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Kinesin spindle protein (KSP) inhibitors. Part V: Discovery of 2-propylamino-2,4-diaryl-2,5-dihydropyrroles as potent, water-soluble KSP inhibitors, and modulation of their basicity by β-fluorination to overcome cellular efflux by P-glycoprotein

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Abstract—Installation of a C2-aminopropyl side chain to the 2,4-diaryl-2,5-dihydropyrrole series of kinesin spindle protein (KSP) inhibitors results in potent, water soluble compounds, but the aminopropyl group induces susceptibility to cellular efflux by P-glycoprotein (Pgp). We show that by carefully modulating the basicity of the amino group by β-fluorination, this series of inhibitors maintains potency against KSP and has greatly improved efficacy in a Pgp-overexpressing cell line. The discovery that cellular efflux by Pgp can be overcome by carefully modulating the basicity of an amine may be of general use to medicinal chemists attempting to transform leading compounds into cancer cell- or CNS-penetrant drugs.

The discovery and widespread use of the taxanes and vinca alkaloids as effective therapies to combat cancer has led to the recognition of the mitotic spindle as a well-validated target for chemotherapeutic agents.[1] As effective as these antimitotic agents have been clinically, they suffer from a number of liabilities, including: (1) the development of resistance through both tubulin mutations and P-glycoprotein (Pgp)-mediated cellular efflux; (2) significant dose-limiting toxicities due to drug action on post-mitotic neurons; and (3) inconvenient dosing regimes including prophylactic treatment to prevent vehicle-associated toxicities.[2]

Small molecule inhibitors of kinesin spindle protein (KSP, or *Hs*Eg5) are part of a new generation of antimitotic agents that do not interact directly with the mitotic spindle, but instead target key regulatory enzymes required during mitosis. KSP is a microtubule motor protein that is essential for the generation of a bipolar mitotic spindle, and its inhibition leads to mitotic arrest followed by apoptotic cell death.[3] The anticipated clinical benefits of KSP inhibitors relative to the taxanes and vinca alkaloids include: (1) a different resistance profile with the potential for activity against refractory tumors; (2) lack of neuronal toxicity, since KSP is not expressed in post-mitotic neurons; and (3) simplified dosing formulations. Several companies are currently investigating KSP inhibitors in the clinic to establish if these benefits will be realized in practice.[4]

Dihydropyrazole **1** and dihydropyrrole **2** are small molecule inhibitors of KSP discovered at Merck that possess moderate potency, but are not suitable for dosing in an aqueous vehicle.[5] We recently disclosed a structure-based optimization of **1** that led to the installation of an aminopropyl side chain to provide dihydropyrazole **3** with improved potency, pharmacokinetics, and good aqueous solubility.[6] In this communication, we describe how the aminopropyl appendage was incorporated into

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the dihydropyrrole series to provide the potent KSP inhibitor (KSPi) **4**, but that this compound, as well as its dihydropyrazole analog **3**, is not potent in a multidrug-resistant (MDR) cell line due to efflux by Pgp. We then go on to illustrate that careful modulation of the basicity of the amine in **4** by β-fluorination provides KSP inhibitors such as **18** that are effective in a Pgp-overexpressing cell line, and have thus overcome cellular efflux by Pgp.

Design and synthesis. Our previous studies have established that the preferred substitution patterns in the dihydropyrazole and dihydropyrrole series are very similar.[5,6] Additionally, X-ray crystallographic evidence indicates that both series bind in a similar mode to the same allosteric site of KSP, making effective use of both hydrophobic and hydrophilic interactions. It was therefore a logical extension that aminopropyl substitution at C2 of dihydropyrrole **2** would lead to a KSPi with a similar increase in potency as seen in going from **1** to **3**, and thus **4** became the target of our synthetic efforts (Fig. 1). Evaluation of the literature revealed no general way to access 2,2-disubstituted-2,5-dihydropyrroles, so we embraced the challenge of designing a route to these targets. As illustrated in Scheme 1, the methodology we developed to reach **4** has the advantage of allowing straightforward SAR investigation of the C4-aryl group, the C2-alkyl substituent, and the acyl group at N1.

The benzyl imine of racemic phenylglycine methyl ester (**5**) was first alkylated with methallyl dichloride under phase transfer conditions to give the monoalkylation product (not shown or isolated). The imine was subsequently hydrolyzed with aqueous hydrochloric acid, and during workup a second, intramolecular alkylation occurred to furnish pyrrolidine **6**. Crude **6** was treated with LAH to afford an amino alcohol, and reaction with 1,1′-carbonyldiimidazole provided oxazolidinone **7** which was treated with ozone to generate bicyclic ketone **8** in an overall yield of 20% from **5**.

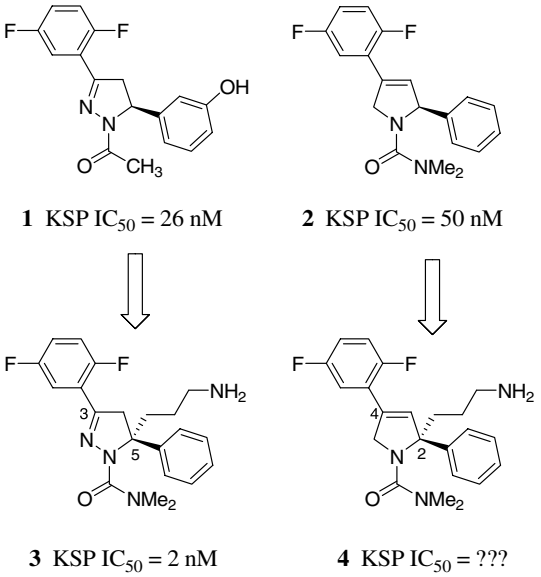


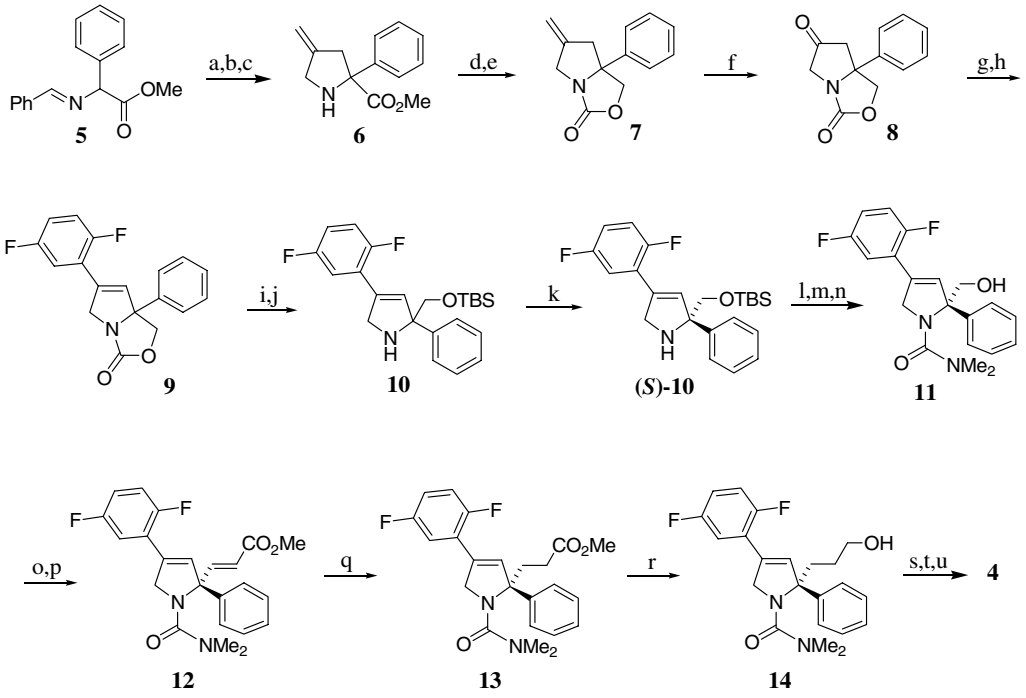
Figure 1. Dihydropyrazole and dihydropyrrole inhibitors of KSP.

The triflate of ketone **8** was generated regioselectively with $PhN(Tf)_2$ and NaHMDS,[5b] and converted to **9** via Suzuki reaction in 64% yield for two steps. The oxazolidinone was hydrolyzed with NaOH and the resulting amino alcohol was treated with *t*-butyldimethylsilyl chloride to provide **10** in nearly quantitative yield. As noted in our previous reports, only the (*S*)-antipode of compounds in this class of inhibitors exhibits KSP activity, so a chiral HPLC separation was carried out at this stage to provide optically pure (***S***)-**10**. Pretreatment of this intermediate with triphosgene, followed by the addition of dimethylamine and subsequent deprotection, led to **11** in 90% yield from (***S***)-**10**.

Installation of the aminopropyl side chain commenced with Dess–Martin oxidation of **11** to the corresponding aldehyde in quantitative yield, followed by Horner–Wadsworth–Emmons reaction with commercially available trimethyl phosphonoacetate to provide **12**. Since the nonconjugated double bond in the core of **12** was reactive to a number of standard hydrogenation conditions, a $NiCl_2$-mediated $NaBH_4$ conjugate reduction of the α,β-unsaturated ester was carried out to provide **13**. Following reduction of the ester to alcohol **14**, mesylate formation, azide displacement, and finally Staudinger reduction led to the targeted amine **4**.

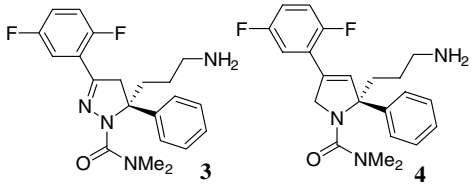
Table 1 summarizes the key properties of dihydropyrazole **3** and the newly synthesized dihydropyrrole **4**. As expected, **4** has excellent potency, both against purified enzyme and in a cell-based assay, as well as good solubility in pH 4 water. While **3** displays weak binding affinity for the hERG potassium channel, **4** shows somewhat greater binding, consistent with earlier reports on compounds in the dihydropyrrole series.[5b] Unfortunately, both inhibitors possessed significantly reduced potency in Pgp-overexpressing cells derived from a KB-3-1 human epidermoid carcinoma cell line, as measured by the MDR ratio. This ratio provides a measure of Pgp-mediated resistance to mitotic arrest, and entails determining the IC_{50} for induction of a G2/M block by the test compound in a cell line that highly overexpresses Pgp, as well as the IC_{50} in the parental line that does not express Pgp. The quotient of these numbers is reported as the MDR ratio. For instance, a ratio of unity indicates that the KSPi is not effluxed by Pgp because it is equipotent in both cell lines. Taxol, by comparison, has an MDR ratio of about 25,000. KSP inhibitors **3** and **4**, with MDR ratios of 490 and 1200, respectively, are therefore subject to Pgp-mediated cellular efflux.[7]

Optimization of Pgp profile. Pgp is a protein encoded in the human MDR1 gene that has been extensively studied as a major aspect of the MDR phenotype.[8] Since many cancer cells have been shown to overexpress Pgp, and it is assumed that Pgp plays a role in clinical resistance to Taxol and other important antimitotic agents,[9] good levels of potency in a Pgp-overexpressing cell line was a selection criterion for advancing compounds in our program. Several small molecule inhibitors of KSP have been reported to retain efficacy in Pgp-overexpressing cell lines,[10] and **1** and **2** both have an MDR ratio of 1. This knowledge, together with the



Scheme 1. Reagents and conditions: (a) $ClCH_2C(CH_2)CH_2Cl$, Bu_4NHSO_4, 10 M NaOH, DCM; (b) 1MHC1; (c) Na_2CO_3/H_2O; (d) LAH, THF, 0 °C to rt; (e) CDI, TEA, DCM; (f) O_3, DCM, −78 °C, then DMS (~20% yield from **5**, trituration from acetone); (g) NaHMDS, THF, −78 °C, then $PhN(Tf)_2$; (h) 2,5-difluoroboronic acid, Na_2CO_3, LiCl, $Pd(PPh_3)_4$, DME (64% for 2 steps); (i) 10 M NaOH, EtOH, 60 °C; (j) TBSC1, imidazole, 5% DMF in DCM (96% for 2 steps); (k) Chiralpak AD, 1% iPrOH in hexanes (0.1% DEA as modifier), first isomer to elute is the desired (*S*)-isomer; (1) triphosgene, TEA, THF; (m) $NHMe_2$; (n) HF-TEA, CH_3CN (90% for 3 steps); (o) Dess–Martin Periodinane, DCM (quant); (p) trimethyl phosphonoacetate, NaH, THF (67%); (q) $NiCl_2$, $NaBH_4$, MeOH, 0 °C; (r) $LiBH_4$, 3:1 THF/MeOH (55% from **12**); (s) MsCl, TEA, DCM; (t) NaN_3, DMF, 50 °C; (u) PPh_3, THF, 55 °C, then H_2O (43% for three steps).

Table 1. Comparison of the key properties of **3** and **4**



Property	Compound **3**	Compound **4**
KSP IC_{50}[a] (nM)	1.9 ± 1.2	2.2 ± 1.2
Cell potency[a] (nM)	5.2 ± 0.3	6.0 ± 1.1
log *P*	1.2	1.1
Solubility[b] (mg/mL)	>12	>12
hERG IC_{50}[c] (μM)	19.3 ± 0.7	7.1 ± 3.7
MDR ratio[d]	490	1200

[a] See Ref. 5b for assay details, average of $n = 3$ or greater.
[b] Solubility of free-base in 0.1 M citrate, initial pH 4.0.
[c] See Ref. 5b for assay details, average of $n = 2$ or greater.
[d] See text and Ref. 7 for discussion of this assay.

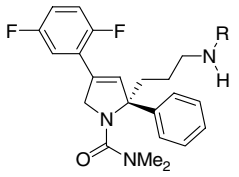
fact that basic amines are a pharmacophore often found in Pgp substrates,[11] implicated the aminopropyl side chain for engendering Pgp susceptibility in **3** and **4**.

Our previous investigation demonstrated that the protonated form of the basic amine in dihydropyrazoles such as **3** is engaged in a hydrogen bonding interaction with the amide backbone in the allosteric site of KSP.[6] Consistent with this observation, a decrease in potency was noted when the basicity of the amine was significantly reduced, eliminating this favorable interaction. A similar trend has been observed in the dihydropyrrole series. In light of these observations, namely that KSP potency is improved by a basic amine while the MDR ratio is improved by a nonbasic amine (or lack of an amine altogether), we sought to determine if a middle ground could be established where both features are maintained in an optimal range.

To this end, we explored β-fluorination of the amine in **4** to carefully modulate its basicity in a step-wise manner while imparting minimal steric effects.[12] As revealed in Table 2, substituting the amine with an ethyl group provides **15a** with a slight reduction in potency relative to **4**, but it remains a good substrate for Pgp efflux. Monofluorination of the ethyl group affords **15b** with no loss in KSP potency relative to **15a**, but with a significant improvement in the MDR ratio. Gratifyingly, difluorination provides **15c** that again retains good KSP potency but has an MDR ratio of almost unity. Finally, the nonbasic trifluoroethyl derivative **15d** continues the trend of reduced MDR ratio, but also suffers a substantial reduction in KSP potency. The final two columns in Table 2 illustrate the dramatic changes to pK_a and log *P* that β-fluorination imparts across this series of inhibitors.

The data presented in Table 2 indicate that when the amine has a pK_a of about 7, KSP activity is maintained while Pgp-mediated efflux is greatly attenuated.

Table 2. Effect of β-fluorination on key properties of C2-propylamino dihydropyrroles



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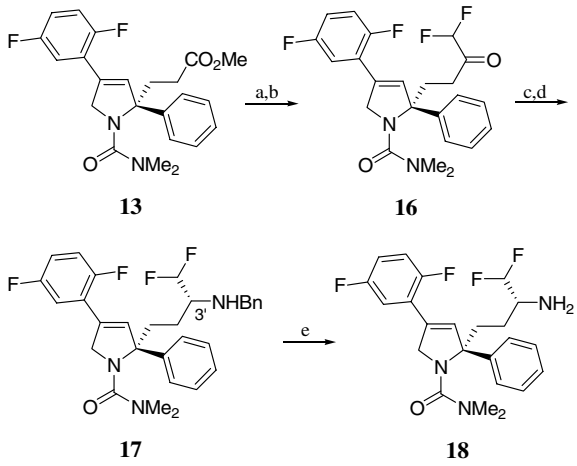
Compound[a]	R	KSP IC_{50}[b] (nM)	MDR ratio[c]	pK_a[d]	log P
4	H	2.2 ± 1.2	1200	10.3	1.2
15a	CH_2CH_3	10.2 ± 2.0	>135	10.7	1.6
15b	CH_2CH_2F	10.2 ± 1.9	32	8.8	2.6
15c	CH_2CHF_2	12.1 ± 2.5	3	7.0	3.4
15d	CH_2CF_3	110 ± 47	1	5.2	>3.2

[a] The synthesis of analogs **15a-d** was performed via $Na(OAc)_3BH$-mediated reductive amination of the appropriate amine with the aldehyde generated by Dess–Martin oxidation of **14**.
[b] See Ref. 5b for assay details, average of $n = 3$ or greater.
[c] See text and Ref. 7 for discussion of this assay.
[d] Values were determined with a Sirius GL pK_a titrator, average of $n = 3$ determinations.

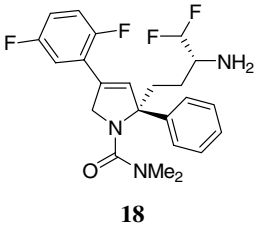
Extensive literature exists on the rational design of compounds to evade Pgp recognition; however, most published examples of altering an amino functionality to circumvent Pgp efflux do so at the expense of adding or subtracting hydrogen bond donors or acceptors, and/or greatly altering the steric environment of the amine.[13] To the best of our knowledge, this is the first demonstration that β-fluorination of an aliphatic amine can overcome Pgp-mediated efflux while maintaining good activity on the target of interest.[14]

Though **15c** possesses a good balance of properties, a fivefold reduction in KSP potency relative to **4** was observed in the process of enlarging the substituent on the amine. Several alternative β-difluorination strategies were therefore undertaken in an attempt to increase potency, the most successful of which resulted in the interior α-difluoromethyl substituted primary amine **18**. Scheme 2 illustrates how **13** was transformed into **18**, beginning with the addition of diethyl (difluoromethyl) phosphonate to produce ketone **16**, followed by $TiCl_4$-mediated reductive amination with benzylamine to generate **17**. Conveniently, the desired 3′R-diastereomer was easily separated from its 3′S-isomer at the stage of **17** by flash chromatography.[15] Transfer hydrogenation in acetic acid cleanly unveiled the primary amine **18**.

Figure 2 shows the key properties of **18**. Both enzymatic and cell-based potency have been improved relative to **15c**. The log P and pK_a are dramatically affected by β-fluorination, but solubility in pH 4 water remains excellent. Additionally, the hERG binding of **18** is reduced relative to the starting point **4**. Consistent with this observation, it has been proposed that decreasing the pK_a of a basic nitrogen disrupts π-cation interactions in the hERG channel binding site due to a reduction in the percentage of molecules in the ionized form at physiological pH.[16]



Scheme 2. Reagents and conditions: (a) LDA, $F_2CHP(O)(OEt)_2$, −78 °C, THF, then **13**; (b) NaOMe, MeOH (77% for two steps); (c) $TiCl_4$, $BnNH_2$, TEA, DCE, stir 24 h, then $NaCNBH_3$ in MeOH; (d) flash chromatography (SiO_2, EtOAc/hexanes), first isomer to elute (45% from **16**); (e) 1,4-cyclohexadiene, 10% Pd/C, AcOH, 50 °C (86%).



KSP IC_{50} = 5.2 ± 0.8 nM
Cell Potency = 22.5 ± 5.6 nM
logP = 3.2; pK_a = 7.0
solubility @ pH 4: > 10 mg/mL
hERG = 15.9 ± 1.7 μM
MDR ratio = 5

18

Figure 2. Important properties of **18**.

As expected, the MDR ratio of **18** is very similar to that observed for **15c**. It is significant to note that a standard monolayer transport assay also indicated **18** is much less susceptible to Pgp efflux than **4**. In an LLC-PK1 cell line transfected with the human MDR1 gene, **18** has a $B-A/A-B$ ratio of 2.5 and a passive permeability of 38×10^{-6} cm/s. By comparison, **4** has a $B-A/A-B$ ratio of 18.5 and a much lower passive permeability of 4×10^{-6} cm/s.[17] Thus, **18** represents an optimized KSPi with good potency, aqueous solubility, and promising activity in an MDR cell line that highly overexpresses Pgp.[18]

Importantly, we have found that modulating the pK_a of a basic nitrogen to the range of 6.5–8.0 to balance Pgp efflux potential with KSP potency is a general trend that holds for several diverse series of KSP inhibitors.[19] Adjusting the pK_a to approximately physiological pH insures that significant concentrations of both the ionized and neutral forms are available for pharmacological activity. This may explain the relationship found herein between pK_a and inhibitor activity, because the ability of the amine to achieve the ionized form is required for good KSP potency. On the other hand, increasing lipophilicity and favoring the neutral form of the amine by halogenation increases membrane permeability, and compounds with very good passive permeability can override the effect of efflux by the Pgp transporter.[20]

However, we cannot exclude the possibility that pK_a is simply a surrogate marker for Pgp efflux potential that is correlated with other physicochemical changes, such as the increased lipophilicity revealed by log P values, that occur due to halogenation. While more detailed studies are required to show how the interaction of our inhibitors with the Pgp transporter changes as the basicity of the amine is modified,[21] that does not limit the usefulness of the relationship we have established between pK_a and activity in Pgp-overexpressing cells. Significantly, the monolayer transport data for **18** corroborates the information provided by the MDR ratio, and suggest that the correlations established herein may be of general use to those involved not only in oncology research, but in CNS drug discovery as well.

In conclusion, we described how addition of a propylamino side chain to the 2,4-diaryl-2,5-dihydropyrrole series provided potent, water-soluble inhibitors of KSP. Additionally, we illustrated that the efficacy of these compounds against a Pgp over-expressing cell line can be dramatically increased by carefully tuning the basicity of the amine by β-fluorination. In a forthcoming paper, we will demonstrate how we applied these lessons learned in balancing Pgp efflux potential with KSP potency to identify an optimal clinical candidate for the treatment of cancer.

Acknowledgments

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14. Recent publications have utilized β-fluorination of aliphatic amines to provide improvements in pharmacokinetics (ref a) or selectivity (ref b) while maintaining good potency against the target of interest; see: (a) van Niel, M. B.; Collins, I.; Beer, M. S.; Broughton, H. B.; Cheng, S. K. F.; Goodacre, S. C.; Heald, A.; Locker, K. L.; MacLeod, A. M.; Morrison, D.; Moyes, C. R.; O'Connor, D. O.; Pike, A.; Rowley, M.; Russell, M. G. N.; Sohal, B.; Stanton, J. A.; Thomas, S.; Verrier, H.; Watt, A. P.; Castro, J. L. *J. Med. Chem.* **1999**, *42*, 2087; (b) Grunewald, G. L.; Seim, M. R.; Lu, J.; Makboul, M.; Criscione, K. R. *J. Med. Chem.* **2006**, *49*, 2939.
15. The stereochemistry at the 3′-center was determined by carrying both diastereomers from the reductive amination through the final step of the sequence described in Scheme 2, and studying the more potent diastereomer, **18**, by X-ray crystallography. The structure of KSP-**18**-ADP was determined as previously described for other inhibitors in our program (see Ref. 6 and references therein), and thus also confirmed the (*S*)-stereochemistry at C2.
16. Jamieson, C.; Moir, E. M.; Rankovic, Z.; Wishart, G. *J. Med. Chem.* **2006**, *49*, 5029.
17. Compounds with a $B - A/A - B$ ratio less than three are considered weak Pgp substrates, and thus have the potential for good brain penetration. For a discussion on the use of the monolayer transport assay in drug design, see Ref. 8a.
18. In the KB-3-1 (parental) cell line, **18** has an IC_{50} = 24 nM, whereas in the KB-V-1 (Pgp-overexpressing) line the IC_{50} = 123 nM. By way of comparison, the values for **4** are 23 nM and 28 μM, respectively.
19. In forthcoming publications we will illustrate that this trend holds for structurally diverse KSP inhibitors where basicity is controlled not only by β-fluorination, but by other means as well.
20. Marbeuf-Gueye, C.; Ettori, D.; Priebe, W.; Kozlowski, H.; Garnier-Suillerot, A. *Biochim. Biophys. Acta* **1999**, *1450*, 374.
21. Several studies have examined the way amine basicity alters recognition of drugs by the Pgp transporter, see: (a) Ecker, G.; Huber, M.; Schmid, D.; Chiba, P. *Mol. Pharmacol.* **1999**, *56*, 791; (b) Frezard, F.; Pereira-Maia, E.; Quidu, P.; Priebe, W.; Garnier-Suillerot, A. *Eur. J. Biochem.* **2001**, *268*, 1561.