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Toward a Better Pharmacophore Description of P-Glycoprotein Modulators, Based on Macrocyclic Diterpenes from *Euphorbia* Species

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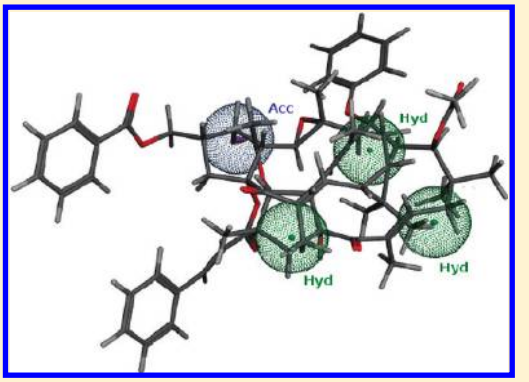
Supporting Information

ABSTRACT: Multidrug resistance related to the increased expression of P-glycoprotein (P-gp) by cancer cells is the major contributor for the failure of chemotherapeutic treatments. Starting from pharmacophores and data already published and in macrocyclic diterpenes isolated from *Euphorbia* species, a comprehensive study of pharmacophore definitions of features was performed in order to obtain a new improved four-point pharmacophore able to detect literature and in-house modulators and simultaneously specific enough to avoid the detection of most nonactive molecules in a universe of 152 (literature), 74 (in-house), and 46 (inactive) molecules. This pharmacophore detects 84.2% of the molecules described in the literature, along with 100% detection of in-house isolated compounds and 19.5% of false positives. The importance of the hydrophobic and electron acceptor moieties as essential features for recognition of different molecules by the P-gp drug-binding site is clarified. The best combination of acceptor, donor, hydrophobic, and aromatic characteristics that contribute for the increased selectivity shown by the described pharmacophore is evaluated, and the protonation state of the molecules is also addressed.



INTRODUCTION

Multidrug resistance (MDR), due to increased expression of P-glycoprotein (P-gp) at the surface of cancerous cells, is the major contributor for the failure of chemotherapeutic regimens.[1,2] This protein, a member of the ABC transporters, is expressed by several tissues such as kidney tubules, intestinal epithelium, blood vessel endothelial cells in the blood-brain barrier, and tissues with excretory and secretory functions[3,4] of metabolic endogenous products. The overexpression by the cancer cells takes advantage of the protein's physiological function, increasing the elimination rate of toxic xenobiotics as antitumoral drugs that hinder growth. This transporter, classified as an energy-dependent transmembranar drug efflux pump, has a sequence of 1280 amino acids (molecular weight of approximately 170 kDa), ordered in two homologous halves with a spatial arrangement in an hexagonal ring with a central asymmetrical pore.[4] Recently, the publication of the murine P-gp crystallographic structure showed a pseudosymmetric organization, with a large central cavity[5] that opens to the cytoplasmic compartment but also to the inner leaflet of the membrane, thus corroborating the hydrophobic vacuum-cleaner model[6] (Figure 1). In addition, several residues in the drug-binding site (DBS) are thought to actively participate on drug recognition. These residues are mainly hydrophobic (15 polar and two potentially charged).[5]

Since later 1980s, several studies demonstrated that some types of cancer actively express P-gp or other related transporters (as MRP1 or BCRP[7]), transporting a wide range of substrates of different chemical structures, with or without pharmacological activity. If the drug concentration required for pharmacological action cannot be achieved in the interior of the cell, the failure of the chemotherapeutic regimen is expected, making the development of P-gp modulators (also referred to as MDR reversers, inhibitors, or chemosensitizers) one of the most promising strategies to overcome MDR.

Therefore, several studies were conducted with the goal of clarifying the efflux and to enable the design of compounds with the ability to inhibit this type of transporters. One of the first studies that identified a molecule with the capability to reverse the MDR in leukemia P388 cells was conducted by Tsuruo et al., with the calcium channel blocker verapamil.[8] However, the first pharmacophore studies, using a series of analogues of reserpine and designed to evaluate which steric features were responsible for the activity in this type of molecules only, began at the end of the 1980s, when it was demonstrated that one basic nitrogen atom, two planar aromatic rings, and the spatial disposition of aromatic rings relatively to nitrogen played an essential role in the P-gp modulating activity.[9] Eight years later, Suzuki proposed a prerequisite of 5 Å between the basic nitrogen (hydrogen bond acceptor) group and the nearest hydrophobic moiety,[10] and Seelig demonstrated that the existence of patterns comprising two or three electron donor groups, with a fixed spatial separation of 2.5 Å (type I) or 4.6 Å to the outer groups (type II), were also

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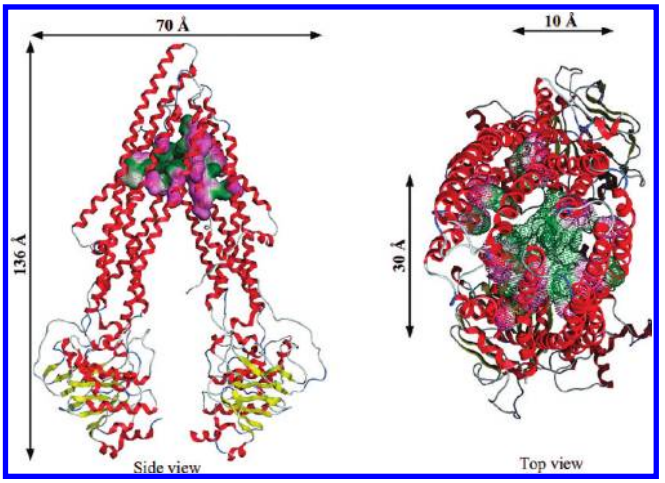


Figure 1. Side and top view of P-gp structure. Dimensions of the total protein (left) and central pore (right) are presented, along with molecular surface of the DBS [adapted from Aller et al.[5]].

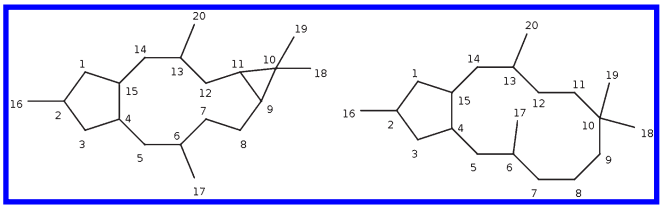
important.[11] One year later, one study referred that the nitrogen atom was not essential, because the interaction with P-gp was nonanionic, being only derived from the sum of the hydrogen acceptor strengths of the drug binding site (DBS).[12]

In the beginning of the 21st century, several pharmacophore patterns were proposed from structurally different drugs, all having in common two hydrophobic points, three hydrogen bond acceptors, and one hydrogen bond donor, charged positively or not.[13−15] It was hypothesized that the DBS could possess different receptor points, in which the substrates interact by different binding modes, being the sum of all the interactions the principal determinant for the binding affinity of the drug.[13] In the same year, other studies obtained three different pharmacophores for substrates involved in the efflux[2] and inhibitors,[16] showing as common features two hydrophobic zones and one hydrogen bond acceptor. In 2008, Tawari et al. studying a series of pyrrolopyrimidines and indolopyrimidines obtained a pharmacophore with five points, including a cationic site.[14] In 2009 Pajeva et al. continued the indolopyrimidine studies, confirming at least four points in the pharmacophore,[15] including the positively charged site previously described by Tawari et al.[14] and already described on antecedent studies.[13]

So far, the majority of the above studies were conducted comprising limited variations on the design of the molecules, only comprehending structural analogues of molecules with known activity as substrates or modulators. Few studies had control groups to evaluate the selectivity of the determined pharmacophores, being impossible to evaluate the validity of the model. The influence of the molecule's protonation state in the P-glycoprotein modulation has also been generally ignored.[17] However, this problem emerges in studies that use one pharmacophoric point defined as a positively charged group derived from protonable atoms such as secondary and tertiary amines.[13,14,18] The absence of a crystallographic structure for this protein until 2009 was also a problem, because stalled the development of inhibitors with increased affinity and selectivity.

In our search for anticancer agents, we have isolated from *Euphorbia* species several macrocyclic lathyrane and jatrophane-type diterpene polyesters, along with some polycyclic rearranged derivatives.[19−23] These diterpenoids have shown to be strong

Scheme 1. Lathyrane (Left) and Jatrophane-type (Right) Macrocyclic Diterpenic Scaffold from *Euphorbia* Species



P-gp modulators on mouse lymphoma cells transfected with human MDR1 gene. Similar results were obtained by other groups.[24,25] These types of cyclic compounds do not have nitrogen atoms and aromatic moieties are frequently absent. They all have a common biosynthetic origin, comprising a 20 carbon scaffold (Scheme 1), organized in a cyclopentane fused with a macrocycle comprising 12 members (jatrophanes), 11 members fused with a cyclopropane ring (lathyranes) or rearranged into tetracyclic systems. More recently, we have isolated several cucurbitane-type triterpenoids[26,27] which were also found to be strong multidrug resistance reversers in cancer cells.

Pharmacophores relying on a nitrogen atom (charged or neutral) or aromatic domains defined as important interaction features cannot be applied to this particular set of natural compounds. Therefore, it is our purpose to define an improved pharmacophore that combines the structural features of macrocycle diterpene derivatives with the majority of compounds known to possess anti-MDR activity. In particular, the present work focuses on the development of a more accurate pharmacophore to achieve two principal objectives:

i) to clarify the molecular properties and structural arrangement common to the in-house and literature modulators, thus defining a base for the development of new modulators or the modification of the existing ones, in a way to increase the activity and specificity toward the P-glycoprotein;

ii) to set a methodology for database screening, allowing the selection of new structures that could act as new modulators.

METHODS AND DATA

Pharmacophore Generation. Three sets of compounds were compiled from the literature. The first set, named literature set, comprises compounds known to interact with the P-glycoprotein and includes modulators or substrates organized in twelve subsets. Each subset was prepared based on the MDR reversal activities as proposed by Wang et al.,[28] adding two additional subsets corresponding to the terpenoids sipholanes[29] and euphodendroidins,[30] also categorized in the literature as anti-MDR modulators. The second set includes only MDR modulators isolated from plants belonging to the *Euphorbia* species[19−23,26,27] and was designed as an in-house set. The third set, named inactive set, contains drugs that are categorized as nonsubstrates regarding to this type of transporter proteins and is included to validate the accuracy of the detection by the various pharmacophores. Pharmacophore generation used the force field MMFF94x within the Molecular Operating Environment (MOE 2009.10).[31] Each compound set was individually fitted with the best pharmacophore that allowed the maximum detection within the set, through two distinct functions in MOE ("pharmacophore elucidation" and "flexible alignment with pharmacophore

consensus"). The inactive set was not used in the development but only for the pharmacophore's refinement and validation.

Molecules Optimization. All molecules were energy minimized using the MMFF94x force field, setting the atomic coordinates to the local minimum of the molecular energy function, with a RMS gradient threshold of 10^{-5}. The forcefield partial charges were also attributed prior to the energy minimization and the total energy was calculated as a sum of the energy contribution of individual terms in the potential energy model. The minimized structures of each subset were added to the correspondent databases, along with the name and the activity, in order to create the three sets employed in this study. The protonation state of secondary and tertiary amines were also taken in account.

Pharmacophore Elucidation. This function utilizes the "Pharmacophore elucidation" module directly from each subset, applying the approximation of the molecular shape as a sum of Gaussian densities, considering the alignment focus on pharmacophore features themselves and emphasizing the alignment of pharmacophoric features rather than the molecular structure. All the possible conformations were determined by systematic specification of torsional angles by bond rotation, with infatuation on the alignment of aromatic and acceptor groups. The system tested random pharmacophore patterns bearing 3, 4, or 5 pharmacophoric points, scoring the cover, overlap, and accuracy of the patterns. In this query three additional groups were added (acceptor and/or donor and hydrophobic atoms) with a radius of 1.4 Å. All other options remained at the program's default. Only the pharmacophore with the best hit percentage inside each set was considered in the determination of the final pharmacophore.

Table 1. Detection Percentages for Each Developed Pharmacophore[a,b]

set (N)	reserpine-scaffold derived	PKC-scaffold derived	literature set derived (ph-A)	literature set derived (ph-B)	present work
literature (152)	42.1%	60.5%	90.8%	66.4%	84.2%
in-house (74)	29.7%	97.2%	95.9%	50.0%	100.0%
inactive (46)	8.6%	13.0%	39.1%	13.0%	19.5%

[a] N is the number of compounds in each set, and PKC is protein kinase C. [b] Information about subsets are included in the Supporting Information.

Flexible Alignment with Pharmacophore Consensus. In each set, all the molecules were superimposed through a stochastic search to determine the relative position of similar groups in the conformational space, emphasizing the hydrophobic nature and the presence of acceptor, aromatic, or donor groups as preferential similarity terms in the function's parametrization. On the alignment presenting the lowest "S" score, that measures the similarity of the superimposition obtained by the sum of the average strain energy in the alignment, a "pharmacophore consensus" was made, defining "tolerance" as the maximum distance allowed for two points that are considered neighbors and "threshold" as the minimum score required to retain a group of points. Only groups with maximum overlap in all the aligned molecules were taken into account when building the final pharmacophore.

RESULTS AND DISCUSSION

In the route to the present pharmacophore proposal, one performance test was made using already a proposed pharmacophore found in the literature (derived from reserpine) and four derived from the molecules contained in the literature set collection. Table 1 summarizes the detection percentages obtained by the different pharmacophores developed.

Reserpine Pharmacophore. Our approach is based on a reserpine-type pharmacophore proposed by Pearce.[9] The pharmacophore described in the literature, with two aromatic domains and one basic nitrogen atom, only detects 26.3% of the literature set (13.6% if taken in account the protonation state of nitrogen at physiological pH) and only 4.0% of the in-house set. The addition of a fourth pharmacophoric point (Figure 2) originated a new pharmacophore, with a hydrogen bond donor point at the aromatic amine — ensuring the spatial orientation of the reserpine analogues — and hydrophobic characteristics in all points except for F1 (defined solely as aromatic). The radius of the selected points varied from 1.0 to 1.9 Å, and the maximum distance between two aromatic domains was set to 13.6 Å.

Although it failed to detect many active molecules in the literature set, some with marked activity as modulators, the success rate of detection in the literature set increased to 42.1% with a false-positive rate of 8.6% alongside with the success rate on the in-house set of only 29.7% (Table 1).

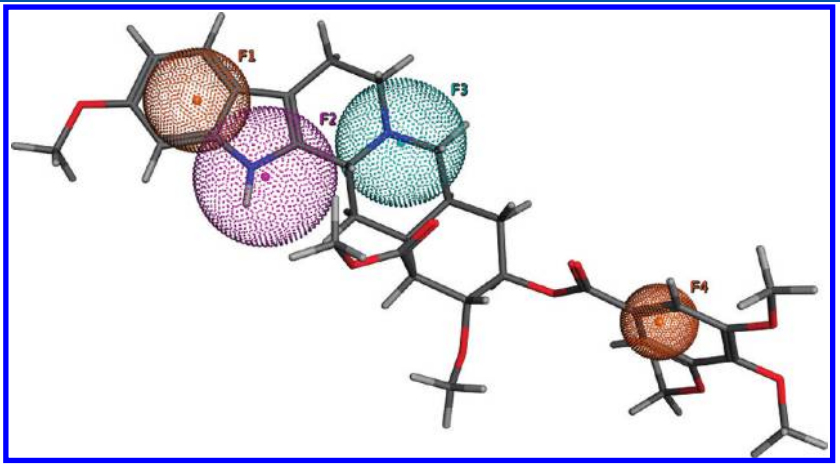


Figure 2. Reserpine-type pharmacophore with pharmacophoric points F1 (aromatic), F2 (donor/hydrophobic), F3 (acceptor/hydrophobic), and F4 (aromatic/hydrophobic) drawn over the reserpine molecule.

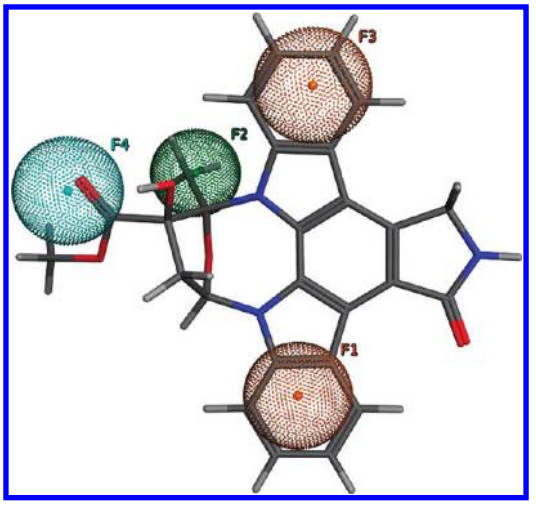


Figure 3. PKC-derived pharmacophore displayed over the K252-a molecule. Pharmacophoric points are F1 (aromatic/hydrophobic), F2 (hydrophobic), F3 (aromatic/hydrophobic), and F4 (acceptor).

As several points had simultaneous characteristics, this introduces bias in the detection accuracy, so the next step was to refine the pharmacophoric points back to a single definition. It is important to note that these low accurate results are a direct consequence of the presence of two aromatic features, because many active molecules do not possess any aromatic moiety.

Ekins et al. determined the presence of hydrophobic features on several pharmacophores, but only one had an aromatic domain.[16] Garrigues et al. and Tawari et al. also determined pharmacophores with only one aromatic feature, defining the other hydrophobic detection points as nonaromatic hydrophobic moieties.[2,14] In an attempt to increase the detection rates other pharmacophores were tested, derived from molecules of different pharmacological families, and compared them with the herein developed reserpine-derived pharmacophore.

Protein Kinase C Pharmacophore. The first developed pharmacophore to detect above 95% of all molecules in the in-house set derived from Protein Kinase C (PKC) inhibitors (subset 7), a group that comprises calphostin C, GF109203X, and K252-a (Figure 3). It simplified the model since only two of the four determined points have simultaneous characteristics. The radius of the determined points was 1.3 Å, and the maximum distance between points did not exceeded 10 Å. It was possible to determine a carbonyl group, hydroxyl, or an oxygen atom as the hydrogen bond acceptor F4, even if a tertiary amine was present, as in dexniguldipine or GF190203X. Interestingly, in the case of the dexniguldipine molecule, the nitro group was detected by the positive partial charge of the nitrogen atom, despite the presence of two oxygen atoms.

The detection percentage in the literature set was higher than the previous obtained for the reserpine pharmacophore (60.5%) with little variation on the false-positive (13.0%). The difference in the detection percentages can probably be explained by a reduction in the length of the major pharmacophoric axis, from 13.5 to 8.1 Å. With these results, it was possible to identify that aromatic/hydrophobic properties are important to the recognition by the transporter and is in accordance with the "hydrophobic vacuum cleaner" model for the P-glycoprotein function.

Suzuki et al. proposed a minimum distance of 5.0 Å between the hydrogen bond acceptor and the first hydrophobic point and

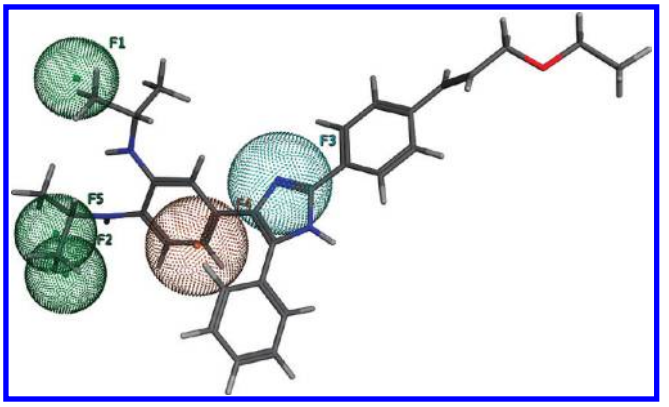


Figure 4. OC144-093 with the literature set derived pharmacophore A (ph-A). The pharmacophoric points F1, F2, and F5 are hydrophobic, F4 is aromatic, and F3 is simultaneously defined as hydrogen bond acceptor and hydrophobic moiety.

Seelig et al. proposed two unit types, two or three electron donor groups with distances of 2.5 Å (type I) or 4.6 Å (type II) to the outer groups, as essential requisites for P-gp recognition.[10,11] Considering both farmacophores above-described, the results suggest an increased detection rate when the maximum distance between points is 8 Å, leading us to propose an additional requirement, that the maximum distance between the hydrogen bond acceptor and any hydrophobic point do not exceed 10 Å and, as only one hydrogen bond acceptor was considered, Seelig's proposition was not proved essential. Additionally, since the nitro groups are often detected by this pharmacophore, the assumption made by Seelig that negative charged groups can inhibit the recognition by the P-gp could not be verified.

Pajeva et al. conducted a study that identified up to six possible groups in the vinblastine molecule that could interact with the transporter, giving special attention to the hydrogen bond acceptor and donor groups.[13] More recent studies from the same author identify up to nine pharmacophoric points but always having at least three hydrophobic centers and a variable number of acceptor and donor groups.[18] Based on the developed reserpine-type and PKC-type, assuming that hydrophobic interactions could be more important for the recognition of the molecule by the DBS than such groups, our next step was the development of pharmacophores comprising five detection points with a minimum of three hydrophobic moieties, evaluate their detection rates, and compare them with the four-point pharmacophore derived from PKC inhibitors, aiming to the increase of the 60.5% detection rate in the literature set.

Literature Set Derived Pharmacophore. Using the literature set, two pharmacophores were developed that include some of the most active molecules such as tariquidar or OC144-093 and high-affinity substrates such as colchicine, daunorrubicin, or teniposide (subset 6). Figure 4 shows the first one determined (ph-A), with three hydrophobic points F1, F2, and F5, one aromatic/hydrophobic atom point F4, and one hydrophobic/hydrogen bond acceptor point F3. The radius varied from 1.4 to 1.8 Å, and the maximum distance between points was 8.2 Å. The detection percentage was 95.9% for the in-house set and 90.8% for the literature set, one of the highest achieved in the current study. However, when the inactive set was tested, 39.1% of false-positives were detected showing that the two overlapping points F2 and F5 originated a less accurate pharmacophore. The same happens by defining a hydrophobic moiety at the hydrogen bond acceptor.

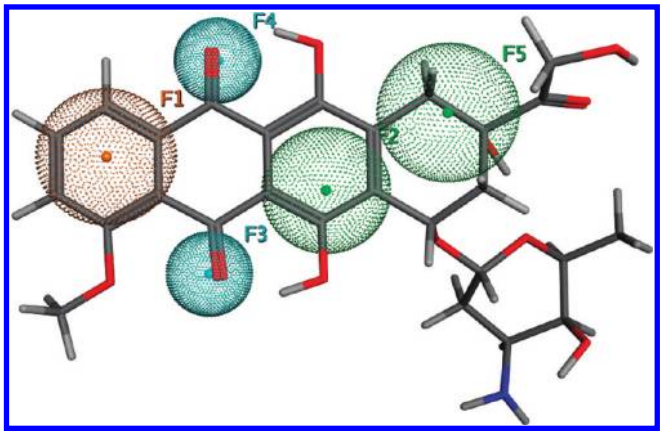


Figure 5. Doxorubicin molecule superimposed on the literature set derived pharmacophore B (ph-B), showing one hydrophobic/aromatic moiety F1, two hydrophobic points F2 and F5, and two acceptor points F3 and F4.

Since from ph-A resulted a high percentage of false-positives, another literature set pharmacophore was derived (subset 5) — pharmacophore B (ph-B, Figure 5) — defined also with five pharmacophoric points (two hydrophobic points, one hydrophobic/aromatic moiety, and two acceptor points) with a distance between the two electron acceptor groups of 6.5 Å, close to the type II unit definition according to Seelig, and a distance acceptor-aromatic groups inferior to the Suzuki prerequisite. The maximum distance is similar to the one in ph-A (about 7.9 Å) and all points are single moieties, except for the aromatic that is defined also as hydrophobic. The hydrophobic points have a maximum radius of 1.6 Å, and the hydrogen bond acceptor points have a radius of 1.0 Å.

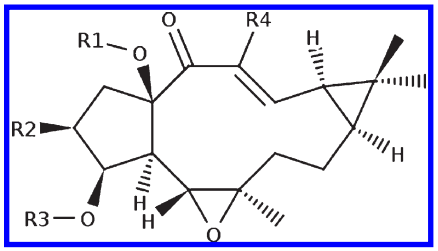
The detection percentage dropped to 66.4% on the literature set, 50.0% on the in-house set, and showed 13% of false positives. The drop on both sets can be explained by the fact that the average distance between two hydrogen acceptor groups is greater than 7 Å in the molecules of the in-house and literature sets. Most of the molecules in the in-house set have one proton acceptor, and only the presence of an additional hydroxyl group or the condensation with glucose or other molecule bearing polar groups provides the necessary distances for these molecules to be detected by this pharmacophore in particular.

More flexible molecules in the in-house set like lathyranes or in literature set as sipholanes and euphodendroidins are detected more than 99% since a second bond acceptor can be found in a flexible side chain. Interestingly, when the detected conformations of vincristine and vinblastine with and without protonation of the basic nitrogens are compared, neutral tertiary nitrogen atoms are always detected as hydrogen bond acceptor points in the vincristine molecule but only in 50% of vinblastine conformations, being the carbonyl or oxygen atoms also associated to the electron acceptor moiety. With the nitrogen protonated, only vinblastine is detected by the pharmacophore, due to the detection of only oxygen-bearing groups.

Despite several attempts, since an increase in the total detection rate or reduction of the inactive detection rate from the previously determined pharmacophores was not possible, a new approach was used, which lead to the development of a pharmacophore based on the modulators isolated from *Euphorbia* species.

Present Pharmacophore Development. Using the lathyrane-type diterpenes isolated from *Euphorbia* species as scaffold[22,23,32] (Scheme 2) and all the previously developed pharmacophores,

Scheme 2. Macrocyclic Lathyrane Diterpenes Used in the Development of the Herein Proposed Pharmacophore



namely the confirmation of the distances and spatial arrangement of the detection points, a four point pharmacophore was developed. A flexible alignment of these molecules (chemical family in the in-house set with less variations in the central macrocyclic skeleton) revealed that most of the prerequisites were present in the structures i.e. we have, according to Seelig's pattern, at least one electron donor type I unit, with a mean distance of 2.6 Å between two hydrogen bond acceptor points, and multiple electron donor type II units of 4.5 Å, along with Suzuki's prerequisite of 5 Å spatial separation between hydrogen bond acceptors and hydrophobic or aromatic domains. The final pharmacophoric points were optimized to obtain the maximum detection percentage within the lathyrane subset.

In this pharmacophore, the points only have one characteristic, the radius of the hydrogen bond acceptor (F1 - Acc) is 1.5 Å and the remaining three hydrophobic points (F2, F3, and F4 - Hyd) have a 1.4 Å radius. The distance between the acceptor moiety and the nearest point is 5.9 Å, and the maximum distance between points is 9.1 Å, with an angle between the hydrophobic points of 57.6° (Figure 6). Although the in-house set contains molecules with several hydrogen bond acceptor groups, a pharmacophore concordant with Seelig's type I or II units was not achieved, and, therefore, it cannot be considered an essential prerequisite.

This pharmacophore positively detected 100% of the molecules of the in-house set, 84.2% on the literature set, and only 19.5% in the inactive set (Table 1). The most active molecules present in the literature set were successfully detected. 36.4% of the nondetected molecules belong to the flavonoids family (Chart 1 — subset 4), i.e. small molecules without hydrophobic groups attached to the chromone scaffold such as chrysin, apigenin, or acacetin. These findings agree with a previous study[33] showing that flavonoids and related structures probably interact within a vicinal ATP-binding site but not with the steroid-interacting region as supposed and neither with the verapamil binding site. If this subset is ignored, the detection percentage rises to 87.5%, one of the highest percentages achieved.

Several studies indicate a common binding site for multiple drugs at the verapamil site. Therefore, the conformational space of the vinblastine,[8] latilagascene E,[23] verapamil,[8] colchicine,[34] and tariquidar[35] molecules was searched, and, for each molecule, the one with the lowest RMSD to the pharmacophore definition is shown in Figure 7. This superimposition highlights the common structural points detected by this pharmacophore. As can be seen in Figure 7, colchicine occupies the space defined by the pharmacophore spatial area, whereas vinblastine, latilagascene E, verapamil, and tariquidar possess groups that protrude several angstroms from the recognition area. Since it has been identified an internal cavity with 6.000 $Å^3$ in murine P-gp,[5] the hydrophobic residues thought to participate on drug recognition will probably interact with the hydrophobic area defined by the three

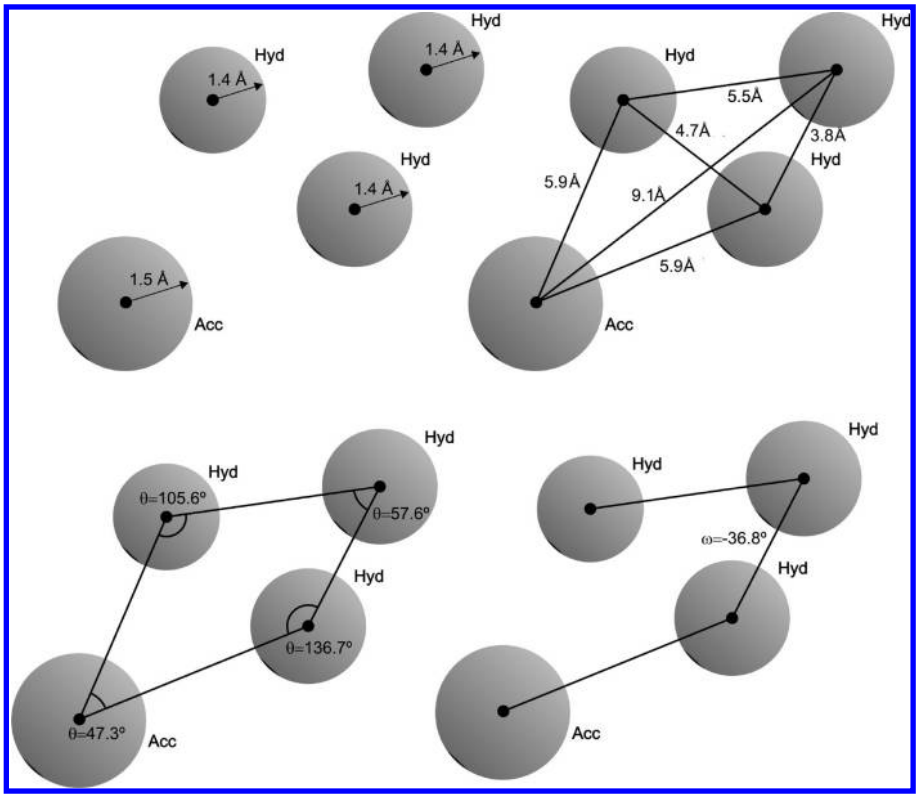
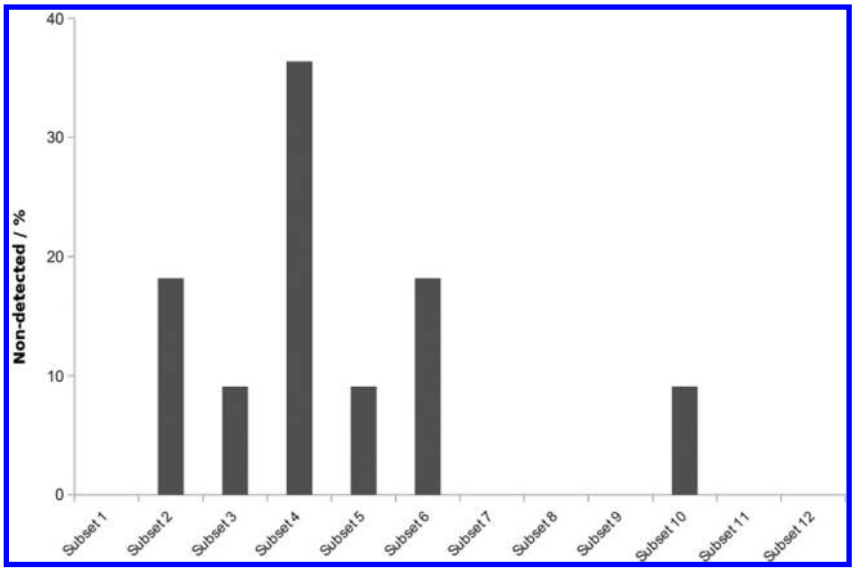


Figure 6. Measurements of the radius, distances, angles, and dihedrals on developed pharmacophore.

Chart 1. Distribution Percentages of the Nondetected Molecules[a]



[a]Information about subsets are included in the Supporting Information.

hydrophobic pharmacophoric points with the acceptor point defining the orientation of the molecule in the recognition area. This means that only a small portion of the molecule is relevant for function and effectively recognized by the transporter's binding site, with the remaining groups actively contributing for the affinity by interaction with other residues identified in the DBS, through additional hydrogen bonds or aromatic interactions. Tariquidar protrudes 20 Å away from the central region having the longest molecular axis, with a flexible chain containing aromatic and hydrogen bond acceptor groups capable of establishing a larger number of hydrophobic interactions or hydrogen bonds, thus explaining the highest affinity.

An attempt was also made to clarify the importance of the hydrogen bond acceptor point defined in this pharmacophore, since several studies consider a hydrogen bond donor, a positively charged feature or an aromatic as important features. However,

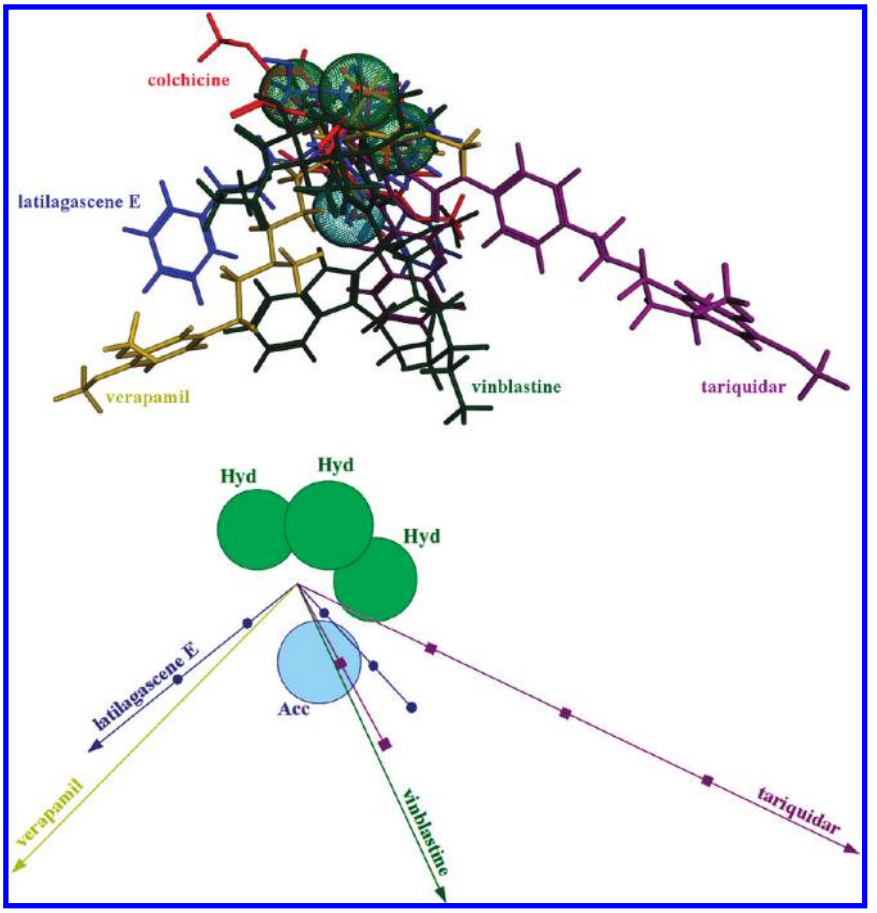


Figure 7. Lowest RMSD conformations and alignment diagram, in agreement with the developed in-house pharmacophore.

Chart 2. Impact on the Detection Percentage by Modification of the Hydrogen Bond Acceptor Point by a Donor, Aromatic, Cationic, or Donor/Acceptor Point

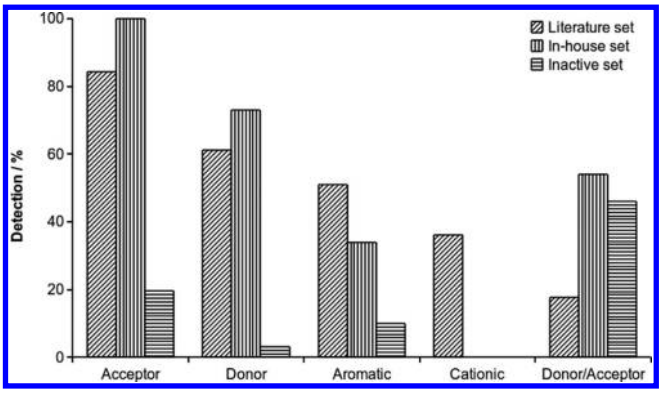
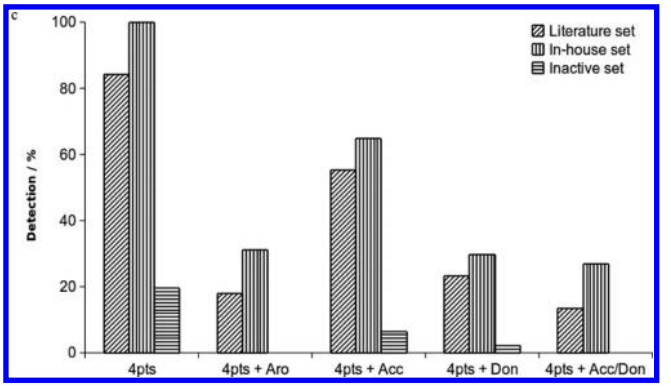


Chart 3. Impact on the Detection by Addition of a Fifth Pharmacophoric Point



the substitution of the acceptor moiety by one of these characteristics proved that the maximum detection was achieved with the acceptor point since all these changes decreased the detection percentages (Chart 2) i.e. changing the acceptor point to a donor or aromatic feature still allows detection superior to 50% in the literature set, but the positively charged point or the donor/acceptor point have much lower detection rates.

Another important point to clarify is the implications of an additional fifth pharmacophoric point in the detection query. Seelig proposed at least two electron donor groups,[11] and Penzotti and Neuhoff proposed the existence of an aromatic moiety[36,37] capable of producing aromatic $\pi-\pi$ stacking with phenylalanine residues, in agreement with a previous proposal by Hait and Aftab.[38] Penzotti and Pajeva also proposed the existence of a hydrogen bond donor contributing to increase the affinity with the transporter protein.[36,13]

Several choices for another pharmacophoric feature added to our 4-point pharmacophore were tested: an aromatic ring (Aro), a second hydrogen bond acceptor (Acc), a hydrogen bond donor

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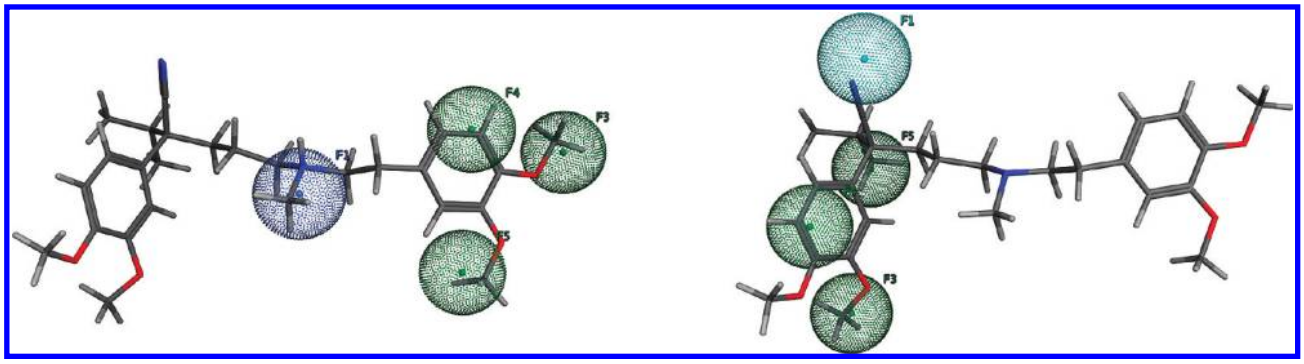

Figure 8. Lowest RMSD conformation of verapamil molecule with a cationic (dark blue; left) and an acceptor (light blue; right) pharmacophoric point.

(Don), or a simultaneous hydrogen bond acceptor or donor (Acc/Don). The pharmacophoric positions were defined in order to obtain the best detection percentages. The best result obtained was with the addition of a second hydrogen bond acceptor at a distance of 7.5 Å from the first one. However, although all the most active modulators along with well-known substrates like vinblastine, verapamil, and colchicine were detected, the detection rate in the literature set decreased to 55.4% (Chart 3). The introduction of an aromatic point next to the acceptor one, as suggested by Pajeva et al.[15] or a donor group or an acceptor/donor groups as some other studies proposed,[36,13] decreased the detection percentage to 17.8%, 22.2%, and 13.4% in the literature set, respectively.

The introduction of the simultaneous acceptor/donor (Acc/Don) point failed to detect many active molecules in all sets, showing that a second hydrogen bond donor or acceptor point is not relevant. In fact, the hydroxyl group corresponding to the acceptor point in the 4-point pharmacophore has proton donor and acceptor characteristics. The addition of a second hydrogen bond donor feature, although decreasing the number of detected molecules, still allows the detection of several of the most active ones showing that the presence of a second hydrogen bond donor group is not essential for modulation but, when present, increases the affinity toward P-gp. In a similar way, when an aromatic domain is added the detection rate in the literature set drops to 17.9%, although still detecting some of the most actives, like tariquidar, OC144-093, and LY335979, meaning that an aromatic point can be, as hydrogen donor groups, an important factor to enhance the affinity of the molecule to the drug binding site, by electrostatic or $\pi-\pi$ electron stacking.

The protonation state of the molecules was also tested and did not significantly change the detection percentages, slightly increasing the number of detected molecules in the literature set, when a hydrogen bond donor point is present in the pharmacophore pattern. An added cationic point drops the detection percentage to only 6.3%, not detecting any molecules on the in-house set. However, the particular case of verapamil is interesting since the detection still occurs but in a different part of the molecule, as shown in Figure 8. This might imply that the DBS may detect protonated and neutral forms of the same molecule but with different binding affinities and possibly in different locations.

CONCLUSIONS

The present study was conducted with the objective of developing an improved pharmacophore for P-glycoprotein modulators that could be used as a working tool to enable the discovery and development of new and more specific multi-resistance modulators, contributing to the comprehension of the efflux mechanism. Using a database containing 272 molecules, the pharmacophore herein developed detected with success compounds with the ability to modulate the P-gp, an efflux protein belonging to the ABC transporter superfamily. The molecules were organized in three different sets (literature, in-house, and inactive), identified by their activity for this type of transporter. Our findings corroborate the importance of a hydrophobic core as an essential feature for the interaction with the DBS, often named the "verapamil binding site". In a recent study of the crystallographic structure of the mouse P-gp, valine, and phenylalanine residues were mapped at the center of the DBS, matching at least three recognition domains, corroborating the here-in developed pharmacophore.[5] The presence of hydrogen bond acceptor groups is also critical, because it allows molecules to have an increased affinity through the formation of hydrogen bonds with amino acids such as histidine, arginine, or serine. An aromatic moiety can also enhance the affinity for the DBS, by means of electrostatic interactions and aromatic $\pi-\pi$ electron stacking with phenylalanine or tyrosine. This one observation is also referred to by Aller et al. on the mouse P-gp mapping, with the upper half of the DBS containing predominantly hydrophobic residues and the lower half having charged, thus more polar amino-acids.[5] This supports our conclusions that the principal mode of interaction with the transporter is mainly hydrophobic, being the aromatic, acceptor, or donor groups responsible for the increased affinity that some molecules display to this type of transmembranar protein.

The herein proposed 4-point pharmacophore comprises three hydrophobic and one hydrogen bond acceptor points. It was constructed based on data from previous studies and pharmacophores described in the literature but has the advantage of detecting an increased number of molecules with different structures, capable of modulating P-gp. It was obtained from P-gp modulators isolated in-house and detects 100% of all molecules in the in-house set and also 84.2% of the molecules described in the literature, being specific enough to largely avoid the detection of inactive molecules. The developed pharmacophore is also in accordance with a very recent study that used fingerprints and ligands for proteins (FLAP) methodology to propose a pharmacophore with one hydrogen bond acceptor and a hydrophobic region that favors hydrophobic interactions.[39] Nevertheless, the referred study considered all nitrogens in its neutral form (thus ignoring protonation) and did not test terpenoid-derived modulators.

The introduction of a fifth pharmacophoric point reduced the detection percentage in the literature and in-house sets, with the best result obtained with a second hydrogen bond acceptor point. However, all of the most active molecules in the literature and the in-house set are still detected in all the 5-point pharmacophores except when an acceptor/donor point is added. This corroborates the importance of the aromatic and donor groups for the interaction with the drug binding site in the P-gp.

The influence of the protonation state of the molecules was also evaluated showing a small influence in the percentage of detection rates.

■ ASSOCIATED CONTENT

Supporting Information. Tables listing the molecules included in each referred set. The number of molecules, correspondent references for the different subsets of literature, in-house and inactive set is also included. This material is available free of charge via the Internet at http://pubs.acs.org.

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