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In vitro activity of novel dual action MDR anthranilamide modulators with inhibitory activity on CYP-450 (Part 2)

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Abstract—Synthesis and in vitro cytotoxicity assays of new anthranilamide MDR modulators have been performed to assess their inhibition potency on the P-glycoprotein (P-gp) transporter. Previous studies showed that the replacement of the aromatic spacer group between nitrogen atoms (N^1 and N^2) in the P-gp inhibitor XR9576 with ethyl or propyl chain is optimal for P-gp inhibition potency. To confirm that observation, the ethyl or the propyl linker arm was replaced with a pyrrolidine or an alicyclic group such as cyclohexyl. In addition, an arylpiperazinyl group and two methoxyl groups onto the anthranilic part were introduced to assess their effect on the anti P-gp activity. Five molecules were prepared and evaluated on CEM/VLB500. All new anthranilamides were more potent than verapamil, most of them exhibited a lower cytotoxicity than XR9576. Compound **5** was the most potent and its inhibition activity was similar to XR9576. Interestingly, in vitro biotransformation studies of compounds **4** and **5** using human CYP-450 isoforms revealed, that conversely to XR9576, compounds **4** and **5** inhibited CYP3A4, an enzyme that colocalizes with P-gp in the intestine and contributes to tumor cell chemoresistance by enhancing the biodisposition of numerous drugs, notably paclitaxel. In that context, **5** might be suitable for further drug development.

1. Introduction

Multidrug resistance (MDR) in tumor cells is a major impediment to successful cancer therapy.[1] Multidrug resistance is a phenomenon related to tumor cells having been exposed to a cytotoxic agent and subsequently developed cross-resistance to a wide range of structurally and functionally unrelated compounds such as anthracyclines, vinca alkaloids, podophyllotoxins, and taxus alkaloids.[2] The chemoresistance that tumor cells develop often results from the overexpression of particular proteins such as multidrug-resistance-associated protein (MRP) and P-glycoprotein (P-gp).[3] P-gp is an ATP-binding cassette transporter encoded by the MDR1 gene.[4] P-gp contributes to drug resistance via ATP-dependent drug efflux of the cytotoxic drugs out of the cancer cells, thus lowering the intracellular concentrations at innocuous levels.[5,6] In addition, MDR genes are expressed intrinsically in some cancer cells without previous exposure to chemotherapy agents.[7] Therefore, molecules able to antagonize P-gp activity are of utmost importance to restore the efficacy of clinically used antineoplastics. To that end, several studies have demonstrated that P-gp activity can be lowered both in vitro and in vivo by a wide variety of structurally heterogeneous molecules such as verapamil,[8] cyclosporin A,[9] tacrolimus,[10] quinidine,[11] dihydropyridines,[12] and other drugs.[13,14] Consequently, numerous clinical trials using clinically relevant P-glycoprotein modulators such as verapamil,[15,16] tamoxiphen,[17] progesterone,[18] and cyclosporin A[19] have been conducted with limited successes mainly because of their intrinsic toxicity or the unfavorable pharmacokinetics of the accompanying anticancer drugs. A third generation of P-gp modulators including cyclosporin D analogues,[20] anthranilamide derivatives (XR9576, tariquidar™),[21–23] acridonecarboxamide derivatives (elacridar™),[24] and cyclopropyldibenzosuberane derivatives (zosuqui-

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dar™)[25] have displayed improved P-gp selectivity and pharmacological properties.[26]

The anthranilamide derivative XR9576 (Fig. 1) was shown to reverse primary doxorubicin, vinblastine, and paclitaxel resistance in advanced breast cancer. Unfortunately, a recent phase II clinical trial on breast carcinoma and a phase III on lung carcinoma assessing the efficacy of tariquidar™ in combination with vinorelbine versus vinorelbine alone were discontinued due to a significant proportion of undisclosed adverse events in the tariquidar™ cohort.[27] In addition, a third clinical trial assessing the efficacy of paclitaxel/carboplatin/tariquidar™ in combination was also terminated due to poor response rates and toxicity.[28] Therefore, there are unmet needs for newer P-gp inhibitors with reduced intrinsic toxicity.

We report here the synthesis of novel P-gp inhibitors based on the anthranilamide derivative XR9576 (Fig. 1). As aforementioned, it is known that anthranilamides abrogate cell chemoresistance at low nanomolar levels. We have already published a study of ten anthranilamide derivatives (Fig. 2) that were designed to assess the importance of the aromatic spacer and the distance between N^1 and N^2 by replacing the aromatic spacer with alkyl chains of various lengths.[29]

In that paper, we have shown that an ethyl or a propyl carbon spacer between N^1 and N^2 is optimal for P-gp inhibition. The most potent P-gp modulator synthesized was **P03** (Fig. 2) and it is fourfold more active than verapamil, but fourfold less active than XR9576. To optimize the anti P-gp activity of compound **P03**, we have rigidified the spacer group between N^1 and N^2 found in portion C of the molecule (Fig. 2). In that context, we have evaluated the effect of modifying the ethyl group of **P03** to the inhibitory activity on P-gp by choosing a group with the same length and more rigidity such as *R* or *S* pyrrolidinyl group and cyclohexyl group (Fig. 3). We have decided also to modify the linker by an arylpiperazinyl group instead of ethyl linker because the distance between N^1 and N^2 is similar to the distance between N^1 and N^2 of XR9576. We prepared arylpiperazinyl derivatives bearing two methoxy-groups on the anthralinidyl moiety (Fig. 3) to be similar to XR9576. All compounds were compared to verapamil (VRP) and XR9576. Herein we describe our efforts to identify new anthranilamide-based P-gp inhibitors.

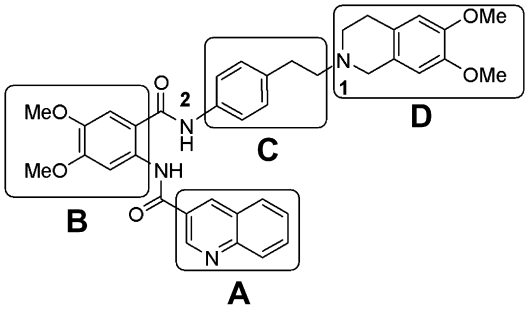
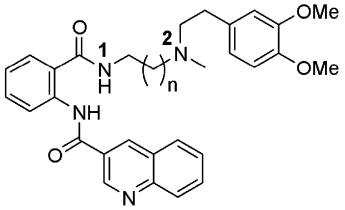


Figure 1. Molecular structure of XR9576 (tariquidar™). The letters A, B, C, and D define the four regions of XR9576.

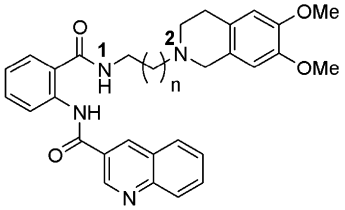
2. Chemistry

The preparation of compound **1S** or **2R** (Scheme 1) was achieved from commercially available enantiomer **6S** or **6R** and isatoic anhydride (**7**) by acylation producing the aniline **8S** or **8R** in good yields. Afterwards, **8S** or **8R** was reacted with 3-quinoloyl chloride (**11**) in the presence of K_2CO_3 in dichloromethane to generate the secondary amine **10S** or **10R** in good yields. Following the deprotection of the secondary amine **10S** or **10R** in acidic condition, nucleophilic substitution reaction of **11S** or **11R** with 4-(2-chloroethyl)-1,2-dimethoxybenzene (**12**) in acetonitrile and K_2CO_3 gave the target compound **1S** or**2R** (Schemes 2 and 3).

The synthesis of compound **3** was achieved from commercially available cyclohexane oxide (**13**) and phenylpiperazine (**14**) producing the corresponding secondary *trans*-alcohol **15** (*rac–trans*) in good yield as a mixture of diastereoisomers. Compound **15** (*rac–trans*) was reacted with methanesulfonyl chloride and Et_3N in dichloromethane to generate the methanesulfonate derivative. Then nucleophilic substitution on the leaving group methanesulfonate was performed using sodium azide to yield the corresponding azido compound **16** (*rac–cis*). Hydrogenation of **16** (*rac–cis*) with Pd/C at 38 psi for 1 h gave the *rac–trans*-(4-phenylpiperazin-1-yl)cyclohexanamine. The *rac–trans*-(4-phenylpiperazin-1-yl)cyclohexanamine was reacted with isatoic anhydride to produce aniline **17** (*rac–cis*) in good yield. 3-quinoloyl chloride and aniline **17** (*rac–cis*) were finally



P01 (n = 4)
P02 (n = 3)
P03 (n = 1)
P04 (n = 2)
P05 (n = 5)



P06 (n = 2)
P07 (n = 4)
P08 (n = 5)
P09 (n = 1)
P10 (n = 3)

Figure 2. Molecular structures of compounds P01-P10.[28]

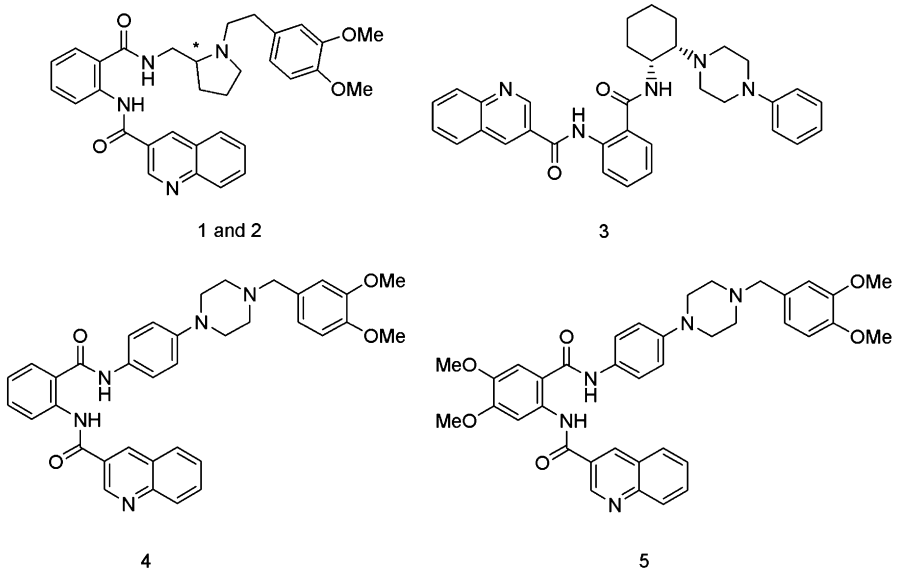
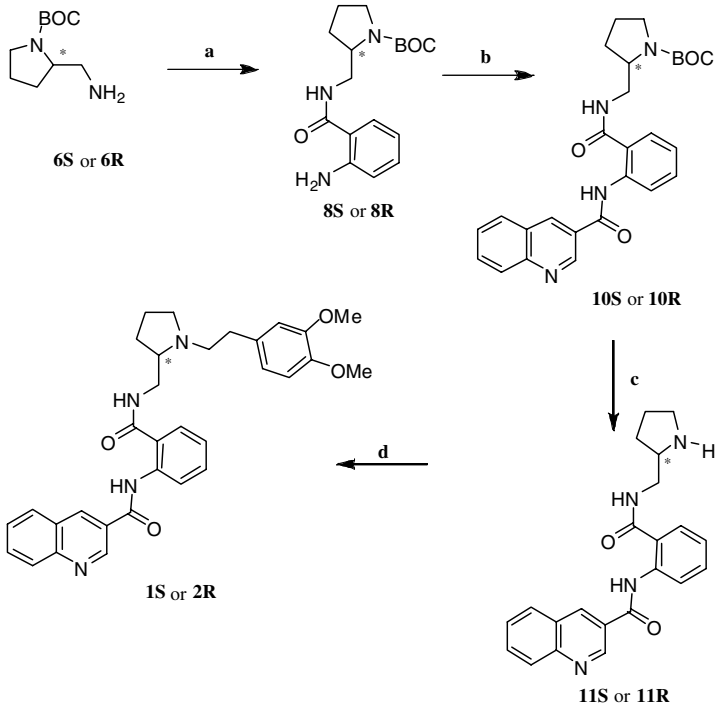


Figure 3. Structures of compounds **1S**, **2R**, **3**, **4**, and **5**.



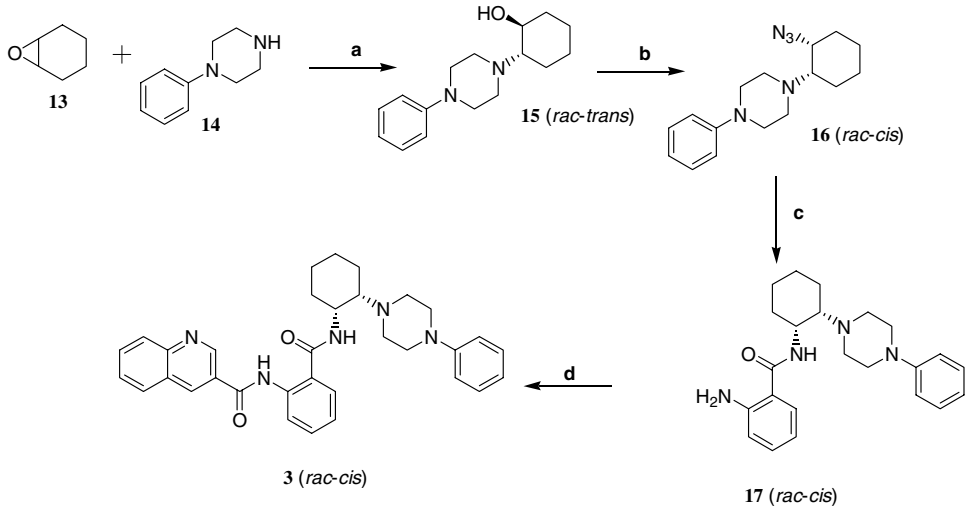
Scheme 1. Preparation of **1S** and **2R**. Reagents and conditions: (a) isatoic anhydride **7**, acetonitrile, reflux, 24 h; (b) 3-quinoline acid chloride **9**, CH_2Cl_2, 0–25 °C, K_2CO_3, 24 h; (c) TFA/CH_2Cl_2; (d) 4-(2-chloroethyl)-1,2-dimethoxybenzene **12**, acetonitrile, K_2CO_3, reflux, 24 h.

reacted in the presence of K_2CO_3 in dichloromethane to generate compound **3** (*rac–cis*).

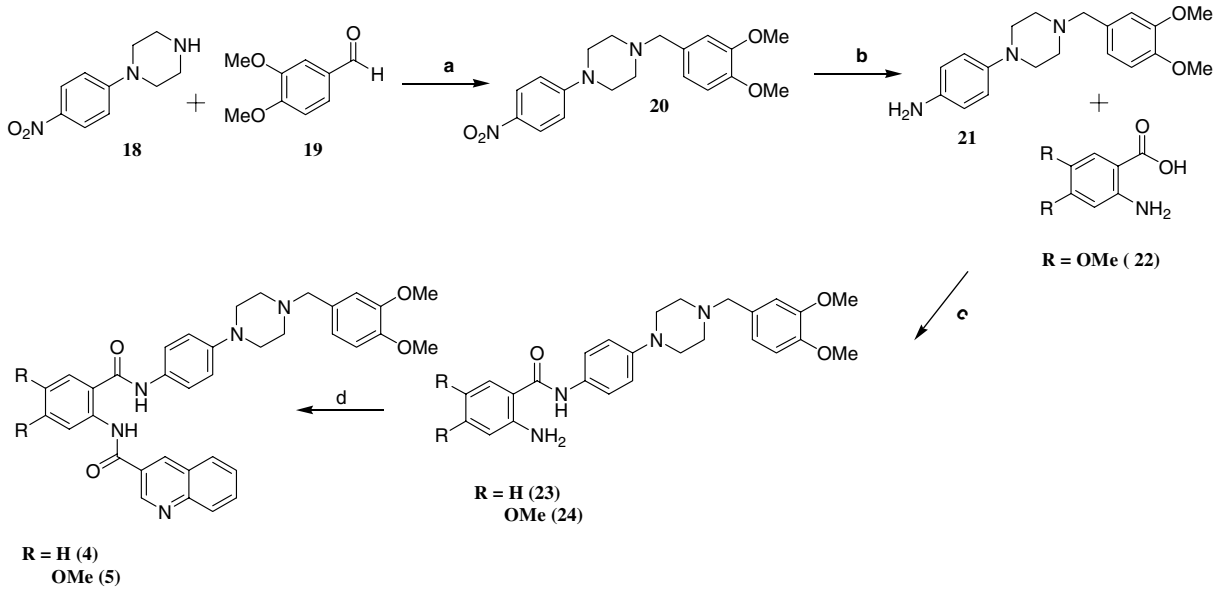
The synthesis of compound **4** was achieved by reacting the 4-nitrophenylpiperazine (**18**) and the 3,4-dimethoxybenzaldehyde (**19**) to produce the alkylated piperazine **20** in quantitative yields. Hydrogenation of compound **20** with Pd/C at 38 psi for 1 h produced the amine **21**. Afterwards, the amine **21** was acylated with isatoic anhydride (**7**) to yield the corresponding aniline **23** or with 3,4-dimethoxyanthranilic acid (**22**) producing the corresponding aniline **24** in moderate yields. Aniline **23** or**24** was reacted with 3-quinoloyl chloride in the presence of K_2CO_3 in dichloromethane to generate compound **4** or **5**.

3. In vitro biological evaluation

The P-gp inhibition potency of compounds **1S**, **2R**, **3**, **4**, and **5** was assessed using CEM/VLB_{500} human leukemia cells. The assay is based on the ability of the drug to revert



Scheme 2. Preparation of compound **3**. Reagents and conditions: (a) H_2O, reflux, 18 h; (b) 1—MsCl, Et_3N, CH_2Cl_2; 2—NaN_3, DMF, reflux, overnight; (c) (1) 10% Pd/C, EtOH, 38 PSI, 1 h; (2) isatoic anhydride **7**, acetonitrile, reflux, overnight; (d) 3-quinoline acid chloride **9**, CH_2Cl_2, 0–25 °C, K_2CO_3, 24 h.



Scheme 3. Preparation of compounds **4** and **5**. Reagents and conditions: (a) $NaBH(OAc)_3$; (b) 10% Pd/C, EtOH, 38 psi 1 h; (c) isatoic anhydride **7** or **22**, acetonitrile, reflux, overnight; (d) 3-quinoline acid chloride **9**, CH_2Cl_2, 0–25 °C, K_2CO_3, 24 h.

the chemoresistance of these cells to vinblastine (VBL). As depicted in Figure 4, CEM/VLB_{500} cells were more than 5500-fold resistant to VLB than their wild-type counterpart (CEM). This ratio was determined by cytotoxicity assays using escalating concentration of vinblastine (0–15 μM) on CEM/VLB_{500} and wild-type CEM cell lines.

The ability of the drugs to reverse MDR in chemoresistant cells was determined by treating the cells with escalating concentrations (0–15 μM) of the inhibitor either alone or in combination with 100 nM of vinblastine that is P-gp substrate. After 3 days of treatment, the GI_{50} of the inhibitor (Table 1) (concentration of inhibitor necessary to kill 50% of the cells) was determined using the resazurin assay.[30] A 'difference score' (EC_{50}) based on the difference between the number of cells that survived in presence of the modulator alone (cytotoxicity of the inhibitor) minus the number of cells that survived in presence of the modulator and the cytotoxic agent[31] was calculated also (Table 1).

4. In vitro CYP450 inhibition studies

Many drug–drug interactions are initiated by the metabolism of drugs by cytochrome P-450 (CYP). Eleven CYPs metabolizing xenobiotics namely CYP1A2, CYP2A6, CYP2B6, CYP2C8/9/18/19, CYP2D6, CYP2E1, and CYP3A4/5 are expressed in a typical human liver. However, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 appear to be involved in the metabolism, and the drug–drug interactions of commonly used drugs.[32] Anthranilamides **4** and **5** exhibited the highest inhibition potency of P-gp on CEM/VLB_{500} cells and

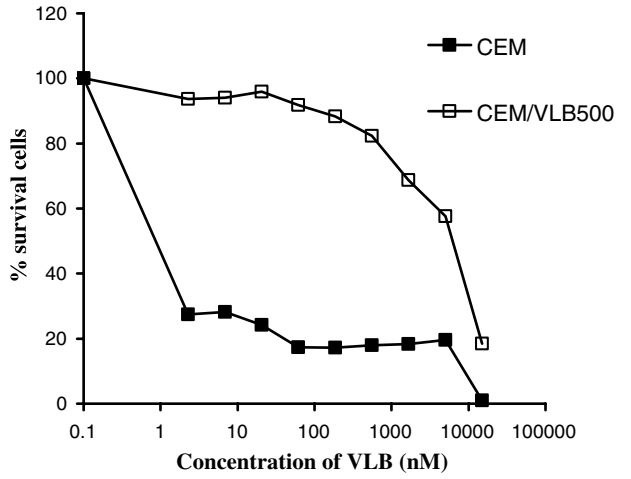


Figure 4. Comparison of the cytotoxic activity of VLB on CEM and CEM/VLB_{500} MDR cells.

Table 1. In vitro inhibition activity of new anthranilamide derivatives modulator of P-gp on CEM/VBL_{500} cells incubated in the presence of vinblastine and their cytotoxic activity

Compound	Inhibitory activity on CEM/VLB_{500} EC_{50} (nM)	Cytotoxicity activity on CEM/VLB_{500} GI_{50} (μM)
1R	329 ± 138[a]	>15[a]
2S	482 ± 226[a]	>15[a]
3	648 ± 208[b]	11.5 ± 1.4[b]
4	124 ± 76[a]	>15[a]
5	59 ± 35[b]	⩾15[b]
P03	270 ± 84[e]	27.7 ± 15.9[e]
XR9576	68 ± 40[c]	13.5 ± 3.1[c]
VRP	1041 ± 443[d]	>15[d]

[a] Mean of three separate experiments made in quadruplicate.
[b] Mean of two experiments made in quadruplicate.
[c] Mean of five experiments made in quadruplicate.
[d] Mean of four experiments made in quadruplicate.
[e] Values come from Ref. 29.

therefore were selected for evaluation on CYP450 to assess their ability to inhibit the catalytic activity of the enzymes. The assays are based on the capacity of a given drug to inhibit or to increase the metabolism of a specific substrate of the CYP yielding in a fluorescent metabolite. In that context, microsomes from insect cells transfected with a baculovirus expressing individual human CYP450 subtypes were prepared.[33] The drugs were incubated with the microsomes, the specific CYP substrate was added, and the kinetics of the substrate metabolism was established using fluorescence. Of interest, inhibition constants are substrate-dependent for CYP3A4 and to avoid potential misinterpretation of the results two substrates (7-benzyloxy-4-trifluoromethylcoumarin and 7-benzyloxyquinoline) were used. The IC_{50} are listed in Table 2.

5. Results and discussion

Three regions of the target compounds were investigated (Fig. 1): (1) region B, (2) region C, and (3) region D. Our results have shown that compounds **1S**, **2R**, **3**, **4**, and **5** exhibit potent MDR reversal activities. In addition, all of our compounds were more active than verapamil and two were almost equipotent to XR9576. Compounds **1S** and **2R** exhibited equivalent potency and were, respectively, 2.5- and 3.5-fold more potent than verapamil when tested in combination with VLB. Compounds **4** and **5** were, respectively, 9- and 20-fold more potent than verapamil with VLB and showed similar potency as XR9576. Overall, the most active P-gp inhibitor was compound **5** (Fig. 3). The selection of a 'hit' or a 'lead compound' cannot be based solely on its ability to inhibit the activity of its target. Therefore, we have evaluated the cytotoxicity of the drug itself and its ability to be involved in drug–drug interactions through CYP inhibition or induction. These preliminary experiments were aimed to determine if a drug candidate might potentially trigger deleterious effects in in vivo experiments. As depicted in Figure 5 and Table 1, compounds **1S**, **2R**, and **4** exhibited minimal cytotoxicity at concentrations up to 6 μM, while compounds **3** and **5** were significantly cytotoxic at that concentration (GI_{50} = 11.5 μM and ⩾15 μM, respectively). Interestingly, compound **5** at 1 μM was not toxic alone, but killed efficiently the cells when in combination with VBL, while verapamil had about 33% of the activity of **5** and is toxic by itself.

Some general trends seem to emerge from the new anthranilamides in terms of the effect of the modifications on the regions B-C-D versus the P-gp inhibition and the cytotoxicity. Indeed, the increase of the rigidity of the spacer group between N^1 and N^2 in region B with cyclohexyl group did not affect the P-gp inhibition compared to **P03**. In addition, the presence of a chiral center

Table 2. Cytochrome-P450 Inhibition Assays of Compounds **4**, **5** and XR9576

P-450 subunit	Inhibitor Ref.	Ref. IC_{50} (μM)	XR9576 IC_{50}(μM)	P03 IC_{50} (μM)	4 IC_{50} (μM)	5 IC_{50} (μM)
CYP1A2	Furafylline	4.98	27.2	>100	>100	6.68
CYP2A6	Tranylcypromine	0.98	>100	>100	>100	n/a
CYP2B6	Tranylcypromine	10.3	>100	>100	>100	100
CYP2C8	Quercetin	1.56	45.3	5.82	13.3	1.08
CYP2C9	Sulfaphenazole	0.56	7.1	2.08	>10	0.0968
CYP2C19	Tranylcypromine	8.74	>10	14.7	23	0.393
CYP2D6	Quinidine	0.0074	100	>100	>100	17.3
CYP2E1	DDTC	17.1	>100	>100	>100	n/c
CYP3A4/BFC	Ketoconazole	0.096	>100	1.62	20.2	0.195
CYP3A4/BQ	Ketoconazole	0.26	>100	7.75	3.58	2.48

n/a, not applicable (i.e., no inhibition was observed over the concentration range tested); n/c, not able to calculate (i.e., inhibition due to DMSO alone was 97%); DDTC, diethyldithiocarbamic acid.

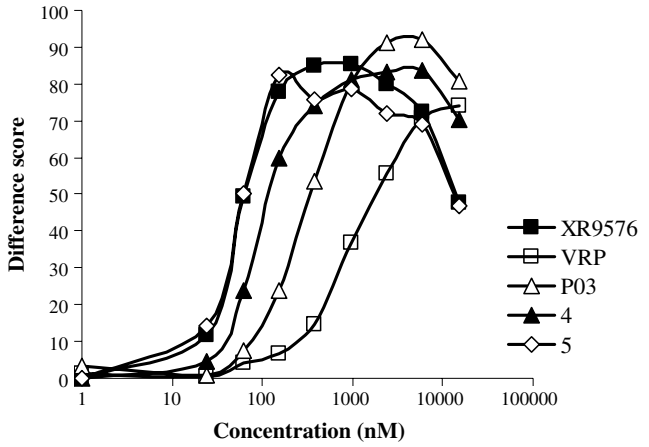


Figure 5. Comparison of P-gp inhibitory activity of **P03**, **4**, **5**, verapamil (VRP), and XR9576 on CEM/VLB_{500} cells using vinblastine as anticancer agent. Difference score = (percent of cell survival with tested agent alone) – (percent of cell survival when treated with the tested agent and 100 nM vinblastine). See Section 7 for details.

introduced via R or S pyrrolidones did not exhibit any significant effect on the inhibitory activity. However, compound **3** bearing a cyclohexyl group instead of an ethyl group as linker was more active than verapamil. These results suggest that active anthranilamides require high level of flexibility between N^1 and N^2 and possibly bring the basic amine group in a proper conformation to bind efficiently to P-gp. This result was expected because it is a common feature for the MDR modulators, which are often flexible molecules that can adapt the groups that interact with the recognition site in a variety of binding modes.[34] The addition of an arylpiperazinyl spacer instead of an alkyl chain led to significantly active derivatives exhibiting pharmacological activities comparable to XR9576. Furthermore, the addition of two methoxyl groups on the anthralinidyl moiety (region B) increased by 200% the inhibition activity of compound **4**. Those results suggest that the efficacy of compound **5** compared to compound **4** is probably due to the presence of an additional acceptor group on the anthralinidyl moiety or an increase of the H-bond acceptor strength of the carbonyl-group para to the methoxyl group. The presence of these methoxyl groups may also increase the electron density of the aromatic ring system enhancing pi/pi interactions. The most potent P-gp modulator was compound **5** and it was 18 times more active than verapamil and as active as XR9576.

Interesting aspects of these molecules are their potency to inhibit the activity of certain CYP-450. Some general trends seem to emerge from these new anthranilamides in terms of the effect of the modifications on the region B-C-D versus CYP-450 inhibition. The modification of the spacer on compounds **4** and **5** compared to XR9576 changed the CYP inhibition profile of these compounds. In fact, it seems that modification of the phenylethyl linker of XR9576 to a phenylpiperazinyl linker of compound **4** or **5** changed drastically the inhibition of CYP3A4 isoform from inactive (compound **P03**) to very potent inhibitor (compound **5**). The ethyl linker (**P03**) has also the same effect on CYP3A4, but to a lower extent than the piperazinyl analogues. Surprisingly, the addition of two methoxyl groups at the anthralinidyl moiety of compound **4** modified completely the inhibitory activity on CYP450. In fact, the presence of these methoxyl groups increases the inhibitory activity on CYP2C9, CYP2C19, and CYP3A4 at low nanomolar levels, and at low micromolar concentrations on CYP1A2, CYP2C8, and CYP2D6. Also, the addition of these methoxyl groups modified the inhibitory activity of compound **4** (>100 μM) to potent inhibitory activity on two CYP isoforms, CYP1A2 and CYP2D6. Studies of the effect of compounds **4**, **5** and XR9576 on human CYP were also conducted using several human cytochrome P-450 enzymes, notably CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4, to determine the ability of these compounds to inhibit the metabolism of cytotoxic drugs thereby increasing their accumulation in cells. Table 2 shows that compound **4** has a CYP inhibition profile similar to **P03**, but different from XR9576. Indeed, compound **4** inhibited CYP2C8 (13.3 μM), CYP2C9 (>10 μM), CYP2C19 (23 μM), and CYP3A4 (20.2 for BFC and 3.58 μM for BQ) at μM level, while XR9576 inhibited CYP1A2, CYP2C8 and CYP2C9 at 27.2, 45.3, and 7.1 μM, respectively. Our results show also that compound **5** has a CYP inhibition profile different from **P03** and XR9576. Compound **5** inhibited the same CYPs as **P03** and **4**, but with higher affinity (nanomolar concentrations). However, it inhibited also CYP1A2 (6.68 μM) and CYP2D6 (17.3 μM) at μM level. These differences suggest that compounds **4** and **5** may have a greater impact on the metabolism of cytotoxic drugs such as paclitaxel, daunorubicin, vinblastine, and etoposide than XR9576. CYP3A4 accounts for approximately 25% of the total hepatic CYP enzymes and is responsible for the metabolism of the majority of cytotoxic drugs.[35] Fortuitously, P-gp shares a broad substrate overlap with CPY3A4 and is co-localized in the small intestine, which can also limit the gastrointestinal absorption of several drugs.[36] The apparent co-localization of CYP3A4 and P-gp could act synergistically to enhance drug metabolization via a recycling process in the gut. Therefore, an inhibitor of P-gp that is also an inhibitor of CYP3A4 might provide a therapeutical advantage by enhancing the bioavailability of the anticancer drugs through a decrease of its excretion and a decrease of its biotransformation. Several investigators reported that poor bioavailability of paclitaxel might be due to CYP450 isoforms and P-gp present in the gut wall.[37–39] To stress the importance of CYP450 inhibition in improving the absorption of paclitaxel, administration of known P-gp inhibitor LY335979 was shown to reduce paclitaxel clearance by approximately 25% in a recent study.[40] This improvement, although modest, is likely due to its weak inhibition of CYP3A4, as LY335979 is about 60-fold more selective for P-gp than for CYP3A4.[41] In our studies, compounds **4** and **5** inhibited both CYP3A4 and P-gp, while XR9576 inhibited only P-gp. Both **5** and XR9576 may increase the accumulation of drugs in the cell, but compound **5** may also enhance their bioavailability due to its inhibitory effect on CYP3A4 at the nM and the μM levels.

6. Conclusions

In this work, we have demonstrated that rigidification of the spacer in the regions B and C of anthranilamides **1S**, **1R**, and **3** provided no improvement on the inhibitory activity when compared to compound **P03** and XR9576 even if they were more potent than verapamil. This suggests that the orientation of the amine N^2 is important to modulate the potency. Interestingly, the use of an arylpiperazinyl group as spacer (compound **4**) enhanced P-gp inhibition. In addition, the methoxyl groups onto the anthralinidyl moiety (region B, compound **5**) improved the inhibition activity of compound **4** by twofold, suggesting that the presence of acceptor groups in region B is important to the activity. Compound **5** was the most potent molecule synthesized and in our study, its potency was 18 times higher than verapamil and equivalent to XR9576. Compound **5** was less cytotoxic than verapamil but as cytotoxic as XR9576 on the cancer cell lines tested. In addition, compound **5** has a different inhibitory activity on CYP compared to XR9576. In contrast to XR9576, **5** inhibited CYP3A4 and a few other CYPs at low nanomolar concentrations. There is also increasing evidence suggesting that the presence of intratumoral enzymes such as CYP3A4, which is known to co-localize with P-gp, may play an important role in the development of cancer chemoresistance.[42] A recent study on 23 patients having breast cancers and treated with docetaxel, which is metabolized by CYP3A4, showed that patients with low CYP3A4 mRNA levels had significantly higher response rates compared to those with high CYP3A4 mRNA levels. This suggests that overexpression of CYP3A4 may provide a protective survival advantage to cancer cells by the inactivation of cytotoxic agents. Compound **5** might be a therapeutically interesting molecule that is able to improve the bioavailability and the half-life of a number of anticancer drugs such as paclitaxel through inhibition of P-gp and bioinactivation of its metabolism.

7. Experimental

7.1. Cell lines and cell culture

CEM and CEM/VLB_{500} human lymphoma cells were kindly provided by Dr. W.T. Beck (Department of Biopharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago). Both cell lines were grown in RPMI 1640 medium supplemented with 2.0 mM glutamine and 10% fetal bovine serum (Hyclone, Road Logan, Utah). Cells were cultured in a moisture-saturated atmosphere at 37 °C in 5% CO_2.

7.2. MDR inhibition assay

Cells were plated in 96-well microtiter plates at 1×10^4 cells/well in media with and without vinblastine (100 nM). Then, anthranilamide modulators dissolved in DMSO were added at escalating concentrations ranging from 0.025 to 15 μM to the cells. The final concentration of DMSO in each well was maintained at maximum 0.5%. The plates were incubated for 3 days. The cell survival was assessed using the resazurin assay.[30] Briefly, cells were washed three times with 200 μL PBS solution. Fifty microliters of a PBS solution containing 20% resazurin in RPMI-1640 was added. Cell survival was calculated from fluorescence (excitation, 485 nm; emission, 590 nm) measured with a FL 600 Reader (Bio-Tek Instruments). Cytotoxicity was expressed as the concentration of the drug required to inhibit cell growth by 50% (GI_{50}). Values are means of at least three-independent determinations. The values were electronically processed to determine 'difference scores,' that is defined as percent survival of cells treated with test agent alone minus percent survival of cells treated with test compound in presence of 100 nM vinblastine. All experiments were performed in quadruplicate. The maximum variation of these data points was ±10%. The difference score was plotted in function of the concentration of the modulator to determine an EC_{50} that represents the concentration that is necessary to reverse 50% chemoresistance by 50%.[31]

7.3. CYP450 inhibition assay

A general description of the assays follows, with enzyme-specific parameters listed separately below for each CYP subtype. The CYP450 inhibition assays were conducted using microsomes (Supersomes®, BD GENTEST, Woburn, MA) prepared from insect cells, each expressing a specific CYP subtype (CYP1A2, CYP2C9, CYP2C19, CYP2D6, or CYP3A4) expressed from the corresponding human CYP cDNA using a baculovirus expression vector. The microsomes also incorporate supplemental cDNA-expressed human reductase and/or cytochrome b5, as these enzymes stimulate the activity of the CYPs, allowing for a reduction in the amount of enzyme required per reaction (BD GENTEST). The assays monitored, via fluorescence detection, the formation of a fluorescent metabolite following incubation of the microsomes with a specific CYP substrate. Two CYP substrates were tested for CYP3A4 (7-benzyloxy-4-trifluoromethylcoumarin and 7-benzyloxyquinoline), as this enzyme has been shown to exhibit complex inhibition kinetics.[43] Reactions (0.2 mL) are performed in 96-well microtiter plates at 37 °C in the presence of an NADPH regenerating system [β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH)] and $MgCl_2$. Inhibition of metabolic product formation by the test compound for each enzyme was tested in the absence and presence of 0.0457–100 μM of test compound. An enzyme-selective inhibitor was also tested (at eight concentrations) in each assay as a positive control. All determinations were performed in duplicate. Assays for all enzymes were performed in the following manner: the NADPH regenerating system, appropriate buffer solution, and vehicle, inhibitor (positive control) solution or test compound solution were dispensed into 96-well microtiter plates. Eight inhibitor and test compound

concentrations were tested using threefold serial dilutions. The microtiter plates containing 0.1 ml/well of the latter mixture were pre-warmed to 37 °C in an incubator. A solution of buffer, microsomes, and substrate were separately prepared and vortex mixed to disperse the protein. The reactions were initiated by the addition of the microsome/substrate solution (0.1 mL) to the wells of the microtiter plates containing the pre-warmed NADPH regenerating system, buffer, and inhibitor or test compound solutions. The final concentration of acetonitrile, the vehicle used for the known inhibitors, was 2%. Following specified incubation times, the reactions were stopped by the addition of 0.075 mL of a STOP solution (see below). Blank (background noise) samples were also assayed by adding the STOP solution prior to the addition of the microsome/substrate mix to the NADPH regenerating system. The amount of metabolic product formed was quantified by fluorescence detection in a fluorescence plate reader utilizing excitation and emission filters that have been optimized for the detection of each metabolite.

CYP1A2	Microsomes: CYP1A2 (0.5 pmol) and supplemental cDNA-expressed human reductase NADPH regenerating system: 1.3 mM $NADP^+$, 3.3 mM G6P, 0.4 U/mL G6PDH, 3.3 mM $MgCl_2$ Buffer: 100 mM potassium phosphate buffer, pH 7.4 (PPB) Inhibitor: furafylline (0.0457–100 μM) Substrate: 3-cyano-7-ethoxycoumarin (5 μM CEC) Metabolic product: 3-cyano-7-hydroxycoumarin (CHC) Incubation time: 15 min STOP buffer: 80% acetonitrile/20% 0.5 M Tris–base Excitation filter: 400 nm, emission filter: 460 nm
CYP2A6	Microsomes: CYP2A6 (1.0 pmol) and supplemental cDNA-expressed human reductase and human cytochrome b5 NADPH regenerating system: 0.065 mM NADP+, 3.3 mM G6P, 0.4 U/mL G6PDH, 3.3 mM $MgCl_2$ Buffer: 100 mM Tris buffer, pH 7.5 Inhibitor: tranylcypromine (0.0457–100 μM) Substrate: coumarin (3 μM) Metabolic product: 7-hydroxycoumarin (7-HC, umbelliferone) Incubation time: 15 min STOP buffer: 80% acetonitrile/20% 0.5 M Tris–base Excitation filter: 400 nm, emission filter: 460 nm
CYP2B6	Microsomes: CYP2B6 (1.0 pmol) and supplemental cDNA-expressed human reductase and human cytochrome b5 NADPH regenerating system: 1.3 mM NADP+, 3.3 mM G6P, 0.4 U/mL G6PDH, 3.3 mM $MgCl_2$ Buffer: 100 mM PPB Inhibitor: tranylcypromine (0.057 to 125 μM) Substrate: 7-ethoxy-4-trifluoromethylcoumarin (2.5 μM EFC) Metabolic product: 7-hydroxy-4-trifluoromethylcoumarin (HFC) Incubation time: 30 min STOP buffer: 80% acetonitrile/20% 0.5 M Tris–base Excitation filter: 400 nm, emission filter: 530 nm
CYP2C8	Microsomes: CYP2C8 (4.0 pmol) and supplemental cDNA-expressed human reductase and human cytochrome b5 NADPH regenerating system: 1.3 mM NADP+, 3.3 mM G6P, 0.4 U/mL G6PDH, 3.3 mM $MgCl_2$ Buffer: 50 mM PPB Inhibitor: quercetin (0.009–20 μM) Substrate: dibenzylfluorescein (1 μM DBF) Metabolic product: fluorescein Incubation time: 30 min STOP buffer: 2 mM NaOH Excitation filter: 485 nm, emission filter: 530 nm

CYP2C9	Microsomes: CYP2C9*1 (1.0 pmol) and supplemental cDNA-expressed human reductase and human cytochrome b5 (CYP2C9*1 is the most common allele in human populations which has been studied to date) NADPH regenerating system: 1.3 mM $NADP^+$, 3.3 mM G6P, 0.4 U/mL G6PDH, 3.3 mM $MgCl_2$ Buffer: 25 mM PPB Inhibitor: sulfaphenazole (0.00457–10 μM) Substrate: 7-methoxy-4-trifluoromethylcoumarin (75 μM MFC) Metabolic product: 7-hydroxy-4-trifluoromethylcoumarin Incubation time: 45 min STOP Buffer: 80% acetonitrile/20% 0.5 M Tris–base Excitation filter: 400 nm, emission filter: 530 nm
CYP2C19	Microsomes: CYP2C19 (0.5 pmol) and supplemental cDNA-expressed human reductase and human cytochrome b5 NADPH regenerating system: 1.3 mM $NADP^+$, 3.3 mM G6P, 0.4 U/mL G6PDH, 3.3 mM $MgCl_2$ Buffer: 50 mM PPB Inhibitor: tranylcypromine (0.229–500 μM) Substrate: 3-cyano-7-ethoxycoumarin (25 μM) Metabolic product: 3-cyano-7-hydroxycoumarin Incubation time: 30 min STOP buffer: 80% acetonitrile/20% 0.5 M Tris–base Excitation filter: 400 nm, emission filter: 460 nm
CYP2D6	Microsomes: CYP2D6*1 (1.5 pmol) and supplemental cDNA-expressed human reductase (CYP2D6*1 is the most common human CYP2D6 allele) NADPH regenerating system: 8.2 μM $NADP^+$, 0.41 mM G6P, 0.4 U/mL G6PDH, 0.41 mM $MgCl_2$ Buffer: 100 mM PPB Inhibitor: quinidine (0.00023–0.5 μM) Substrate: 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (1.5 μM AMMC) Metabolic product: 3-[2-(*N*,*N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin (AHMC) Incubation time: 30 min STOP buffer: 80% acetonitrile/20% 0.5 M Tris–base Excitation filter: 400 nm, emission filter: 460 nm
CYP2E1	Microsomes: CYP2E1 (2.0 pmol) and supplemental cDNA-expressed human reductase and cytochrome b5 NADPH regenerating system: 1.3 mM NADP+, 3.3 mM G6P, 0.4 U/mL G6PDH, 3.3 mM $MgCl_2$ Buffer: 100 mM PPB Inhibitor: diethyldithiocarbamic acid (0.0457–100 μM DDTC) Substrate: 7-methoxy-4-trifluoromethylcoumarin (100 μM MFC) Metabolic product: HFC Incubation time: 45 min STOP buffer: 80% acetonitrile/20% 0.5 M Tris–base Excitation filter: 400 nm, emission filter: 530 nm
CYP3A4/ BFC	Microsomes: CYP3A4 (1.0 pmol) and supplemental cDNA-expressed human reductase and cytochrome b5 NADPH regenerating system: 1.3 mM $NADP^+$, 3.3 mM G6P, 0.4 U/mL G6PDH, 3.3 mM $MgCl_2$ Buffer: 200 mM PPB Inhibitor: ketoconazole (0.00229–5 μM) Substrate: 7-benzyloxy-4-trifluoromethylcoumarin (50 μM BFC) Metabolic product: 7-hydroxy-4-trifluoromethylcoumarin Incubation time: 30 min STOP buffer: 80% acetonitrile/20% 0.5 M Tris–base Excitation filter: 400 nm, emission filter: 530 nm

CYP3A4/ BQ	Microsomes: CYP3A4 (3.0 pmol) and supplemental cDNA-expressed human reductase and cytochrome b5 NADPH regenerating system: 1.3 mM $NADP^+$, 3.3 mM G6P, 0.4 U/mL G6PDH, 3.3 mM $MgCl_2$ Buffer: 200 mM PPB Inhibitor: ketoconazole (0.00229–5 μM) Substrate: 7-benzyloxyquinoline (40 μM BQ) Metabolic product: quinolinol Incubation time: 30 min STOP Buffer: 80% acetonitrile/20% 0.5 M Tris–base Excitation filter: 400 nm, emission filter: 530 nm

7.4. Chemistry

Melting points were determined on an electrothermal melting point apparatus. All nominal and accurate mass electronic impact (EI) measurements were made using a JEOL HX110 double focusing mass spectrometer. All nominal and accurate mass electrospray ionization (ESI) measurements were made with a Waters/Micromass QTOF Ultima Global instrument of QQTof geometry. IR spectra were recorded on a Bomem MB-100 spectrometer and reported in cm^{-1}. ^1H NMR spectra were determined at 200 MHz using a Varian XL-200 or at 300 MHz using a Bruker AC-300 spectrometer; ^{13}C NMR spectra were determined at 50.3 MHz using a Varian XL-200 or at 75.5 MHz using a Bruker AC-300 spectrometer; chemical shifts (δ) are given using chloroform-d as a reference. The silica gel used for flash chromatography was Kieselgel 60 70–230 mesh Merck. Unless otherwise noted all reagents and solvents obtained from commercial suppliers were used without further purification.

7.4.1. Preparation of (S) *N*-((1-Boc-pyrrolidin-2-yl)-methyl)-2-aminobenzamide (8S). A mixture of compound **6S** (0.779 mmol) and isatoic anhydride (**7**) (0.818 mmol) in acetonitrile was stirred and refluxed overnight. The mixture was diluted with 20 mL EtOAc and extracted with a solution of HCl 1 N. The aqueous layer was combined and alkalized with NaOH pellets. The white solution was extracted with EtOAc. The organic phases were combined, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained after column chromatography using a 0–5% methanol/dichloromethane as eluant to give 155.2 mg of colorless oil. Yield: 62%. IR (NaCl): 3450, 3342 (NHCO + NH_2), 2974, 2932, 2878 (C–H), 1667 (CO), 1637 (NH_2 deformation), 1578, 1525, (C=C Ar), 1400 (CH_2 deformation), 855, 749 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 8.16 (s, 1H, NH), 7.42 (d, 2H, ArH, J = 7.68 Hz), 7.11 (m, 1H, ArH), 6.57 (m, 2H, ArH), 5.56 (br s, 1H, NH_2), 4.14 (m, 1H, CH), 3.38 (m, 4H, CH_2), 1.90 (m, 3H, CH_2), 1.80 (m, 1H, CH_2), 1.65 (s, 9H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): 169.7, 149.0, 132.0, 127.8, 117.1, 116.4, 115.4, 80.2, 55.9, 47.1, 46.7, 29.4, 28.4, 23.9.

7.4.2. Preparation of (R) *N*-((1-Boc-pyrrolidin-2-yl)-methyl)-2-aminobenzamide (8R). A mixture of compound **6R** (0.774 mmol) and isatoic anhydride (**7**) (0.812 mmol) in acetonitrile was stirred and refluxed overnight. The mixture was diluted with 20 mL EtOAc and extracted with a solution of HCl 1 N. The aqueous layer was combined and alkalized with NaOH pellets. The white solution was extracted with EtOAc. The organic phases were combined, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product could be obtained after column chromatography using a 0–5% methanol/dichloromethane as elution solvent to give 121.2 mg of colorless oil. Yield: 49%. IR (NaCl): 3449, 3331 (NHCO + NH_2), 2971, 2918, 2871 (C–H), 1670 (CO), 1637 (NH_2 deformation), 1578, 1525 (C=C Ar), 1399 (CH_2 deformation), 852, 751 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 8.24 (br s, 1H, NH), 7.50 (d, 1H, ArH, J = 7.7 Hz), 7.18 (t, 1H, ArH, J = 7.7 Hz), 6.66 (m, 2H, ArH), 4.19 (m, 1H, CH), 3.38 (m, 4H, CH_2), 1.98 (m, 3H, CH_2), 1.72 (m, 1H, CH_2), 1.46 (s, 9H, CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): 169.7, 149.0, 132.0, 127.8, 117.1, 116.4, 115.4, 80.2, 55.9, 47.1, 46.7, 29.4, 28.4, 23.9.

7.4.3. Preparation of (S) *N*-(2-((1-Boc-pyrrolidin-2-yl)-methylcarbamoyl)phenyl)quinoline-3-carboxamide (11S). To a solution of dichloromethane (10 mL) containing compound **8S** (0.429 mmol) were added 3-quinoline acid chloride (**9**) (0.472 mmol) and K_2CO_3 (0.858 mmol). The mixture was diluted in dichloromethane and extracted three times with 10 mL of solution of 1 N NaOH. The organic phases were combined, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum to yield **10S**. The crude of **10S** was diluted in a solution of dichloromethane with 10% TFA and stirred for 3 h. The mixture was diluted in dichloromethane and extracted three times with 10 mL of solution of 1 N NaOH. The organic phase was combined, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained by flash chromatography on silica gel using a gradient concentration of solvent from 0% to 20% MeOH in dichloromethane to give 68.4 mg of yellow solid. Yield: 42%. Mp 123–124 °C; IR (NaCl): 3320, 3166, 3071 (NH), 2956, 2851 (C–H), 1676, 1596 (C=O), 1525, 1446, 1198 (C=C Ar), 909, 864, 787 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 12.72 (s, 1H, NHCO), 9.52 (d, 1H, ArH, J = 12.6 Hz), 8.74 (m, 1H, ArH), 8.16 (d, 1H, ArH, J = 8.5 Hz), 7.98 (d, 1H, ArH, J = 8.1 Hz), 7.80 (t, 1H, ArH, J = 7.6 Hz), 7.64 (m, 2H, ArH), 7.51 (t, 1H, ArH, J = 7.3 Hz), 7.12 (t, 1H, ArH, J = 7.6 Hz),

3.68 (m, 2H, CH_2); 3.50 (m, 1H, CH_2), 3.28 (m, 1H, CH_2); 2.97 (m, 2H, CH_2), 2.89 (s, 1H, NH); 1.86 (m, 2H, CH_2), 1.52 (m, 1H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 169.3, 163.8, 149.3, 148.9, 139.9, 135.9, 132.9, 131.4, 129.3, 129.2, 128.2, 127.5, 127.4, 126.9, 123.3, 121.5, 120.0, 57.7, 46.3, 43.3, 29.0, 25.7.

7.4.4. Preparation of (R) *N*-(2-((1-Boc-pyrrolidin-2-yl)methylcarbamoyl)phenyl)quinoline-3-carboxamide (11R). To a solution of dichloromethane (10 mL) containing compound **8R** (0.344 mmol) were added 3-quinoline acid chloride (**9**) (0.378 mmol) and K_2CO_3 (0.688 mmol). The mixture was diluted in dichloromethane and extracted three times with 10 mL of solution of 1 N NaOH. The organic phases were combined, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum to yield **10R**. The crude of **10R** was diluted in a solution of dichloromethane with 10% TFA and stirred for 3 h. The mixture was diluted in dichloromethane and extracted three times with 10 mL of solution of 1 N NaOH. The organic phase was combined, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained by flash chromatography on silica gel using a gradient concentration of solvent from 0% to 20% MeOH in dichloromethane to give 50.4 mg of yellow solid. Yield: 35%. Mp 123–124 °C; IR (NaCl): 3320, 3166, 3071 (NH), 2956, 2851 (C–H), 1676, 1596 (C=O), 1525, 1446, 1198 (C=C Ar), 909, 864, 787 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 12.72 (s, 1H, NHCO), 9.52 (d, 1H, ArH, $J = 12.6$ Hz), 8.74 (m, 1H, ArH), 8.16 (d, 1H, ArH, $J = 8.5$ Hz), 7.98 (d, 1H, ArH, $J = 8.1$ Hz), 7.80 (t, 1H, ArH, $J = 7.6$ Hz), 7.64 (m, 2H, ArH), 7.51 (t, 1H, ArH, $J = 7.3$ Hz), 7.12 (t, 1H, ArH, $J = 7.6$ Hz), 3.68 (m, 2H, CH_2); 3.50 (m, 1H, CH_2), 3.28 (m, 1H, CH_2); 2.97 (m, 2H, CH_2), 2.89 (s, 1H, NH); 1.86 (m, 2H, CH_2), 1.52 (m, 1H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 169.3, 163.8, 149.3, 148.9, 139.9, 135.9, 132.9, 131.4, 129.3, 129.2, 128.2, 127.5, 127.4, 126.9, 123.3, 121.5, 120.0, 57.7, 46.3, 43.3, 29.0, 25.7.

7.4.5. Preparation of (S) *N*-(2-((1-(3,4-dimethoxyphenethyl)pyrrolidin-2-yl)methylcarbamoyl)phenyl)quinoline-3-carboxamide (1S). To a solution of acetonitrile (10 mL) containing compound **11S** (0.129 mmol) were added 3-4-dimethoxyphenethyl chloride (**12**) (0.142 mmol) and K_2CO_3 (0.258 mmol). The mixture was diluted in dichloromethane and extracted three times with a solution of 1 N HCl (8 mL). The aqueous layers were combined and NaOH pellets were further added to alkalinize the mixture. The solution was extracted with dichloromethane. The organic layers were combined and washed with brine, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained by flash chromatography on silica gel using a gradient concentration of solvent from 2% to 5% MeOH in dichloromethane to give 22.2 mg of yellow solid. Yield: 32%. Mp 92–93 °C; IR (NaCl): 3396 (NHCO), 2947, 2829 (C–H); 1667, 1598 (CO), 1513, 1447 (C=C Ar), 1265, 1230 (tertiary amine), 788, 760 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 12.84 (s, 1H, NHCO), 9.55 (s, 1H, NHCO), 8.86 (d, 1H, ArH, $J = 8.4$ Hz), 8.79 (s, 1H, ArH), 8.17 (d, 1H, ArH, $J = 8.4$ Hz), 8.00 (d, 1H, ArH, $J = 8.0$ Hz), 7.81 (t, 1H, ArH, $J = 7.5$ Hz), 7.56 (m, 2H, ArH), 7.12 (m, 2H, ArH), 6.68 (m, 4H, ArH), 3.82 (s, 3H, OMe), 3.72 (s, 3H, OMe), 3.39 (m, 1H, CH_2), 3.26 (m, 1H, CH_2), 3.05 (m, 1H, CH_2), 2.82 (m, 3H, CH_2), 2.60 (m, 1H, CH_2), 2.35 (m, 1H, CH_2), 1.80 (m, 5H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 169.3, 163.9, 149.4, 149.1, 148.9, 147.4, 140.0, 135.8, 132.8, 131.3, 129.4, 129.2, 127.6, 127.4, 127.0, 126.6, 123.3, 121.4, 120.2, 111.9, 111.3, 55.9, 55.8, 53.8, 39.6, 34.6, 27.9, 23.2; MS: 539.24 $[M+H]^+$. HRMS $[M+H]^+$: calcd for $C_{32}H_{35}N_4O_4$ 539.2658, found: 539.2667

7.4.6. Preparation of (R) *N*-(2-((1-(3,4-dimethoxyphenethyl)pyrrolidin-2-yl)methylcarbamoyl)phenyl)quinoline-3-carboxamide (2R). To a solution of acetonitrile (10 mL) containing compound **11R** (0.133 mmol) were added 3-4-dimethoxyphenethyl chloride (**12**) (0.146 mmol) and K_2CO_3 (0.456 mmol). The mixture was diluted with dichloromethane and extracted three times with a solution of 1 N HCl (8 mL). The aqueous layers were combined and NaOH pellets were added to alkalinize the mixture. The solution was extracted with dichloromethane. The organic layer was combined and washed with brine, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained by flash chromatography on silica gel using a gradient concentration of solvent from 2% to 5% MeOH in dichloromethane to give 32.2 mg of yellow solid. Yield: 45%. Mp 92–93 °C; IR (NaCl): 3378 (NHCO), 2942, 2841 (C–H), 1675, 1598 (CO), 1516, 1446 (Ar), 1264, 1237 (tertiary amine), 861, 788, 760 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 12.84 (s, 1H, NHCO), 9.55 (s, 1H, NHCO), 8.86 (d, 1H, ArH, $J = 8.5$ Hz), 8.79 (s, 1H, ArH), 8.17 (d, 1H, ArH, $J = 8.3$ Hz), 8.00 (d, 1H, ArH, $J = 8.0$ Hz), 7.81 (t, 1H, ArH, $J = 7.9$ Hz), 7.56 (m, 2H, ArH), 7.12 (m, 2H, ArH), 6.67 (m, 4H, ArH), 3.82 (s, 3H, OMe), 3.71 (s, 3H, OMe), 3.39 (m, 1H, CH_2), 3.25 (m, 1H, CH_2), 3.05 (m, 1H, CH_2), 2.81 (m, 3H, CH_2), 2.60 (m, 1H, CH_2), 2.35 (m, 1H, CH_2), 1.80 (m, 5H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 169.3, 163.9, 149.4, 149.1, 148.9, 147.4, 140.0, 135.8, 132.8, 131.3, 129.2, 127.6, 127.4, 127.0, 126.6, 123.3, 121.4, 120.2, 119.7, 111.9, 111.3, 55.9, 55.8, 55.5, 53.8, 39.6, 34.6, 27.9, 23.2; MS: 539.27 $[M+H]^+$. HRMS $[M+H]^+$: calcd for $C_{32}H_{35}N_4O_4$ 539.2658, found: 539.2672.

7.4.7. Preparation of *rac–trans*-2-(4-phenylpiperazin-1-yl)cyclohexanol (15 (*rac–trans*)). To a solution of water (6 mL) containing compound **13** (3.93 mmol) was added phenylpiperazine (**14**) (1.96 mmol). The mixture was refluxed during 18 h. After cooling down the mixture, the precipitate was filtered and the pure product was obtained to give 528.6 mg of white solid. Yield: 52%. Mp 138–139 °C; IR (NaCl): 3432 (OH); 2932, 2859, 2826 (C–H); 1577, 1498 (C=C Ar), 1447 (CH_2 deformation), 1233 (tertiary amine); 761 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 7.27 (t, 2H, ArH, $J = 7.4$ Hz), 6.87 (m, 3H, ArH), 3.98 (br s, 1H, OH), 3.42 (m, 1H, CH), 3.23 (m, 4H, CH_2), 2.90 (m, 2H, CH_2), 2.60 (m, 2H, CH_2), 2.29 (m, 2H, CH_2), 2.16 (m, 2H, CH_2), 1.80

(m, 3H, CH_2), 1.25 (m, 4H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 151.3, 129.1, 119.9, 116.2, 70.3, 68.7, 49.8, 48.7, 33.3, 25.5, 24.4, 22.4.

7.4.8. Preparation of *rac–cis*-1-(2-azidocyclohexyl)-4-phenylpiperazine (16 (*rac–cis*)). To a solution of dichloromethane (6 mL) containing compound **15** (*rac–trans*) (0.499 mmol) and triethylamine (0.998 mmol) was added methanesulfonyl chloride (0.998 mmol) at 0 °C. The mixture was stirred and warmed up to room temperature overnight. The mixture was diluted in dichloromethane and extracted three times with 1 N NaOH (8 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Part of the crude product (0.265 mmol) was dissolved in DMF (3 mL) and NaN_3 (1.33 mmol) was added. The mixture was refluxed overnight. The mixture was diluted in dichloromethane and extracted three times with NaOH 1 N (8 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained by flash chromatography on silica gel using a gradient concentration of solvent from 0% to 25% EtOAc in hexane to give 68.6 mg as a sticky yellow solid. Yield: 91% IR (NaCl): 2911, 2853 (CH_2), 2097 (N_3), 1501, 1600 (C=C Ar), 1450 (CH_2 deformation), 1260, 1234 (tertiary amine), 864, 805 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 7.27 (t, 2 H, ArH, $J = 7.5$ Hz), 6.93 (d, 2H, ArH, $J = 8.2$ Hz), 6.86 (t, 1H, ArH, $J = 7.2$ Hz), 3.34 (m, 1H, CH), 3.22 (m, 4H, CH_2), 2.89 (m, 2H, CH_2), 2.73 (m, 2H, CH_2), 2.49 (m, 1H, CH), 2.01 (m, 2H, CH_2), 1.80 (m, 2H, CH_2), 1.73 (m, 4H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 151.6, 129.1, 119.7, 116.2, 68.3, 60.7, 49.5, 48.6, 32.4, 25.0, 24.7, 23.9.

7.4.9. Preparation of *rac–cis*-2-amino-*N*-(2-(4-phenylpiperazin-1-yl)cyclohexyl)benzamide (17 (*rac–cis*)). In a hydrogenated bottle, compound **16** (*rac–cis*) (0.221 mmol) was dissolved in 10 mL EtOH. To this solution, approximately 10 mg of 10% Pd/C was added. The bottle was placed in a hydrogenator machine and the system was purged three times with hydrogen. The system was poured under 38PSI for 6 h. The reaction mixture was filtered over Celite™. The Celite™ was washed three times with EtOH and the filtrate was evaporated under reduced pressure. A part of the crude (0.212 mmol) was dissolved in ACN and isatoic anhydride (0.223 mmol) was added. The mixture was refluxed overnight. The mixture was diluted in dichloromethane and extracted three times with 1 N NaOH (8 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained by flash chromatography on silica gel using a gradient of solvent from 2% to 5% MeOH in dichloromethane to give 52.3 mg of yellow oil. Yield: 65% IR (NaCl): 3443, 3342 ($NHCO + NH_2$), 2929, 2855, 2822 (C–H), 1638 (CO), 1588 (NH_2 deformation), 1600, 1515, (C=C Ar), 1449 (CH_2 deformation), 1260, 1235 (tertiary amine), 864, 802, 755 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 7.40 (d, 1H, ArH, $J = 7.8$ Hz), 7.27 (m, 2H, ArH), 7.17 (t, 1H, ArH, $J = 7.6$ Hz), 6.88 (m, 4H, ArH), 6.63 (t, 1H, ArH, $J = 7.9$ Hz), 5.45 (br s, 2H, NH_2), 3.78 (m, 1H, CH), 3.14 (m, 4H, CH_2), 2.89 (m, 2H, CH_2), 2.55 (m, 4H, CH_2), 2.01 (m, 1H, CH_2), 1.87 (m, 1H, CH_2), 1.77 (m, 1H, CH_2), 1.26 (m, 4H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 169.5, 151.3, 148.3, 132.0, 129.1, 127.5, 119.8, 117.2, 116.9, 116.1, 115.7, 67.4, 50.6, 49.9, 47.9, 33.1, 25.6, 24.7, 23.3.

7.4.10. Preparation of *rac–cis*-*N*-(2-(2-(4-phenylpiperazin-1-yl)cyclohexylcarbamoyl)phenyl)quinoline-3-carboxamide (3 (*rac–cis*)). To a solution of dichloromethane (10 mL) containing compound **17** (*rac–cis*) (0.106 mmol) were added 3-quinoline acid chloride (**9**) (0.128 mmol) and K_2CO_3 (0.212 mmol). The mixture was stirred overnight, diluted in dichloromethane, and extracted three times with 10 mL of solution of 1 N NaOH. The organic phase was combined, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. The mixture was diluted in dichloromethane and extracted three times with 10 mL of 1 N NaOH. The organic phase was combined, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained by flash chromatography on silica gel using a gradient of solvent from 0% to 5% MeOH in dichloromethane to give 40.7 mg of a yellow solid. Yield: 72%. Mp 146–147 °C; IR (NaCl): 3319 (NHCO), 2930, 2859, 2812 (C–H), 1678 (CO), 1600, 1515, (C=C Ar), 1447 (CH_2 deformation), 1262, 1234 (tertiary amine), 802, 758 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 12.7 (s, 1H, NH), 8.84 (s, 1H, ArH), 8.78 (d, 1H, ArH, $J = 8.3$ Hz). 8.66 (s, 1H, NH), 8.18 (t, 1H, ArH, $J = 8.3$ Hz), 8.01 (d, 1H, $J = 7.9$ Hz), 7.83 (t, 1H, ArH, $J = 7.6$ Hz), 7.64 (m, 3H, ArH), 7.41 (t, 1H, ArH, $J = 7.8$ Hz), 7.19 (t. 2H, $J = 7.9$ Hz), 7.01 (t, 1H, ArH, $J = 7.3$ Hz), 6.83 (t, 1H, ArH, $J = 7.3$ Hz), 6.78 (d, 2H, ArH, $J = 8.2$ Hz), 3.96 (m, 1H, CH), 3.23 (m, 4H, CH_2), 3.00 (m, 3H, CH), 2.76 (m, 1H, CH_2), 2.56 (m, 1H, CH_2), 1.94 (m, 4H, CH_2), 1.36 (m, 4H, CH_2), NMR ^{13}C (75.5 MHz, $CDCl_3$): 169.5, 163.6, 151.1, 150.6, 149.3, 148.7, 139.9, 138.3, 136.2, 132.7, 131.4, 130.9, 129.4, 129.2, 129.0, 127.8, 123.3, 121.4, 120.3, 116.2, 66.6, 50.6, 49.9, 47.7, 32.7, 25.2, 24.6, 23.4. MS: 533.3 $[M]^+$. HRMS $[M+H]^+$: calcd for $C_{33}H_{36}N_5O_2$ 534.2869, found: 534.2797.

7.4.11. Preparation of 1-(3,4-dimethoxybenzyl)-4-(4-nitrophenyl)piperazine (20). Sodium triacetoxyborohydride (8.205 mmol) was added in one portion under nitrogen to a cloudy mixture of **18** (5.47 mmol) and **19** (6.01 mmol) in CH_2Cl_2 (20 mL). The gelatinous mixture formed initially over a 15 min period. The resulting light yellow solution was stirred for 18 h before it was partitioned between EtOAc and saturated aqueous $NaHCO_3$. The aqueous layer was extracted with EtOAc and the combined organic layers were dried over $MgSO_4$ anhydrous and concentrated under vacuum to give 2.0 g, as an orange solid. Yield: 100%. Mp 144–146 °C; IR (NaCl): 3337 (NH_2), 2924, 2835 (C–H), 1597, 1325 (NO_2), 1509 (C=C Ar), 1250, 1231 (tertiary amine), 811, 775 (CH deformation Ar), NMR ^1H (300 MHz, $CDCl_3$): 6.84 (m, 6H, ArH), 6.60 (d, 1H, ArH,

J = 8.5 Hz), 3.87 (s, 3H, OMe), 3.85 (s, 3H, OMe), 3.49 (s, 2H, CH_2), 3.04 (s, 2H, NH_2), 3.04 (s, 4H, CH_2) 2.58 (s, 4H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 148.9, 148.8, 148.2, 144.6, 140.1, 130.7, 121.4, 118.5, 116.2, 112.3, 110.8, 62.8, 55.9, 53.2, 50.9.

7.4.12. Preparation of 4-(4-(3,4-dimethoxybenzyl)piperazin-1-yl)benzenamine (21). In a hydrogenated bottle, compound **20** (1.78 mmol) was dissolved in 40 mL EtOH and Pd/C 10% (65 mg) was added. The bottle was placed in a hydrogenator machine and the system was purged three times with hydrogen. The system was poured under 38 PSI and stirred for 6 h. The reaction mixture was filtered over Celite™. The Celite™ was washed three times with EtOH and the filtrate was evaporated under reduced pressure. The mixture was diluted in dichloromethane and washed with water, brine, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum to give 584.2 mg of yellow oil. The crude product was pure enough to be used for the next step. Yield: 100%. Mp 120–121 °C; IR (NaCl): 3432, 3353 (NH_2), 2940, 2829 (C–H), 1625 (NH_2), 1513 (C=C Ar), 1253 (tertiary amine), 1212 (OCH_3), 811, 775 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 6.84 (m, 6H, ArH), 6.60 (d, 1H, ArH, J = 8.5 Hz), 3.87 (s, 3H, OMe), 3.85 (s, 3H, OMe), 3.49 (s, 2H, CH_2), 3.04 (s, 2H, NH_2), 3.04 (s, 4H, CH_2) 2.58 (s, 4H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 148.9, 148.8, 148.2, 144.6, 140.1, 130.7, 121.4, 118.5, 116.2, 112.3, 110.8, 62.8, 55.9, 53.2, 50.9.

7.4.13. Preparation of *N*-(4-(4-(3,4-dimethoxybenzyl)piperazin-1-yl)phenyl)-2-aminobenzamide (23). To a solution of acetonitrile (10 mL) containing compound **21** (0.409 mmol) was added isatoic anhydride (**7**) (0.450 mmol) and the mixture was refluxed overnight. The mixture was diluted in dichloromethane and extracted three times with 1 N NaOH (8 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained by flash chromatography on silica gel using a gradient of solvent from 2% to 5% MeOH in dichloromethane to give 81.2 mg of yellow oil. Yield: 44%. Mp 121–123 °C; IR (NaCl): 3468, 3370, 3298 (NHCO + NH_2), 2925, 2841 (C–H), 1639 (CO), 1513 (C=C Ar), 1261 (tertiary amine), 1222 (OCH_3), 816, 742 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 7.75 (s, 1H, NHCO) 7.42 (m, 3H, ArH), 7.21 (m, 1H, ArH, J = 7.5 Hz), 6.85 (m, 5H, ArH), 7.21 (m, 2H, ArH), 5.47 (br s, 1H, NH_2), 3.88 (s, 3H, OMe), 3.86 (s, 3H, OMe), 3.50 (s, 2H, CH_2), 3.16 (m, 4H, CH_2), 2.59 (m, 4H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 167.6, 148.9, 148.6, 148.2, 132.5, 130.6, 130.0, 127.2, 122.2, 121.4, 117.4, 116.7, 116.6, 116.5, 116.4, 112.3, 110.9, 62.8, 55.9, 53.0, 49.5, 49.3.

7.4.14. Preparation of *N*-(4-(4-(3,4-dimethoxybenzyl)piperazin-1-yl)phenyl)-2-amino-4,5-dimethoxybenzamide (24). To a solution of dichloromethane (25 mL) containing compound **21** (2.415 mmol) were added 2-amino-4,5-dimethoxybenzoic acid (**22**) (2.19 mmol), *N*-cyclohexyl-*N*-(2-morpholinoethyl)-carbodiimide methyl-*p*-toluene sulfonate (2.52 mmol), and 1-hydroxybenzotriazole (2.52 mmol). The mixture was stirred at room temperature during 2 days. The mixture was diluted in dichloromethane and extracted three times with 1 N NaOH (8 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 anhydrous, filtered, and concentrated under vacuum. Pure product was obtained by flash chromatography on silica gel using a gradient of solvent from 2% to 5% MeOH in dichloromethane to give 777.8 mg of yellow solid. Yield: 70%. Mp 94–95 °C; IR (NaCl): 3467 (NH_2), 3349 (NHCO), 2936, 2829 (C–H), 1655 (C=O), 1513, 1442 (C=C Ar), 1236 (tertiary amine), 1206 (OCH_3); 811 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 7.84 (s, 1H, NHCO), 7.40 (d, 1H, ArH, J = 8.76 Hz), 6.80 (m, 6H, ArH), 6.19 (s, 1H, ArH), 5.30 (br s, 1H, NH_2), 3.87 (s, 3H, OMe), 3.86 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.49 (s, 2H, CH_2), 3.15 (m, 4H, CH_2), 2.58 (m, 4H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 167.1, 153.5, 148.9, 148.4, 148.3, 144.9, 141.1, 130.4, 121.3, 121.5, 121.4, 118.6, 116.2, 112.5, 112.4, 111.4, 110.9, 107.8, 101.0, 62.7, 57.1, 55.9, 53.1, 52.9, 50.7, 49.5.

7.4.15. Preparation of *N*-(2-(4-(4-(3,4-dimethoxybenzyl)piperazin-1-yl)phenylcarbamoyl)phenyl)quinoline-3-carboxamide (4). To a solution of dichloromethane (10 mL) containing compound **23** (0.157 mmol) were added 3-quinoline acid chloride (**9**) (0.189 mmol) and K_2CO_3 (0.316 mmol). The mixture was stirred for 24 h at room temperature. The mixture was diluted in dichloromethane and extracted three times with 10 mL of solution of 1 N NaOH. The organic phase was combined, dried over Na_2SO_4 anhydrous, filtered, and evaporated. Pure product was obtained by flash chromatography on silica gel using a gradient of solvent from 2% to 5% MeOH in dichloromethane to give 65.4 mg as a yellow solid. Yield: 69%. Mp 206–207 °C; IR (NaCl): 3297 (NH); 2932, 2818 (C–H), 1684, 1592 (C=O), 1514, 1447, 1407 (C=C Ar), 1306 (N aromatic), 1223 (tertiary amine), 1141 (OCH_3), 910, 861, 818 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 12.16 (s, 1H, NHCO), 9.49 (s, 1H, NHCO), 8.84 (s, 1H, ArH), 8.72 (s, 1H, ArH), 8.59 (d, 1H, ArH, J = 8.3 Hz), 8.13 (d, 1H, ArH, J = 8.4 Hz), 7.94 (d, 1H, ArH, J = 8.0 Hz), 7.79 (t, 1H, ArH, J = 7.9 Hz), 7.60 (m, 4H, ArH), 7.33 (t, 1H, J = 7.8 Hz), 6.85 (m, 6H, ArH), 3.87 (s, 3H, OMe), 3.86 (s, 3H, OMe), 3.50 (s, 2H, CH_2), 3.18 (s, 4H, CH_2), 2.59 (s, 4H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 167.2, 164.1, 149.5, 149.0, 148.9, 148.8, 148.2, 139.3, 135.9, 132.6, 131.5, 129.5, 129.4, 129.2, 127.5, 127.3, 126.9, 123.4, 122.2, 121.9, 121.4, 116.5, 112.3, 110.8, 62.8, 55.9, 52.9, 49.2. MS: 602.26 $[M+H]^+$.

7.4.16. Preparation of *N*-(2-(4-(4-(3,4-dimethoxybenzyl)piperazin-1-yl)phenylcarbamoyl)-4,5-dimethoxyphenyl)quinoline-3-carboxamide (5). To a solution of dichloromethane (20 mL) containing compound **25** (1.028 mmol) were added 3-quinoline acid chloride (**9**) (1.28 mmol) and K_2CO_3 (2.06 mmol). The mixture was stirred for 24 h at room temperature. The mixture was diluted in dichloromethane and extracted three times with 10 mL of solution of 1 N NaOH. The organic

phase was combined, dried over Na_2SO_4 anhydrous, filtered, and evaporated. Pure product was obtained by flash chromatography on silica gel using a gradient of solvent from 2% to 5% MeOH in dichloromethane to give 413.9 mg as a yellow solid. Yield: 61%. Mp: 226–227 °C; IR (NaCl): 3266 (NHCO), 2939, 2828 (C–H), 1640, 1596 (C═O), 1514, 1452 (C═C Ar), 1317 (N aromatic), 1242 (tertiary amine), 1210 (OCH_3), 923, 861, 822 (CH deformation Ar); NMR ^1H (300 MHz, $CDCl_3$): 12.49 (s, 1H, NHCO), 9.50 (s, 1H, NHCO), 8.69 (s, 1H, ArH), 8.48 (s, 1H, ArH), 8.44 (s, 1H, ArH), 8.13 (d, 1H, ArH, $J = 8.4$ Hz), 7.94 (d, 1H, ArH, $J = 8.0$ Hz), 7.79 (t, 1H, ArH, $J = 7.26$ Hz), 7.60 (t, 1H, ArH, $J = 7.5$ Hz), 7.52 (d, 1H, $J = 8.7$ Hz), 7.06 (s, 1H, ArH), 6.84 (m, 5H, ArH), 3.87 (s, 3H, OMe), 3.86 (s, 3H, OMe), 3.85 (s, 3H, OMe), 3.64 (s, 3H, OMe), 3.50 (s, 2H, CH_2), 3.18 (s, 4H, CH_2), 2.59 (s, 4H, CH_2); NMR ^{13}C (75.5 MHz, $CDCl_3$): 167.4, 163.9, 152.5, 149.4, 149.0, 148.9, 148.3, 144.5, 135.7, 135.4, 131.4, 129.6, 129.3, 127.4, 127.2, 126.9, 122.5, 121.4, 116.4, 112.4, 112.3, 110.9, 109.8, 104.9, 62.7, 56.2, 56.1, 55.9, 52.9, 49.2; MS: 661.2 $[M]^+$. HRMS $[M+H]^+$: calcd for $C_{38}H_{40}N_5O_6$ 662.2979, found: 662.2993.

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