Propafenone analogue with additional H-bond acceptor group shows increased inhibitory activity on P-glycoprotein

Anna Cseke1* | Theresa Schwarz1* | Sankalp Jain1,2 | Simon Decker1 | Kerstin Vogl1 | Ernst Urban1 | Gerhard F. Ecker1

1Department of Pharmaceutical Chemistry, University of Vienna, Vienna, Austria
2National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD, USA

Correspondence
Gerhard F. Ecker, Department of Pharmaceutical Chemistry, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria.
Email: gerhard.f.ecker@univie.ac.at

Funding information
European Union’s Horizon 2020 Research and Innovation Programme, Grant/Award Number: 681002 (EU-ToxRisk); Austrian Science Fund, Grant/Award Numbers: #F03502, SFB35

Abstract
P-glycoprotein (P-gp) is an ATP-dependent efflux pump that has a marked impact on the absorption, distribution, and excretion of therapeutic drugs. As P-gp inhibition can result in drug–drug interactions and altered drug bioavailability, identifying molecular properties that are linked to inhibition is of great interest in drug development. In this study, we combined chemical synthesis, in vitro testing, quantitative structure–activity relationship analysis, and docking studies to investigate the role of hydrogen bond (H-bond) donor/acceptor properties in transporter–ligand interaction. In a previous work, it has been shown that propafenone analogs with a 4-hydroxy-4-piperidine moiety exhibit a generally 10-fold higher P-gp inhibitory activity than expected based on their lipophilicity. Here, we specifically expanded the data set by introducing substituents at position 4 of the 4-phenylpiperidine moiety to assess the importance of H-bond donor/acceptor features in this region. The results suggest that indeed an H-bond acceptor, such as hydroxy and methoxy, increases the affinity by forming a H-bond with Tyr310.

KEYWORDS
inhibitor, molecular docking, P-glycoprotein, propafenone, quantitative structure–activity relationship

1 | INTRODUCTION

P-glycoprotein (P-gp) is an extensively studied efflux pump belonging to the ABC (ATP binding cassette) transporter superfamily. This transporter protein is integrated into the plasma membrane and uses the energy of adenosine triphosphate (ATP) hydrolysis to extrude a wide variety of substances from the intracellular to the extracellular compartment. In the human body, P-gp is physiologically expressed in tissues with barrier and/or excretory functions, including the liver, kidney, gastrointestinal tract and blood–brain barrier, where it mediates the uptake and elimination of endogenous compounds and xenobiotics. In addition, overexpression of P-gp in cancerous cells has been recognized as a major cellular mechanism of multidrug resistance.

On the basis of its expression profile and broad substrate specificity, P-gp is involved in the absorption, distribution, and excretion of therapeutic drugs. Co-administration of a transported drug and a P-gp inhibitor can lead to elevated drug levels in the plasma and in the organs defended by blood–tissue barriers, which poses the risk of organ toxicity. On this account, both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) recommend drug candidates to be routinely screened for their
capacity to inhibit P-gp and the analogous breast cancer-resistant protein BCRP.\[9-11\]

Over the past four decades, a considerable effort has been made to characterize the molecular basis of the interaction between P-gp and its inhibitors.\[12-13\] Although within analogous sets of compounds (e.g., propafenone, tetrahydroisoquinoline, taritardar and chalcone derivatives, alkaloids, and flavonoids), a clear structure-activity relationship (SAR) pattern was observed, a general and conclusive model is still missing.\[14-19\] Numerous SAR studies suggested that there is a strong correlation between lipophilicity and P-gp inhibitory potency and that active compounds commonly possess at least one aromatic ring, a basic nitrogen atom, and several hydrophobic regions.\[12\] Preference of P-gp toward lipophilic compounds can be explained by the widely accepted model of substrate transport, which proposes that P-gp extracts its ligands directly from the inner leaflet of the plasma membrane.\[20,21\] In addition, ligand- and structure-based approaches suggest that hydrogen-bond (H-bond) acceptor properties are important for P-gp inhibitors.\[21,22\]

Propafenone analogs are potent inhibitors of P-gp mediated drug efflux, exhibit a well-defined SAR pattern, and therefore, represent an excellent tool for investigating the molecular features triggering P-gp inhibition.\[23\] Chiba et al.\[24\] performed quantitative structure-activity relationship (QSAR) analyses on a series of highly related propafenone analogs and found a significant correlation between lipophilicity and inhibitory activity (log[1/EC50]). They also revealed that piperazine and piperidine analogous propafenone derivatives, which bear a 4-hydroxy-4-phenylpiperidine moiety, are generally 10-fold more active than equililipophilic compounds without a hydroxy group in position 4 of the piperidine ring. On the basis of the docking study of a small set of propafenone analogs into P-gp homology models, Klepsch et al.\[23\] proposed the formation of an H-bond between the 4-hydroxy group of the 4-hydroxy-4-phenylpiperidine compound GPV062 (4g) and the Y310 moiety of P-gp. This additional H-bond could account for a stronger interaction with P-gp, and thus for the high relative activity of 4g when compared to its calculated logP value (EC50 = 0.07 µM and logP = 3.98). In the present study, we further investigate the structural requirements for P-gp inhibition, focusing on the role of potential H-bond acceptors at the 4-hydroxy-4-phenylpiperidine moiety. To this end, we synthesized six novel propafenone derivatives sharing the same scaffold with 4g, but bearing varying functional groups in position 4 of the 4-phenylpiperidine substituent and tested their inhibitory activity on P-gp in vitro.

2 | RESULTS AND DISCUSSION

2.1 | Synthesis

The propafenone derivatives 3a–g were synthesized by ring-opening reaction of oxirane 1 with appropriate substituted 4-phenylpiperidines 2a–g and subsequently transformed to their hydrochlorides 4a–g, as described previously by Chiba et al.\[24\] (see Scheme 1). While 4-phenylpiperidines 2d–g were commercially available, 4-phenylpiperidines 2a–c were synthesized either from 4-hydroxy-4-phenylpiperidine 6 or 1-benzyl-4-piperidine 10 (see Schemes 2 and 3).

Following Barker et al.\[26\] the commercially acquired amine 6 was protected by Boc2O to yield carbamate 7. The free hydroxy group of 7 was activated using sodium hydride and methylated by methyl iodide to

![Scheme 1](image-url) Synthetic route to compounds 4a–g

|   | a | b | c | d | e | f | g |
|---|---|---|---|---|---|---|---|
| R | OMe | F | CH3 | H | COCH3 | CN | OH |

![Scheme 2](image-url) Synthesis of 4-methoxy-4-phenylpiperidine and 4-fluoro-4-phenylpiperidine
give 8. For deprotection of carbamates, the method of DeGoey et al.[27] proved to give the best results. Thus, 8 was deprotected by HCl in dioxane to yield 4-methoxy-4-phenylpiperidine (2a).

Substitution of the hydroxy group in carbamate 7 by diethylamino sulfur trifluoride led to 4-fluoro-4-phenylpiperidine 11. Deprotection of 11 by HCl in dioxane yielded 4-fluoro-4-phenylpiperidine (2b).

1-Benzyl-4-piperidone 10 was used as a starting material to synthesize 4-methyl-4-phenylpiperidine (2c). The addition of methyl-lithium to 10 gave 1-benzyl-4-propylpiperidine-4-ol (11), which was activated by aluminum chloride and reacted with benzene to yield the Friedel–Crafts alkylation product 12. Finally, the protective group was removed by catalytic transfer hydrogenation in accordance with Salon et al.[28] to give 4-methyl-4-phenylpiperidine (2c).

### 2.2 | In vitro studies

To evaluate the P-gp inhibitory activity of 4a–g, the intracellular accumulation of daunorubicin in the P-gp overexpressing CCRF VCR1000 cells was measured by flow cytometry. As shown in Table 1, all compounds proved to be strong P-gp inhibitors with IC$_{50}$ values in the low nanomolar range (see Supporting Information for Figure S1 showing the dose–response curves). Among the new compounds, 4a and 4d showed the highest activity with IC$_{50}$ values of 0.03 ± 0.01 and 0.03 ± 0.00 µM, respectively, while the cyano derivative 4f exhibited somewhat lower values (0.15 ± 0.04 µM). The IC$_{50}$ value of 4g (0.05 ± 0.01 µM) was in good agreement with IC$_{50}$ values reported earlier for this compound (0.07,[24] 0.06,[29] and 0.06 µM[30]), which demonstrates a high comparability with earlier experiments on series of propafenone analogs and thus allows to pool all compounds into one data set for subsequent QSAR analysis.

**TABLE 1** Results of in vitro IC$_{50}$ evaluation of P-glycoprotein inhibition for the compounds 4a–g (Scheme 1)

| Compound | clogP$^a$ | R$^b$ | IC$_{50}$ (µM)$^c$ |
|----------|----------|-------|---------------------|
| 4a       | 4.65     | OMe   | 0.03 ± 0.01         |
| 4b       | 4.90     | F     | 0.06 ± 0.01         |
| 4c       | 5.61     | CH$_3$ | 0.05 ± 0.01        |
| 4d       | 5.31     | H     | 0.03 ± 0.00         |
| 4e       | 5.13     | COCH$_3$ | 0.04 ± 0.01   |
| 4f       | 4.86     | CN    | 0.15 ± 0.04         |
| 4g       | 4.01     | OH    | 0.05 ± 0.01         |

$^a$Calculated partition coefficient logP(octanol/water); determined for newly synthesized compounds using the MarvinSketch software (ChemAxon).

$^b$Substituent.

$^c$IC$_{50}$ values represent the mean ± standard deviation of n = 3 independent experiments performed in triplicate; IC$_{50}$ values were calculated as described in Section 4.

### 2.3 | QSAR analysis

As previously reported,[24] a hydroxy group located at the 4-phenylpiperidine position of propafenone derivatives increases P-gp inhibitory activity, compared to other analogs with the same lipophilicity. Docking studies of analog 4g into a protein homology model of P-gp indicated that this affinity increase is possibly due to an H-bond formed between the hydroxy group and Y310.[23] However, as all four compounds synthesized by Chiba et al.[24] bear a 4-phenyl-4-hydroxy moiety, other reasons than hydrogen bonding might be responsible for the affinity increase. Thus, to broaden the chemical space of the substituent at this position, we synthesized a set of compounds with different substituents on the 4-phenylpiperidine moiety. Substituents included the two H-bond acceptors methoxy and acetyl, as well as hydrogen, fluoro, methyl, and cyano, which are devoid of H-bonding capabilities.

To evaluate the SAR of our newly synthesized compound set (4a–f) and 4g in a broader context, we involved the propafenone analogs 5a–p synthesized and characterized by Chiba et al.[24] in the analysis (Table 2 and Scheme 4). A previous work showed that the activities (IC$_{50}$ values) of the sodium channel blocker propafenone and its analogs exhibit an excellent correlation with their octanol/water partition coefficient (logP value).[31] As it is

**TABLE 2** Calculated logP and IC$_{50}$ values of compounds 5a–p (Scheme 4)[24]

| Compound | clogP$^a$ | IC$_{50}$ (µM)$^b$ |
|----------|----------|--------------------|
| 5a       | 3.54     | 1.08               |
| 5b       | 3.89     | 0.68               |
| 5c       | 4.46     | 0.34               |
| 5d       | 2.82     | 3.75               |
| 5e       | 5.29     | 0.05               |
| 5f       | 4.62     | 0.11               |
| 5g       | 4.62     | 0.12               |
| 5h       | 4.62     | 0.18               |
| 5i       | 4.78     | 0.21               |
| 5j       | 4.83     | 0.14               |
| 5k       | 4.1      | 0.42               |
| 5l       | 2.73     | 3.84               |
| 5m       | 2.31     | 6.84               |
| 5n       | 1.73     | 2.83               |
| 5o       | 2.43     | 0.30               |
| 5p       | 3.63     | 0.19               |

$^a$Calculated partition coefficient logP(octanol/water) using the MarvinSketch software (ChemAxon).

$^b$IC$_{50}$ values experimentally determined by Chiba et al.[24]
difficult to quantify the H-bonding capacity, we used an indicator variable for groups at the 4-piperidine position with H-bond donor or acceptor properties ($I = 1$; else $I = 0$) as a descriptor for multiple linear regression analysis:

$$pIC_{50} = 0.66(\pm 0.04)clogP + 0.73(\pm 0.10)I - 2.29(\pm 0.19),$$

($r^2 = 0.93; SD = 0.20; n = 23$).

The results indicate that indeed an H-bond donor or acceptor in position 4 of the 4-phenylpiperidine is favorable for activity, which is apparent in the case of the 4-hydroxy-4-piperidines 5n–p and 4g, as well as for the 4-methoxy-4-piperidine derivative 4a (Figure 1). However, it is less pronounced for the acetyl analog 4e. This might be due to a less favorable positioning of the carboxy oxygen in the binding site. We therefore docked compounds 4a and 4e into the binding pocket of a homology model of P-gp.
Due to the unavailability of the crystal structure of human P-gp, we used our recently published homology model\textsuperscript{\cite{32}} for docking experiments. As can be seen from Figure 2, both the methoxy analog 4a and the acetyl derivative 4e are positioned in the same way as reported previously for the hydroxy compound 4g.\textsuperscript{\cite{23}}\textsuperscript{20} However, though the methoxy-oxygen is perfectly located for an H-bond with Tyr310, the position of the acetyl-oxygen is less suited for exploiting the full potential of a H-bond. This might be the reason for 4e not perfectly fitting the regression line for 4-hydroxy-4-phenylpiperidines (IC\textsubscript{50} measured, 0.04 µM; IC\textsubscript{50} predicted, 0.015 µM). Further evidence could be obtained from binding affinity score predictions obtained from LigandScout 4.4,\textsuperscript{\cite{[33]}} which provided a score of −46.27 for 4e, and a slightly better score of −48.16 for 4a. The hydroxy analog 4g showed a binding affinity score of −45.67, which is in line with the ranking observed in the biological experiments. These results thus strengthen the hypothesis that indeed hydrogen bonding with Tyr310 is the main driving factor for the increase of the pIC\textsubscript{50}/logP ratio observed for 4-hydroxy/alkoxy-4-phenylpiperidine analogous propafenone derivatives.

3 | CONCLUSIONS

The ABC transporter P-gp is one of the driving molecular mechanisms for the development of multiple drug resistance in tumor therapy. Although thousands of compounds are known to inhibit the transporter, knowledge of the molecular basis of interaction for distinct inhibitor classes is still far from being complete. In the light of our studies on propafenone-type inhibitors of P-gp, we previously hypothesized the importance of an H-bond acceptor close to the basic nitrogen atom of a 4-phenylpiperidine moiety. In this contribution, synthesis of a set of compounds with varied substituents at position 4 of the piperidine, followed by biological testing and ligand-based modeling studies further support this hypothesis. QSAR studies performed also once more outline the importance of lipophilicity as a main basic descriptor for activity, which might compensate for missing interaction features. Thus, SAR of compound series derived for P-gp should always be discussed in light of pIC\textsubscript{50}/logP ratios rather than on pIC\textsubscript{50} values alone.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All moisture-sensitive reactions were conducted in anhydrous solvents (Sigma-Aldrich) under dry argon atmosphere. All solvents and reagents were obtained from Sigma-Aldrich, ABCR, TCI, or Acros and used without further purification. 1-(2-((Oxiran-2-yl)methoxy)-phenyl-3-phenylpropan-1-one was synthesized following a literature procedure.\textsuperscript{\cite{[24]}} All other reagents are commercially acquired and were used without further purification. Thin-layer chromatography (TLC) testings were done on Macherey-Nagel TLC sheets ALUGRAM Xtra SIL G/UV254. TLC silica gel 60 with fluorescent indicator UV\textsubscript{254} was visualized with UV-light (254 nm) or rather ninhydrin and/or Seebach stain. Silica gel 60 M 0.040–0.63 mm from Macherey-Nagel was used for flash chromatography to optimize yields. The InChI codes of the investigated compounds and the biological activity data are provided as Supporting Information Material.

4.1.2 | Nuclear magnetic resonance spectroscopy

\textsuperscript{1}H- and \textsuperscript{13}C-NMR-spectra were measured by Bruker Avance 500 Nuclear Magnetic Resonance spectrometer at 500 MHz using deuterated chloroform (CDCl\textsubscript{3}) as the solvent. Chemical shifts are specified in parts per million (ppm) relative to tetramethylsilane and coupling constants (J) are given in Hertz (Hz). Multiplicities are described as s (singlet), brs (broad singlet), d (doublet), t (triplet), q (quartet), quint (quintet), dec (decet) and m (multiplet). Spectra were adjusted to the solvent signal of CDCl\textsubscript{3}. δ\textsuperscript{(1H)} = 7.26 ppm and δ\textsuperscript{(13C)} = 77.00 ppm, respectively. The \textsuperscript{1}H- and \textsuperscript{13}C-NMR-spectra of newly synthesized compounds are given in the Supporting Information. For data of compounds 4g and 5a–p.\textsuperscript{[24]}

4.1.3 | High-resolution electrospray ionization mass spectrometry (HR-ESI-MS)

High-resolution mass spectra (HRMS) were obtained on a Bruker-Daltonics maXis HD ESI-Qq-TOF mass spectrometer using the direct...
infusion. The ESI ion source was operated as follows: capillary voltage: 1.0 to 4.0 kV (individually optimized), nebulizer: 0.4 bar (N2), dry gas flow: 41/min (N2), and dry temperature: 200°C. Mass spectra were recorded in the range of m/z 50–1.550 in the positive-ion mode. The sum formulas were determined using Bruker Compass Data Analysis 4.2 based on the mass accuracy (Δm/z ≤ 2 ppm) and isotopic pattern matching (SmartFormula algorithm).

4.1.4 | Synthesis of propafenone derivatives (general procedure)

Oxirane 1 and the 4-phenylpiperidine derivatives were dissolved in MeOH under argon. The mixture was stirred at reflux for 2 hr. Then the solvent was removed under reduced pressure.

1-(2-(2-Hydroxy-3-(4-methyl-4-phenylpiperidine-1-yl)propoxy)-phenyl)-3-phenylpropan-1-one (3a)

Phenylnipriderone 2a (200 mg; 1.05 mmol; 1 eq.) and oxirane 1 (290 mg; 1.03 mmol; 1 eq.) in methanol (5 ml) gave after chromatographic purification (silica gel, DCM, MeOH, conc. NH3 200 + 1 to 200 + 5 + 1) 400 mg 3a, yellow oil, 9% yield. 1H-NMR (500 MHz, CDCl3): δ = 1.93 (m, 1H), 1.95 (m, 1H), 2.00 (m, 1H), 2.01 (m, 1H), 2.37 (t, J = 11.0 Hz, 1H), 2.49 (m, 1H), 2.53 (m, 2H), 2.68 (td, J = 11.0, 3.0 Hz, 1H), 2.75 (t, J = 11.0 Hz, 2H), 2.97 (s, 3H), 3.04 (t, J = 7.7 Hz, 2H), 3.38 (td, J = 7.8, 3.3 Hz, 2H), 4.08 (m, 1H), 4.09 (m, 2H), 6.97 (d, J = 8.2 Hz, 1H), 7.02 (t, J = 7.6 Hz, 1H), 7.18 (t, J = 6.9 Hz, 1H), 7.27 (m, 4H), 7.30 (m, 1H), 7.38 (m, 2H), 7.41 (m, 2H), 7.45 (t, J = 7.9 Hz, 1H), and 7.72 (d, J = 7.9 Hz, 1H) ppm. 13C-NMR (125 MHz, CDCl3): δ = 30.27 (CH2), 34.70 (CH2), 34.86 (CH2), 45.55 (CH3), 47.85 (CH3), 49.38 (CH3), 50.76 (CH3), 60.66 (CH3), 65.21 (CH), 70.91 (Cq), 75.19 (Cq), 112.58 (CH), 120.97 (CH), 125.85 (CH), 125.94 (2C, CH), 127.26 (CH), 128.25 (Cq), 128.35 (2C, CH), 128.39 (4C, CH), 130.44 (CH), 133.46 (Cq), 141.63 (Cq), 144.38 (Cq), 157.81 (Cq), and 201.37 (Cq) ppm. HRMS (ESI): calcd for C30H36NO3 m/z 474.2639 [M+H]+, found m/z 474.2641 [M+H]+.

1-(2-(3-(4-Fluoro-4-phenylpiperidine-1-yl)-2-hydroxypropoxy)-phenyl)-3-phenylpropan-1-one (3b)

Phenylnipriderone 2b (124 mg; 0.54 mmol; 1 eq.) and oxirane 1 (324 mg; 1.15 mmol; 1 eq.) in methanol (8 ml) gave after chromatographic purification (silica gel, DCM, MeOH, conc. NH3 200 + 1 to 200 + 5 + 1) 70.9 mg 3b, yellow oil, 27% yield. 1H-NMR (500 MHz, CDCl3): δ = 1.98 (m, 1H), 1.99 (m, 1H), 2.08 (m, 1H), 2.13 (m, 1H), 2.35 (t, J = 11.5 Hz, 1H), 2.56 (m, 2H), 2.58 (m, 1H), 2.68 (t, J = 10.9 Hz, 1H), 2.82 (d, J = 11.0 Hz, 1H), 3.05 (t, J = 7.9 Hz, 2H), 3.38 (m, 2H), 4.09 (m, 1H), 4.10 (m, 2H), 6.98 (d, J = 8.2 Hz, 1H), 7.03 (t, J = 7.4 Hz, 1H), 7.18 (t, J = 6.8 Hz, 1H), 7.26 (m, 4H), 7.32 (m, 1H), 7.40 (m, 4H), 7.47 (t, J = 7.3 Hz, 1H), and 7.73 (d, J = 7.9 Hz, 1H) ppm. 1H-NMR (125 MHz, CDCl3): δ = 30.30 (CH2), 36.80 (d, J13C–19F) = 21.6 Hz, CH2), 37.11 (d, J13C–13C = 20.9 Hz, CH3), 45.70 (CH2), 47.82 (CH2), 50.98 (CH2), 60.78 (CH2), 65.40 (CH), 70.85 (CH), 112.63 (CH), 121.05 (CH), 123.89 (d, J13C–19F) = 9.2 Hz, 2C, CH), 125.93 (CH), 127.64 (CH), 128.22 (Cq), 128.39 (6C, CH), 128.89 (2C, CH), 130.49 (CH), 133.51 (CH), 141.65 (Cq), 144.19 (d, J13C–13C = 20.9 Hz, Cq), 157.80 (Cq), and 201.21 (Cq) ppm. HRMS (ESI): calcd for C29H24FNO3 m/z 444.2533 [M+H]+, found m/z 444.2536 [M+H]+.

1-(2-(2-Hydroxy-3-(4-methyl-4-phenylpiperidine-1-yl)propoxy)-phenyl)-3-phenylpropan-1-one (3c)

Phenylnipriderone 2c (95 mg; 0.54 mmol, 1 eq.) and oxirane 1 (124 mg; 0.54 mmol, 1 eq.) in methanol (8 ml) gave after chromatographic purification (silica gel, DCM, MeOH, conc. NH3 200 + 1 to 200 + 5 + 1) 136 mg 3c, yellow oil, 94% yield. 1H-NMR (500 MHz, CDCl3): δ = 1.23 (s, 3H), 1.76 (m, 2H), 2.12 (m, 1H), 2.27 (m, 1H), 2.34 (m, 1H), 2.54 (m, 1H), 2.64 (m, 1H), 3.02 (t, J = 7.8 Hz, 2H), 3.35 (t, J = 8.1 Hz, 2H), 4.00 (m, 1H), 4.04 (m, 1H), 4.08 (m, 1H), 6.95 (d, J = 8.2 Hz, 1H), 7.01 (t, J = 7.5 Hz, 1H), 7.16 (m, 1H), 7.21 (m, 1H), 7.23 (m, 4H), 7.34 (m, 2H), 7.35 (m, 2H), 7.44 (td, J = 7.8, 2.0 Hz, 1H), and 7.71 (dd, J = 7.7, 1.8 Hz, 1H) ppm. 13C-NMR (125 MHz, CDCl3): δ = 29.68 (Cq), 30.26 (CH2), 36.00 (Cq), 37.08 (2C, CH2), 45.58 (CH2), 50.29 (2C, CH2), 60.85 (CH2), 65.06 (CH), 70.91 (CH2), 112.55 (CH), 120.96 (CH), 127.52 (3C, CH), 128.56 (CH), 128.21 (Cq), 128.34 (4C, CH), 128.40 (2C, CH), 130.42 (CH), 133.47 (CH), 141.62 (Cq), 148.70 (Cq), 157.78 (Cq), and 201.30 (Cq) ppm. HRMS (ESI): calcd for C30H23NO3 m/z 458.2690 [M+H]+, found m/z 458.2690 [M+H]+.
1-(2-Hydroxy-3-(2-(3-phenylpropanoyl)phenoxy)propyl)-4-phenylpiperidine-4-carbonitrile (3f)

Phenylpiperidine 2f (236 mg; 1.06 mmol; 1 eq.) and oxirane 1 (300 mg; 1.06 mmol; 1 eq.) in methanol (8 ml) gave after chromatographic purification (silica gel, DMF, MeOH, conc. NH₄OH 200 + 1 + 1 to 200 + 10 + 1) 69 mg 3f, yellow oil, 13% yield. ¹H-NMR (500 MHz, CDCl₃): δ = 2.09 (td, 4H), 2.44 (td, J = 12.1, 2.1 Hz, 1H), 2.59 (m, 2H), 2.74 (m, 2H), 2.99 (d, J = 12.0 Hz, 1H), 3.05 (t, J = 8.5 Hz, 2H), 3.37 (m, 2H), 4.08 (m, 1H), 4.09 (m, 2H), 6.97 (d, J = 8.5 Hz, 1H), 7.03 (t, J = 7.4 Hz, 1H), 7.18 (t, J = 7.3 Hz, 1H), 7.25 (m, 2H), 7.28 (m, 2H), 7.36 (t, J = 7.4 Hz, 1H), 7.43 (m, 2H), 7.46 (m, 1H), 7.50 (d, J = 7.9 Hz, 2H), and 7.73 (dd, J = 7.6, 1.2 Hz, 1H) ppm. ¹³C-NMR (125 MHz, CDCl₃): δ = 30.29 (CH₂), 36.45 (CH₂), 36.69 (CH₂), 42.44 (Cq), 45.61 (CH₂), 49.36 (CH₂), 52.31 (CH₂), 60.50 (CH₂), 65.54 (CH), 70.78 (CH₂), 112.77 (CH), 121.12 (CH), 121.79 (CH), 125.55 (2CH), 129.95 (2CH), 128.11 (Cq), 128.40 (2CH), 128.43 (2CH), 129.08 (2CH), 130.47 (CH), 133.52 (CH), 139.78 (Cq), 141.64 (Cq), 157.83 (Cq), and 201.16 (Cq) ppm. HRMS (ESI): calcld for C₃₁H₃₆NO₄ 469.2486 [M+H]+, found m/z 469.2485 [M+H]+.

4.1.5 | Synthesis of hydrochlorides (general procedure)

The propafenone derivatives were dissolved in ether under argon then HCl in ether (2 M) was added and stirred for 1 hr at room temperature. The mixture was filtered, washed with ether, and dried.

1-(2-Hydroxy-3-(4-methoxy-4-phenylpiperidine-1-yl)propoxy)-3-phenylpropan-1-one hydrochloride (4a)

3a (100 mg; 0.21 mmol; 1 eq.), ether (6 ml), HCl in ether (0.21 ml; 2 M; 2 eq.) gave 4a (73 mg white to gray powder, 61% yield). HRMS (ESI): calcld for C₃₀H₃₆NClO₄ m/z 474.2639 [M+H]+, found m/z 474.2642 [M+H]+.

1-(2-(3-(4-Fluoro-4-phenylpiperidine-1-yl)-2-hydroxypropoxy)-phenyl)-3-phenylpropan-1-one hydrochloride (4b)

3b (71.0 mg; 0.15 mmol; 1 eq.), ether (8 ml), HCl in ether (0.15 ml; 2 M; 2 eq.) gave 4b (39.5 mg white powder, 56% yield). HRMS (ESI): calcld for C₂₉H₂₃FNO₃ m/z 462.2439 [M+H]+, found m/z 462.2442 [M+H]+.

1-(2-Hydroxy-3-(4-methyl-4-phenylpiperidine-1-yl)propoxy)-phenyl)-3-phenylpropan-1-one hydrochloride (4c)

3c (90.0 mg; 0.20 mmol; 1 eq.), ether (8 ml), HCl in ether (0.20 ml; 2 M; 2 eq.) gave 4c (79 mg white powder, 88% yield). HRMS (ESI): calcld for C₃₉H₃₉NClO₄ m/z 548.2690 [M+H]+, found m/z 548.2694 [M+H]+.

1-(2-(3-(4-Acetyl-4-phenylpiperidine-1-yl))-2-hydroxypropoxy)-phenyl)-3-phenylpropan-1-one hydrochloride (4d)

3d (55.0 mg; 0.21 mmol; 1 eq.), ether (8 ml), HCl in ether (0.12 ml; 2 M; 2 eq.) gave 4d (49.6 mg white powder, 90% yield). HRMS (ESI): calcld for C₃₁H₃₉NClO₄ m/z 486.2639 [M+H]+, found m/z 486.2640 [M+H]+.

1-(2-Hydroxy-3-(2-(3-phenylpropanoyl)phenoxy)propyl)-4-phenylpiperidine-4-carbonitrile hydrochloride (4f)

3f (69.0 mg; 0.13 mmol; 1 eq.), ether (8 ml), HCl in ether (0.14 ml; 2 M; 2 eq.) gave 4f (35 mg white to gray powder, 51% yield). HRMS (ESI): calcld for C₃₀H₃₈NClO₄ m/z 469.2486 [M+H]+, found m/z 469.2486 [M+H]+.

4.1.6 | Synthesis of substituted 4-phenylpiperidines

tert-Butyl 4-hydroxy-4-phenylpiperidine-1-carboxylate (7)

4-Hydroxy-4-phenylpiperidine (6) (15 g, 84.6 mmol; 1 eq.) and di-tert-butyl dicarbonate (20.25 g, 93.15 mmol; 1.1 eq.) were dissolved in DCM (450 ml) under argon and stirred at room temperature for 2 hr. Then saturated aqueous NaHCO₃ solution was added. The aqueous phase was washed three times with DCM and the organic phases were dried over Na₂SO₄, filtered and concentrated. Yield: 245.3 g, yellow to light brown oil, 105% (was used without further purification). ¹H-NMR (500 MHz, CDCl₃): δ = 1.48 (s, 9H), 1.73 (d, J = 12.6 Hz, 2H), 2.00 (td, J = 13.2, 4.7 Hz, 2H), 3.24 (td, J = 12.9, 2.1 Hz, 2H), 4.03 (dt, J = 13.2, 2.0 Hz, 2H), 7.29 (t, J = 7.6 Hz, 1H), 7.37 (t, J = 7.6, 2H), and 7.48 (d, J = 7.9 Hz, 2H) ppm. ¹³C-NMR (125 MHz, CDCl₃): δ = 28.46 (3C, CH₃), 30.08 (2C, CH₂), 39.84 (2C, CH₂), 71.53 (Cq), 79.49 (Cq), 124.40 (2C, CH), 127.26 (CH), 128.47 (2C, CH), 147.94 (Cq), and 154.87 (Cq) ppm.
(0.64 ml, 10.8 mmol; 1.5 eq.) and the reaction was stirred overnight at room temperature. On the next day, more sodium hydride (0.15 g, 6.3 mmol, 0.88 eq.) and methyl iodide (0.33 ml; 5.2 mmol, 0.72 eq.) were added, and the mixture was stirred overnight at room temperature again. Saturated aqueous NaCl solution was added to the reaction, and the aqueous phase was washed with petroleum ether and EtOAc 3 + 1 to 1 + 2. Yield: 2.4 g.

4-Methoxy-4-phenylpiperidine (2a)

tert-Butyl 4-methoxy-4-phenylpiperidine-1-carboxylate 8 (2.06 g, 7.07 mmol; 1 eq.) was dissolved in dioxane and HCl (4.7 ml, 4 M; 2.5 eq.) and stirred for 4 hr under argon. More HCl (1 ml, 6 M; 0.85 eq.) was added and the mixture was stirred overnight at room temperature. Then saturated aqueous NaHCO₃ solution was added. The aqueous phase was washed three times with EtOAc. The organic phases were dried over Na₂SO₄, tested for peroxide and concentrated. Yield: 1.56 g.

4-Fluoro-4-phenylpiperidine (2b)

Hydrogen chloride solution (0.97 ml; 2 M) was added to tert-butyl-4-fluoro-4-phenylpiperidine-1-carboxylate 9 (0.20 g, 0.72 mmol) in dioxane (5 ml) under argon and stirred for 4 hr at room temperature. Because NMR showed there was no reaction, hydrogen chloride solution (5 ml; 2 M) was added and stirred overnight at room temperature. Afterward, the mixture was concentrated. Yield: 0.17 g.

1-Benzyl-4-methylpiperidine-4-ol (11)

Methyl lithium (30.4 ml, 48.6 mmol) was added dropwise to a solution of 1-benzyl-4-methylpiperidine 10 (5 ml; 27 mmol) in THF (40 ml) at −78°C and stirred for 1 hr 30 min. Ether and water were added and the phases were separated. The aqueous phase was extracted with diethyl ether, combined with the organic phase, dried with Na₂SO₄ and concentrated. The product was purified by flash chromatography with petroleum ether and EtOAc 3 + 1 to 1 + 1. Yield: 2.4 g. Brown oil, 43% yield.

1-Benzyl-4-methylpiperidine-4-carboxylic acid (12)

1-Benzyl-4-methylpiperidine-4-ol 11 (1 g, 4.9 mmol) and aluminum chloride (3.24 g; 24.3 mmol) were dissolved in benzene (21 ml) under argon and stirred at reflux for 18 hr. Benzene (10 ml) was added again and the mixture was poured cautiously into ice water. The pH of the aqueous phase was adjusted to 11–12 by the addition of aqueous sodium hydroxide solution (6 M) at a temperature of 0°C. The aqueous phase was extracted with EtOAc, the organic fractions were combined, dried over Na₂SO₄ and concentrated. The product was purified by flash chromatography with petroleum ether and diethyl ether 19 + 1 to 1 + 1. Yield: 0.550 g. Brown oil, 77% yield.

1H-NMR (500 MHz, CDCl₃): δ = 1.49 (d, J = 3.8 Hz, 9H), 1.74 (d, J = 12.6 Hz, 2H), 1.92–2.07 (m, 2H), 3.21 (dt, J = 3.57, 12.9, 2.2 Hz, 2H), 4.07 (dd, J = 42.2, 13.6 Hz, 2H), 7.27–7.34 (m, 1H), 7.34–7.39 (m, 2H), 7.43 (dd, J = 50.9, 7.4 Hz, 2H) ppm. 13C-NMR (50 MHz, CDCl₃): δ = 32.43 (3C, CH₃), 36.50 (d, J(13C-19F) = 23.2 Hz, 2C, CH₂), 39.68 (2C, CH₂), 79.71 (Cq), 94.23 (d, J(13C-19F) = 174.5 Hz, Cq), 123.81 (d, J(13C-19F) = 1.9 Hz, 2C, CH), 127.68 (d, J(13C-19F) = 1.3 Hz, CH), 128.39 (d, J(13C-19F) = 10.0 Hz, 2C, CH), 144.01 (d, J(13C-19F) = 21.3 Hz, Cq), and 154.78 (Cq) ppm.

1H-NMR (500 MHz, CDCl₃): δ = 1.22 (d, J = 3.8 Hz, 3H), 1.74–1.85 (m, 2H), 2.10–2.20 (m, 2H), 2.36–2.46 (m, 2H), 2.47–2.57 (m, 2H), 3.47 (s, 2H), 7.19 (t, J = 7.1 Hz, 1H), 7.22–7.27 (m, 1H), and 7.29–7.37 (m, 5H) ppm. 13C-NMR (125 MHz, CDCl₃): δ = 29.39 (CH₃), 38.55 (2C, CH₂), 49.60 (2C, CH₂), 62.99 (CH₂), 67.83 (Cq), 127.04 (Cq), 128.19 (2C, CH), 128.26 (2C, CH), and 138.04 (Cq) ppm.
4-Methyl-4-phenylpiperidine (2c)
Methanolic formic acid solution (12 ml; 4.4 wt%; 5.5 eq.) was freshly prepared and added to 1-benzyl-4-methyl-4-phenylpiperidine (550 mg; 2.07 mmol; 1 eq.) and Pd on activated carbon (350 mg; 10 wt%) and stirred overnight. The mixture was washed three times with DCMe, dried over Na2SO4 and concentrated. Yield: 0.307 g (2c), light brown oil, 85% yield. 1H-NMR (500 MHz, CDCl3): δ = 1.25 (s, 3H), 1.66–1.79 (m, 2H), 2.02–2.14 (m, 2H), 2.78–2.89 (m, 2H), 2.90–2.99 (m, 2H), 7.14–7.24 (m, 1H), and 7.29–7.41 (m, 4H) ppm. 13C-NMR (125 MHz, CDCl3): δ = 29.64 (CH3), 36.60 (Cq), 38.07 (2C, CH2), 42.91 (2C, CH2), 125.60 (CH), 125.67 (2C, CH), 128.35 (2C, CH), and 149.30 (Cq) ppm. HRMS (ESI): calcd for C12H18N2O2·H2O [M+H]+: found m/z 176.1433 [M+H]+.

4.2 | In vitro inhibition studies

4.2.1 | Materials
Rosewell Park Memorial Institute-1640 (RPMI-1640) cell culture media was purchased from Thermo Fisher Scientific (Waltham, MA). Supplements for cell culture, including fetal bovine serum (FBS), the antibiotics penicillin and streptomycin, as well as vincristine for selection of the CCRF VCR1000 cells were obtained from Sigma-Aldrich (St. Louis, MO). The fluorescent substrate daunorubicin as well as verapamil, DMSO, phosphate-buffered saline (PBS) and all compounds used for RPMI buffer preparation were also purchased from Sigma-Aldrich.

4.2.2 | Cell culture
The human T-lymphoblast cell line CCRF VCR1000, overexpressing P-gp, was generated by stepwise selection of CCRF-CEM cells in the vincristine-containing medium and was kindly provided by V. Gekeler (Altana-Pharma AG, formerly Byk–Gulden, Konstanz, Germany). CCRF VCR1000 cells were cultured in RPMI-1640 medium containing 20% FBS and were treated regularly with vincristine (1 µg/ml) for 72 hr, followed by centrifugation (300 g, 5 min, RT) and resuspension in a normal cell culture medium. Cells were maintained at 37°C in an atmosphere containing 5% CO2 with 95% relative humidity.

4.2.3 | Steady-state daunorubicin accumulation experiments
For P-gp inhibition studies, the steady-state accumulation of daunorubicin (3 µM) was performed as previously described, optimized to a 96-well plate format. Cells were harvested, pelleted (300 g, 5 min, 4°C), and diluted to a concentration of 12×10^6 cells/ml in RPMI (10 mM Hepes, 120 mM NaCl, 5 mM KCl, 0.4 mM MgCl2, 0.04 mM CaCl2, 10 mM glucose, 10 mM NaHCO3, 5 mM Na2HPO4, pH 7.4 with NaOH). For each data point, 15 µl of cell suspension was preincubated for 5 min at 37°C with 15 µl of 2% DMSO in RPMI alone (DMSO control) or containing different concentrations of test compound solutions. Incubation was done by placing the 96-well plate (nerbe plus GmbH, Winsen/Luhe, Germany) into an Eppendorf ThermoMixer® C device (Eppendorf, Hamburg, Germany). Thereafter, cells were loaded with 30 µl of daunorubicin, for 30 min at 37°C, to reach a final daunorubicin concentration of 3 µM. The final DMSO concentration was 0.5%. Loading was stopped by chilling the cells on watery ice for 5 min followed by the addition of 120 µl ice-cold PBS. Cells were pelleted (300 g, 5 min, 4°C), the supernatant was aspirated and cell pellet was resuspended in 120 µl ice-cold PBS. Until measurement cells were kept on ice and in the dark. Immediately before cell fluorescence measurement by flow cytometry (MACSQuant, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), 40 µl of DAPI solution (4 µg/ml in PBS) was added to gate out dead cells. Cells were kept at 4°C during measurement. Verapamil (100 µM) was included as a positive control for P-gp inhibition. The background fluorescence of the cell suspension was measured in the presence of 0.5% DMSO.

4.2.4 | IC50 value measurements and calculations
Flow cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR). IC50 values were estimated measuring eight different compound concentrations and performing nonlinear regression analyses (GraphPad Prism 6; “log(Agonist) vs. response–Variable slope” GraphPad Software, La Jolla, CA), for which the following equation was used:

\[
Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log(\text{IC50 apparent}) - X) \cdot \text{HillSlope}}}
\]  

where X is the log of compound concentration, Y is the response in fluorescent intensity units, "bottom" and "top" are the lower and the higher plateaus of the nonlinear fit curve, respectively, and HillSlope is a factor that describes the steepness of the curve.

To correct for the dependency of IC50 values on the expression level of P-gp and the pump-leak kinetics as reviewed by Stein, the final IC50 values were calculated using the following Equation:

\[
\text{IC50} = \text{IC50 apparent} \cdot \frac{\text{Bottom}}{\text{Top}}
\]  

where "bottom" and "top" are the lower and the higher plateaus of the nonlinear fit curve, respectively, and therefore they refer to the fluorescence intensity at zero and infinite inhibitor concentration, respectively. The mean IC50 values ± standard deviations given were calculated from three independent experiments for each compound.

4.3 | Calculation of lipophilicity and QSAR studies
The logP values were calculated using the “consensus” method of the MarvinSketch software (ChemAxon). The performance of the
"consensus" method was verified on a set of 19 propafenone derivatives whose distribution coefficient was experimentally determined by Chiba et al., using high-performance liquid chromatography (HPLC). The experimentally obtained and the calculated values were in good agreement (r = 0.98). QSAR studies carried out comprised multiple linear regression analyses performed in MS Excel.

4.4 Molecular docking analysis

To further understand the association between the activity of the compounds (4a and 4e) and the molecular structure, we performed molecular docking studies. The LigPrep module of Schrödinger Suite was used to generate the correct protonation states for the ligands, which were then used for the docking studies. The OPLS_2005 force field was applied for the minimization of the structures and different ionization states were generated by adding or removing protons from the ligand at a target pH of 7.0 ± 2.0 using Epik, version 3.1. Tautomers were also generated for each ligand. To generate stereoisomers, the information on chirality from the complexes. Top scored poses were then inspected for the hydrogen atoms were added, and optimal protonation states and ASN/GLN/HIS flips were determined. The binding site was defined as the complete transmembrane region. Docking was performed using the genetic algorithm-based docking program GOLD. All side-chains were kept rigid and the ligand was treated flexible by performing 100 genetic algorithm runs per molecule. The implemented Gold scoring function, GoldScore, was used for the evaluation of the complexes. Top scored poses were then inspected for the presence of protein–ligand interactions as reported by Klepsch et al. and successively a final pose for each ligand (4a and 4e) was selected.

ACKNOWLEDGMENTS

The authors thank V. Gekeler for providing the CCRF VCR1000 cells. They also thank D. Dobusch for performing the HRMS analysis. They wish to acknowledge the helpful discussions provided by M. Grandits and E. Heiß and thank ChemAxon for kindly providing them with their software. This project has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement no. 681002 (EU-ToxRisk) and the Austrian Science Fund (SFB35, #F03502).

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

REFERENCES

[1] E. E. Chufan, H.-M. Sim, S. V. Ambudkar ABC Transporters and Cancer, Vol. 125 (Eds: J. D. Schuetz, T. Ishikawa), Academic Press, New York, NY 2015, Ch. 3.
[2] F. J. Sharom, Essays Biochem. 2011, 50, 161. https://doi.org/10.1042/bse0500161
[3] R. Silva, V. Vilas-Boas, H. Carmo, R. J. Dinis-Oliveira, F. Carvalho, M. de Lourdes Bastos, F. Remião, Pharmacol. Ther. 2015, 147, 1. https://doi.org/10.1016/j.pharmthera.2014.11.013
[4] J. König, F. Müller, M. F. Fromm, Pharmacol. Rev. 2013, 65, 944. https://doi.org/10.1124/pr.113.0107518
[5] Z. Chen, T. Shi, L. Zhang, P. Zhu, M. Deng, C. Huang, T. Hu, L. Jiang, Cancer Lett. 2016, 370, 153. https://doi.org/10.1016/j.canlet.2015.10.010
[6] J. Keogh, B. Hagenbuch, C. Rynn, B. Stieger, G. Nicholls Role and Importance in ADME and Drug Development, Vol. 1 (Eds: G. Nicholls, K. Youdim), Royal Society Of Chemistry, Cambridge, UK 2016, Ch. 1.
[7] D. Balayssac, N. Authier, A. Cayre, F. Coudoure, Toxicol. Lett. 2005, 156, 319. https://doi.org/10.1016/j.toxlet.2004.12.008
[8] F. Montanari, G. F. Ecker, Adv. Drug Deliv. Rev. 2015, 86, 17. https://doi.org/10.1016/j.addr.2015.03.001
[9] Food and Drug Administration, Clinical drug interaction studies—Study design, data analysis, and clinical implications guidance for industry, Silver Spring, MD, 2017. https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM581965.pdf (accessed: November, 2018).
[10] European Medicines Agency, Guideline on the investigation of drug interactions, London, UK, 2012. https://www.ema.europa.eu/documents/scientific-guideline/guideline-investigation-drug-interactions_en.pdf (accessed: November, 2018).
[11] M. Lund, T. S. Petersen, K. P. Dahlhoff, Drugs 2017, 77, 859. https://doi.org/10.1007/s40265-017-0729-x
[12] H. Liu, Z. Ma, B. Wu, Xenobiotica 2013, 43, 1018. https://doi.org/10.3109/00498254.2013.791003
[13] R. Callaghan, Biochem. Soc. Trans. 2015, 43, 995. https://doi.org/10.1042/BST20150131
[14] T. Schwarz, F. Montanari, A. Cseke, K. Wisch, L. Visvader, S. Palme, P. Chiba, K. Kuchler, E. Urban, G. F. Ecker, ChemMedChem 2016, 11, 1380. https://doi.org/10.1002/cmdc.201500592
[15] M. Contino, S. Guglielmo, M. G. Perrone, R. Giampietro, B. Rolando, A. Carrieri, D. Zaccaria, K. Chegaev, V. Borio, C. Riganti, K. Zabielska-Kozyczews, N. A. Colabufo, R. Fruttero, MedChemComm 2018, 9, 862. https://doi.org/10.1039/c8md00075a
[16] I. K. Pajeva, M. Hani, M. Wiese, K. Steggemann, F. Marighetti, M. Wiese, ChemMedChem 2013, 8, 1701. https://doi.org/10.1002/cmdc.201300233
[17] X. Ma, M. Hu, H. Wang, J. Li, Eur. J. Med. Chem. 2018, 159, 381. https://doi.org/10.1016/j.ejmech.2018.09.061
[18] S. B. Syed, M. S. Coumar, Curr. Top. Med. Chem. 2016, 16, 2484. https://doi.org/10.2174/1568026616666160212123814
[19] A. Ferreira, S. Pousinho, A. Fortuna, A. Falcão, G. Alves, Phytochem. Rev. 2015, 14, 233. https://doi.org/10.1007/s11101-014-9358-0
[20] F. J. Sharom, Front. Oncol. 2014, 4, 41. https://doi.org/10.3389/fonc.2014.00041
[21] Y. Xu, E. Eigo, X. Li-Blatter, R. Müller, G. Merino, S. Bernèche, A. Seelig, Biochimie 2015, 54, 6195. https://doi.org/10.1012/acs. biochem.5b00649

ORCID

Anna Cseke https://orcid.org/0000-0002-1801-0317
Gerhard F. Ecker https://orcid.org/0000-0003-4209-6883
Additional supporting information may be found online in the Supporting Information section.