition at

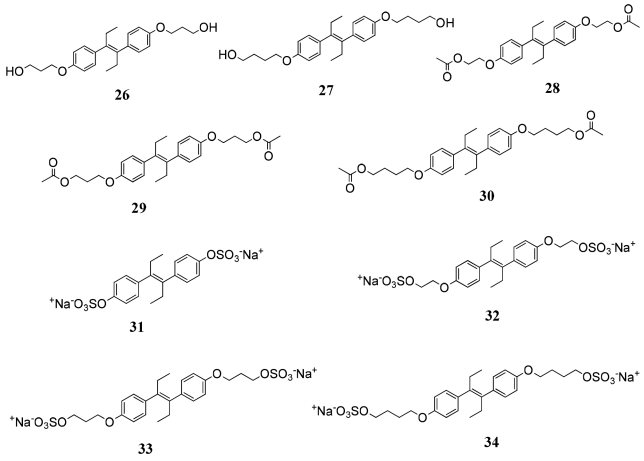

Figure 2. Chemical structures of the diethylstilbestrol derivatives: **26**−**34**.

progressive 2-fold dilutions from 25 μM. Cotreatment of Lucena 1 with **26**−**30** resulted in enhanced DOX accumulation (p < 0.001−0.05), with FIR values ranging from 1.04 to 1.32. While **28**−**30** increased DOX-associated MFI from 3.12 μM (p < 0.01−0.05), **26** and **27** were active from 1.56 μM (p < 0.05), with no differences with respect to the same concentrations of verapamil and CsA (p > 0.05) (Table 1 and Figure S2A). Dose-dependency was observed for **26** (b = 0.0082; p = 0.0106; CI 95% = 0.0032 to 0.0133), **27** (b = 0.0120; p = 0.0025; CI 95% = 0.0071 to 0.0169), **28** (b = 0.0071; p = 0.0109; CI 95% = 0.0031 to 0.0110), **29** (b = 0.0077; p = 0.0001; CI 95% = 0.0068 to 0.0087), and **30** (b = 0.0056; p = 0.0009; CI 95% = 0.0009 to 0.0103). The lack of activity on K562 was confirmed indicating that the effect was due to P-gp inhibition (Table S2).

In addition to the DOX accumulation study, a similar assay using the dye rhodamine 123 (Rho123), a classic P-gp substrate, was further carried out by flow cytometry. As observed in Table S3 and Figure S2B, compounds **26** and **27** increased the intracellular Rho123 from 50 μM and 12.5 μM, respectively, and were significantly different from verapamil and CsA, both at 25 and 12.5 μM, respectively (p < 0.05), but were comparable with tamoxifen at the same concentrations (p > 0.05).

To evaluate the capabilities of the most effective compounds, **26** and **27**, more thoroughly, a multidrug resistance reversal assay was carried out (see Supporting Information). The different parameters measured by both types of technique allowed us to address different aspects related to their pharmacological profile to determine the effectiveness of the compounds.[25] The accumulation assay enables assessment of the quantity of substrate accumulated inside the cells resulting from inhibition of the efflux, while the multidrug resistant reversal assay determines the intracellular activity of a cytotoxic drug applied in combination with P-gp modulators compared with its individual activity.[2] It is worth mentioning that Lucena 1 cells displayed 34-fold more resistance to DOX toxicity than parental K562 cells, with IC_{50} values of 48.49 ± 3.8 and 1.42 ± 0.18 μM, respectively. As expected, **26** and **27** showed their ability to reverse the P-gp-mediated DOX resistance from 1.56 and 0.78 μM, respectively (Table 1 and Figure 3), displaying

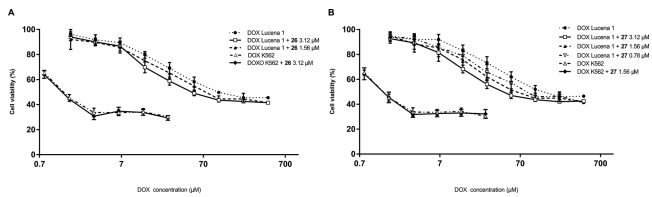

Figure 3. Dose−response curves for cytotoxicity of doxorubicin (DOX) in Lucena 1 and K562 cells with and without compounds **26** (A) and **27** (B) as determined by the multidrug resistance reversal assay. Values are expressed as mean ± SE of at least three independent experiments.

fold reversal (FR) values of 1.30 and 1.16 (p < 0.05), respectively (Table 1). These results match those found in the accumulation assay. To determine whether the DOX cytotoxicity restoring effect was specific to P-gp, K562 cells were treated with 3.12−0.78 μM of **26** or **27**. No sensitization to DOX on K562 was observed with compound **26** at 3.12 μM, while **27** was not able to increase DOX toxicity in these cells at 1.56 (Table S2). These results showed that both molecules appear as chemosensitizers specific to P-gp modulation.

As previously mentioned by Syed et al.,[26] P-gp modulators submitted to clinical trials present octanol/water partition coefficients ranging from 3.64 to 6.81. The partition coefficients (ClogP, calculated with ChemDraw Ultra software) found for compounds **7**−**9** and **26**−**30** are mostly in agreement with these values (see Table S4) suggesting that the assayed compounds show a similar partition into the lipid bilayer to that observed with the anti-P-gp candidates. In addition, the ClogP values obtained are higher than 2.92/3.25, the minimum ClogP values established for a compound to be considered as an effective P-gp inhibitor.[27,28] In the present work, the most potent compounds **26**−**30** showed the highest calculated logP values (5.17 to 7.22, Table S4) in agreement with many authors[20,29,30] who proposed that lipophilicity is a key factor for a better inhibition of P-gp.

The binding mode of **1** was previously described,[2] with the main contacts occurring with residues S222, A223, K234, F303, Y307, Y310, L339, A342, and F343, most of which are amino acids from TMH 4, 5, and 6 at the top of the inverted "V" shaped by the transmembrane α-helices. All these residues were proposed as essential for binding on theoretical and experimental bases, as previously stated.[2,15] The inhibitors **7**−**9** and **26**−**30**, which were found active by DOX accumulation assay and by docking, overlap their binding regions with that of the parent compound **1**, which in turn overlaps with the reference inhibitor tariquidar.[2] In particular, compounds **26**, **27**,

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and **30** showed the maximum superposition with tariquidar, with **27** being the only compound of the **26**−**30** series, which also showed contacts with TMH 3 (T199, F200, and G203). The superimposition of **1**, **26**, and **27** with tariquidar is shown in Figure 4. Further details on the binding mode of **26** and **27** are

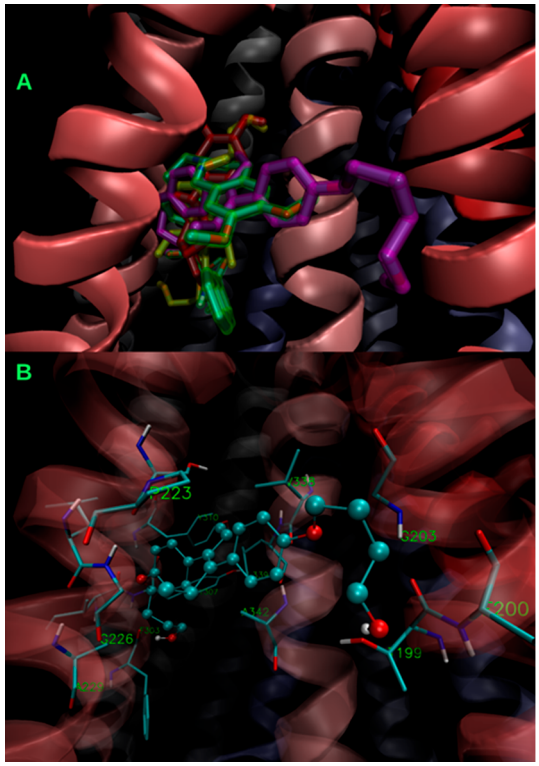

Figure 4. (A) Superimposition of the lowest energy poses of the inhibitors **1** (highlighted in red), **26** (in yellow), **27** (in violet), and the reference inhibitor tariquidar (in green). (B) Binding of the most active compound **27** (balls and sticks) showing its main contacts (licorice). The cartoon representation of the α-helices is colored according to the sequence, from TMH 1 (red) to TMH 12 (blue).

available in Figures S3 and S4. One of the most plausible reasons behind the difference in activity of **26** and **27** with respect to DOX and Rho123 accumulation techniques, which were also observed in treatments with (±) pinoresinol,[2] concerns the affinity of each substrate to the binding sites of P-gp. This issue may be explained based on the results obtained by performing docking studies. The lowest energy docked poses of both **26** and **27** bind to the two main sites of DOX, the latter with −8.23 and −8.16 kcal/mol binding energies (Figure S5). As shown in Figure S5, also other higher energy conformations of **26** and **27** (up to 1.2 kcal/mol above the lowest one) overlapped these sites as well. However, none of these conformations are able to overlap the primary and one of the secondary sites of Rho123 (which have similar affinities within 0.3 kcal/mol), as illustrated in Figures S6 and S7. Clearly, neither **26** nor **27** would be able to inhibit the Rho123 efflux (at least not in the same competitive way) as it does with DOX. In sharp contrast, the much more flexible and bigger molecule CsA binds in a region that occlude the three main Rho123 binding sites (binding energies −10.0 to −9.0 kcal/mol), as shown in Figure S8. Although smaller than CsA, verapamil still showed a similar pattern (Figure S9, conformations from −9.1 to −8.1 kcal/mol shown) These differences in the binding preferences of both target compounds, of the two model substrates, and of the reference inhibitors are consistent with the experimental observations regarding the preference of **26** and **27**, and also of tamoxifen, for DOX, whereas CsA and verapamil showed a similar behavior with Rho123 or DOX as substrates.

While compounds **9**, **26**, and **27**, differing only in the length of the hydroxyalkoxy side chains (2 to 4 carbon atoms), appear as encouraging lead compounds for P-gp inhibition, the sulfate ester derivatives **32**−**34** had no effect. As observed in Table 1, the increase in the length of the side chain in **26**−**27** with respect to **9** enhanced effectiveness. However, the addition of an *O*-acetyl group in the side chain (compounds **29**−**30**) decreased the activity compared to the hydroxy compounds **26**−**27**. The presence of $O-CH_3$ groups at positions 4 and 4′ of compound **8** slightly increased P-gp modulation activity with respect to bis-hydroxy compound **7**, while the addition of the 2-hydroxyethyl group in the lateral chain of **7** to give **9** markedly increased the MDR-modulating efficiency.

To further determine the suitability of compounds **26** and **27** as potential inhibitors of P-gp function, their toxicity on nontumoral peripheral blood mononuclear cells (PBMC) was evaluated. With an MTT assay,[31,32] **26** and **27** showed 34.67 ± 0.88 and 41.00 ± 0.58% cytotoxicity at 25 μM, while a complete absence of toxicity was observed at 6.25 μM. A compound can be considered as cytotoxic when the IC_{50} values are lower than 10 μM.[33]

With these results, the newly identified compounds **26**−**27** appear as promising starting points for the development of inhibitors of the P-gp-mediated transport of chemotherapeutic drugs such as DOX. The information obtained about the structural requirements for activity sheds light on the design of further synthetic pinoresinol-related agents able to reverse the MDR/P-gp phenotype.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00324.

Detailed experimental procedures and biological assays, summary of docking results for PXR ligands and ZINC compounds (Table S1), effect of PXR and ZINC compounds on P-gp function in the K562 cell line by DOX accumulation assay (Table S2), effect of **26** and **27** on P-gp function by rhodamine 123 accumulation assay (Table S3), ClogP values (Table S4), overlays of PXR-ligands and ZINC compounds with **1** according to ROCS (Figure S1), representative histograms of DOX and Rho 123 accumulation assays (Figure S2), and poses of binding to P-gp of the most active compounds determined by docking (Figures S3−S9) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BCRP, breast cancer resistance protein; ClogP calculated octanol, calculated octanol/water partition coefficient; DOX, doxorubicin; FR, fold reversal; FIR, fluorescence intensity ratio; MEC, minimum effective concentration; MFI, medium fluorescence intensity; PBMC, peripheral blood mononuclear cells; P-gp, P-glycoprotein; PXR, pregnane X receptor; Rho123, rhodamine 123

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