Journal of Medicinal Chemistry

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.5b01644 • Publication Date (Web): 04 Mar 2016

ACS Publications

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Pyrrolopyrimidine derivatives as novel inhibitors of Multidrug Resistance-associated Protein 1 (MRP1, ABCC1)

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Key words:

ABC transporter; MRP1; ABCC1; MDR; pyrrolopyrimidine; inhibitor

Abstract: Five series of pyrrolo[3,2-*d*]pyrimidines were synthesized and evaluated with respect to potency and selectivity toward Multidrug Resistance-associated Protein 1 (MRP1, ABCC1). This transport protein is a major target to overcome multidrug resistance in cancer patients. We investigated differently substituted pyrrolopyrimidines using the doxorubicin selected and MRP1 overexpressing small cell lung cancer cell line H69 AR in a calcein AM and daunorubicin cell accumulation assay. New compounds with high potency and selectivity were identified.

Piperazine residues at position 4 bearing large phenylalkyl side chains proved to be beneficial for MRP1 inhibition. Its replacement by an amino group led to decreased activity. Aliphatic and aliphatic-aromatic variations at position 5 and 6 revealed compounds with IC_{50} values in high nanomolar range. All investigated compounds had low affinity toward P-glycoprotein (P-gp, ABCB1). Pyrrolopyrimidines with small substituents showed moderate inhibition against Breast Cancer Resistance Protein (BCRP, ABCG2)

Introduction. The 190 kDa Multidrug Resistance-associated Protein 1 (MRP1, ABCC1) is a member of the ATP-binding cassette (ABC) superfamily of transport proteins. Besides P-gp and BCRP it represents an important transporter in cancer research, conferring resistance to common chemotherapeutic agents. This phenomenon is called multidrug resistance (MDR) as many structurally unrelated drugs are affected like the *Vinca* alkaloids vincristin and vinblastin, the anthracyclines doxorubicin, daunorubicin or epirubicin as well as the intercalating agent mitoxantrone or the podophyllotoxine etoposid.[1,2,3,4,5,6] Utilizing the energy of ATP hydrolysis, this active transport leads to decreased intracellular drug accumulation and often involves co-transportation of glutathione (GSH).[2,3,4,7,8] MRP1 is reported to be nearly ubiquitously expressed in human tissue[9,10] as well as in different cancer types like small (SCLC) and non-small (NSCLC) cell lung cancer[11,12], colon carcinomas or leukemia.[13,14,15] It is associated with reduced retention of xenobiotics and chemotherapeutics since these are extruded as glucuronate or sulfate conjugates.[16,17,18] The pharmacological function of MRP1 might be the regulation of cellular distress as it has high affinity toward endogenous mediators connected to oxidative stress and cell apoptosis like leukotriene C_4 (LTC_4), GSH and its oxidized form GSSG.[19,20,21,22,23,24]

Many structurally diverse compounds have been identified to inhibit MRP1 since its discovery and depiction between 1987 and 1992 by *Cole* et al.[25,26] The co-transportation of the ATPase modulator GSH[27,28] with many cytostatic compounds led to glutathion-conjugate analogs as inhibitors of MRP1.[29] Related to LTC_4, another endogenous substrate of MRP1, the leukotriene D_4 (LTD_4) receptor antagonist MK-571 (Verlukast, Figure 1) was found to reverse vincristine resistance at a 30 µM concentration in the H69 AR cell line overexpressing MRP1.[30] Similar results were

obtained by ONO-1078 (Pranlukast), another leukotriene receptor antagonist.[23,31] Reversal of MDR affecting doxorubicin and paclitaxel was also observed with the pyridine analog PAK-140P in different sarcoma and leukemia cell lines.[32,33] Especially drugs from clinical pharmacology were evaluated as MRP1 inhibitors, for example the anion transport inhibitor probenecid,[34] the nonsteroidal anti-inflammatory drug indometacin (Figure 1)[35,36] or the immunosuppressive agent cyclosporine A and its chemosensitizing derivative PSC-833 (Valspodar).[37] The MRP1-mediated transport of cytostatics is also affected by the calcium antagonist verapamil[37,38] and different reverse transcriptase inhibitors.[39,40] Due to the structural and functional analogy of MRP1 and P-gp, many compounds were discovered due to their ability to inhibit the latter transport protein.[41] Modifications of their scaffolds resulted in new inhibitors designed to affect MRP1 selectively and to elucidate the structure-activity relationships of different partial structures. *Rosenbaum* et al. reported on indometacin-analogs to increase cell death in combination with doxorubicin.[42] Another approach dealt with the development of verapamil analogs that affect LTC_4 and GSH transport,[43] after verapamil was found to stimulate GSH transport.[43,44,45] Other compounds were developed as dual inhibitors of P-gp and MRP1, e.g. the quinolone derivative MS209,[46] chalcogenpyrylium compounds[47] or quinazolinones (Figure 1).[48] The latter were derived from a tricyclic isoxazole scaffold[48,49,50,51] by Elli Lilly, who reported already on the drug export inhibiting cromen derivative LY294002 in 1996.[52] The pipecolinate derivative Biricodar (VX-710) was evaluated in clinical trials regarding its dual inhibition of P-gp and MRP1.[53,54] Natural products were also found to be inhibitors of MRP1, e.g. Agosterol A, a compound isolated from the marine sponge *Spongia* sp.[55,56] as well as flavonoids, which were firstly known as P-gp inhibitors.[57,58] Further investigations of flavonoids like apigenin proved these compounds to be potent MRP1 ATPase stimulators.[59,60,61] Synthesis of flavonoid-

analogs provided potent inhibitors of this transport protein.[62,63,64] α,β-unsaturated carbonyl compounds like curcumin and derivatives were also found to have inhibitory activity against MRP1.[65] *Leyers* and *Häcker* et al. showed in 2008 and 2009, respectively, that compounds bearing a thiourea moiety affect transport of calcein AM in the 2008/MRP1 cell line.[66,67] More recently it was reported that the tyrosine kinase inhibitors (TKI) ibrutinib and lapatinib also affect MRP1 in an inhibitory way.[68,69] TKIs have only been known as inhibitors of BCRP before.[70,71,72,73]

Wang et al., which had reported on quinazolinones to be potent inhibitors in nanomolar range,[48] found pyrrolo- and indolopyrimidines (Figure 1) as raloxifene-derived compounds to be the most active inhibitors toward MRP1 known until now.[74,75] The aim of our investigation was to identify important substructures of the pyrrolopyrimidine scaffold and to analyze the effect of different substitutions at positions 4, 5 and 6 regarding length and size of side chains.

Results and Discussion

Chemistry

All compounds were prepared as illustrated in schemes 1 and 2. The malononitrile derivatives were derived by nucleophilic substitution of malononitrile with *O*-methylated lactams (**2a-b**) as well as nucleophilic substitution of ethoxymethylene-malononitrile with differently substituted primary amines (**7a-i**). Cyclization with ethyl bromoacetate yielded pyrrol derivatives **3a-b** and **8a-i.** Addition of dimethyl formamide dimethyl acetale provided compounds **4a-b** and **9a-i**. Cyclization was conducted using gaseous ammonia in ethanol to build up the pyrimidine ring system

of compounds **5a-b** and **10a-i**. Aromatization was accomplished by treating the lactam structure-bearing substances with phosphoryl chloride giving the chlorinated precursors **6a-b** and **11a-i**. The desired compounds **12-38** were obtained by nucleophilic substitution at position 4 by 1*H-piperazine*- and different primary amines using microwave-assisted synthesis. The identity of intermediates **2a-b**, **3a-b**, **4a-b**, **5a-b**, **6a-b** as well as compounds **7a-i**, **8a-i**, **9a-i**, **10a-i** and **11a-i** was confirmed using ^{1}H NMR spectroscopy. The purity of the desired compounds **12-38** was determined using ^{1}H and ^{13}C NMR spectroscopy as well as LC-MS analysis.

Biological Investigation

Calcein AM and Daunorubicin Accumulation Assays. In order to analyze the influence of different substituents of the pyrrolopyrimidine structure we investigated variably substituted derivatives belonging to various structural classes.

Our first aim was to analyze the contribution of the aliphatic linker between the piperazine substructure at position 4 and the terminal phenyl ring with regard to MRP1 inhibition. Compound **12** (Figure 1) served as starting structure bearing a phenethyl group. This compound has already been described by *Wang* et al.[74] and possessed an IC_{50}-value of 0.370 µM in the calcein AM assay and 0.197 µM in the daunorubicin assay, respectively. This makes it a rather potent inhibitor of MRP1 compared to standard inhibitors like indomethacin or cyclosporine A (Figure S1 and S2).

A stepwise reduction of the phenethyl residue to a benzyl (**13**) and phenyl (**15**) substituent lowered the activity for each reduction about three-fold, as shown in Figure 2 and table 1. Thus the ethylene linker is well accepted in contrast to the shortened linkers. The correlation of the predicted partition coefficient (log P) and the

activity of the compounds (Figure 3) shows that high lipophilicity is beneficial for MRP1 inhibition. Compound **14** bearing a diphenylmethylpiperazine side chain represents a downward outlier. It shows a potency two-fold less than the benzylpiperazine derivative **13** although it has a high calculated log P value**.** Obviously, the sterically demanding diphenylmethyl residue is tolerated but contributes less to activity than could be expected. But the necessity of a lipophilic terminal part of the substituent becomes obvious from the comparison of compounds **16** and **17**, having a methylpiperazine and piperazine substructure, respectively. Therefore a lipophilic group containing an aromatic ring system seems to be required for an effective MRP1 inhibition. Figure 4 shows a comparison of representative dose-response curves of compounds **12**, **16** and **17**.

The second compound set consisted of four substances (**18-21**) in which compound **19** has been described by *Wang* et al. (Figure 1) before[74]. The piperazine linker was replaced by an amino group, resulting in decreased activity values. The correlation of inhibitory activity and lipophilicity is obvious, showing that the downsizing of the aliphatic linker leads to less lipophilic properties and simultaneously to a decrease of MRP1 inhibition of about one order of magnitude (table 1).

The next step was to see if similar changes in the side chains at positions 5 and 6 have similar results regarding MRP1 activity. The enlargement of the cyclohexyl to a cycloheptyl ring of compounds **22**-**25** (third compound set, table 2) showed that all cycloheptyl derivatives are inferior compared to their cyclohexyl counterparts (**12**-**15**). Nevertheless, compound **22** has still high affinity toward MRP1 with an IC_{50} value of 0.833 µM in the calcein AM assay and 0.643 µM in the daunorubicin assay. The replacement of the cyclic ring system by aliphatic side chains makes up the fourth compound set (**26**-**30**). These pyrrolopyrimidine derivatives proved to be potent

MRP1 inhibitors with IC_{50} values in high nanomolar range as it is summarized in table 2. Compound **30** was equally potent as standard compound **12**. With respect to the activity-log P relationship, all compounds form a cluster without correlation.

The fifth compound set was destined to determine the influence of large residues at position 5. Therefore, the aliphatic residue was enlarged with a benzene ring at the end of the chain. Within this set of compounds no. **31** bearing a phenyl residue was the most active one possessing an IC_{50} value of 0.750 µM in the calcein AM assay and 0.340 µM in the daunorubicin assay. All compounds are potent inhibitors of MRP1 in the low micromolar or high nanomolar range (table 3) analogous to compound set 4. No correlation was observed with regard to lipophilicity and calcein AM or daunorubicin activity data.

With regard to the molecular weight of all compounds, one can observe a correlation relating to MRP1 activity as can be seen in Figure 5. Concerning pyrrolopyrimidines, hydrophobic residues influencing the partition coefficient toward high lipophilicity are preferred up to 400 Da. The increase of molecular weight over this point did not lead to more active compounds, but kept the inhibitory level around 1 µM.

The results of both accumulation assays are summarized in Figure 6, showing a linear correlation of activities toward MRP1 within two and half orders of magnitude.

Calcein AM Accumulation Assay to Screen for P-gp Inhibition. Because of the functional and structural analogy between MRP1 and P-gp[41] we screened all compounds for their capability to inhibit the latter transport protein. The results are depicted in Figure 7 and tables 1 to 3. All compounds were poor inhibitors of P-gp with IC_{50}-values higher than the first generation inhibitor verapamil and about hundred fold less potent than tariquidar or elacridar in the same assay.[76]

Pheophorbide A Accumulation Assay to Determine the Inhibitory Activity toward BCRP. All compounds were screened for their capability to inhibit BCRP to ensure selectivity toward MRP1. As can be seen in Figure 8, most compounds from sets one to three show inhibition of BCRP of more than 25% at 10 μM in comparison to 10 μM Ko143 and were further characterized by generating full dose-response curves. The calculated IC_{50}-values are given in tables 1 and 2. Activity toward BCRP was not influenced by shortening of the side chain length in set one (**12-15**). Only compound **14** showed less potency as it contains a bulky diphenylmethylpiperazine residue. Analogous results were partially obtained for the cycloheptyl counterparts (**22**-**25**), although compound **25** was inactive in contrast to compound **15**. The methylpiperazine residue bearing compound **16** shows even better inhibitory activity with an IC_{50} value of 4.25 μM and represents a good scaffold for BCRP inhibition (table 1). Compound **17** is a downward outlier though its structural similarity to compound **16**. The replacement of the piperazine by an amino group in compound set two (**18**-**21**) did not change the activity except of the inactive phenethylamino derivative **19**. The substances of the third, fourth and fifth compound set revealed no BCRP activity.

MTT Assay to Determine Cytotoxicity of Selected Compounds. To evaluate the cytotoxic potential of the pyrrolopyrimidines we investigated representatives of all compound sets with regard to toxicity on H69 AR cells. As Figure 9 shows, nearly all representatives show only a slight influence on cell viability at 10 μM. The only exception is compound **31** from the fifth compound set that reduced the cell viability by 40%. The GI_{50} values of selected test compounds are depicted in table 4.

MTT-efficacy Assay to Determine Capability of Compounds to Restore Daunorubicin Sensitivity. Due to their mostly nontoxic characteristics, potent and less potent

representatives of all compound sets were investigated regarding their capability to reverse multidrug resistance mediated by MRP1. For this purpose the daunorubicin MTT-efficacy assay was conducted. All of the chosen compounds were able to reverse multidrug resistance, as can be seen in table 5. Compound **31** was most efficient in reversing MDR, but its intrinsic toxicity (Figure 9) might have contributed to this effect. Compound **30** proved to be non-toxic and increased the cytotoxicity of daunorubicin in H69 AR cells ten-fold, as depicted in Figure 10.

Conclusion

We elucidated the structure-activity relationship of pyrrolopyrimidines with variations at positions 4, 5 and 6 regarding the three major transport proteins involved in multidrug resistance of cancer cells. Long phenylalkyl residues linked to piperazine at position 4 are preferred as compounds with shortened side chains show reduced activity. The enlargement of the aliphatic side chain at position 5 increased the capability to inhibit MRP1 with great potency, as compound **30** demonstrates. Even large aromatic residues are accepted at this position. Especially small molecules of the pyrrolopyrimidine compound sets like compounds **15**, **16** or **18** are good representatives of dual inhibitors for MRP1 and BCRP or future BCRP inhibitors. Except of compound set 5 (**31**-**38**) all compound proved to be non-toxic, showing that the decrease in daunorubicin resistance in H69 AR cells correlates with their MRP1 inhibition capability.

Experimental Section

Chemistry. *Materials*. All chemicals were supplied by Acros Organics (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany), Applichem GmbH (Darmstadt, Germany), Fisher Scientific GmbH (Waltham, MA, USA), Merck Millipore (Billerica,

MA, USA), Sigma-Aldrich (St. Louis, MO, USA) and VWR International GmbH (Darmstadt, Germany) and used for synthesis without further purification. Analytical thin layer chromatography with silica gel F_{254} coated aluminum plates (Merck Millipore) were used to monitor reaction progress using methylene chloride/acetone (18:1 and 9:1, respectively) or methylene chloride/acetone/methanol (9:1:1) as eluent and an UV cabinet for compound detection at 254 nm. All compounds were purified with column chromatography using silica gel 60 (43-60 µm, Merck Millipore) with gradient elution (petroleum ether/methylene chloride 1:1, methylene chloride, methylene chloride/acetone 18:1, methylene chloride/acetone 9:1, methylene chloride/acetone/methanol 18:1:1, methylene chloride/acetone/methanol 9:1:1, in each case 200 mL). Identity of intermediates was determined by ^{1}H and ^{13}C NMR spectroscopy. The purity of the desired compounds **12-38** was confirmed by ^{1}H and ^{13}C NMR spectroscopy as well as LC-MS analysis. All NMR spectra were recorded in DMSO-d_6 on a Bruker Advance 500 MHz (500/126 MHz) and chemical shifts (δ) are expressed as ppm calibrated to the solvent signal (^{1}H NMR δ 2.50; ^{13}C NMR δ 39.5). ^{13}C signals were assigned employing distortion less enhancement by polarization transfer (DEPT) and attached proton test (APT) techniques and spin multiplicities are depicted as singlet (s), doublet (d), doublet of doublets (dd), doublet of triplets (dt), triplet of doublets (td), triplet (t), quartet (q), quintet (quint) and multiplet (m). LC-MS analysis was performed using an Agilent 1100 series with photo diode array (DAD) detector (Agilent Technologies, Santa Clara, CA, USA) and a Nucleodur column 100-5 C18 (Macherey-Nagel, Düren, Germany) followed by ESI mass spectrometry using an API 2000 Triple Quadrupole mass spectrometer (Applied Biosystems, Waltham, MA, USA) and Sciex Analyst Software version 1.5.1. The purity of all investigated compounds in biological testing was determined as ≥ 95% unless otherwise stated.

General Procedure for the Preparation of Compounds **6a**-**b**. The title compounds were prepared as described in the literature[77] with minor modifications as depicted in schemes 1 and 2. A solution of piperidin-2-one or azepan-2-one in benzene was heated and an equimolar amount of dimethyl sulfate was added dropwise. The reaction mixture was refluxed for 3 to 6 hours. To eliminate excess dimethyl sulfate, a 4 M solution of sodium hydroxide was added after cooling to room temperature and stirred for 30 minutes. The title compounds were extracted three times with 50 mL benzene from the aqueous phase and dried over magnesium sulfate. After filtration and solvent evaporation, to the resultant oil containing *6-methoxy-2,3,4,5-tetrahydropyridine* (**1a**) or *7-methoxy-3,4,5,6-tetrahydro-2H-azepine* (**1b**) a saturated, equimolar solution of malononitrile in ethanol was added under stirring. After 30 minutes, the precipitate was rinsed with ethanol and diethyl ether and dried. The resultant *2-(piperidin-2-ylidene)malononitrile* (**2a**) or *2-(azepan-2-ylidene)malononitrile* (**2b**) was dissolved in dimethyl formamide and potassium carbonate was added. The reaction mixture was heated to 100 °C and an equimolar amount of ethyl bromoacetate was added dropwise. After a reaction time of 3 to 6 hours, the mixture was given to ice cold water (200 mL) under stirring and filtered after 1 hour. The resultant *ethyl 2-amino-1-cyano-5,6,7,8-tetrahydroindolizine-3-carboxylate* (**3a**) or *ethyl 2-amino-1-cyano-6,7,8,9-tetrahydro-5H-pyrrolo[1,2-a]azepine-3-carboxylate* (**3b**) was used without further purification. Three to five equivalents of dimethyl formamide dimethyl acetale were added to a solution of compounds (**3a**) or (**3b**) in dimethyl formamide and heated to 100 °C. After a reaction time of 2 to 5 hours, the solvent was evaporated and the resultant viscous liquid was stirred under petroleum ether until full crystallization, providing *ethyl (E)-1-cyano-2-(((dimethylamino)methylene)amino)-5,6,7,8-tetrahydroindolizine-3-carboxylate* (**4a**) or ethyl *(E)-1-cyano-2-(((dimethylamino)methylene)amino)-6,7,8,9-tetrahydro-5H-*

pyrrolo[1,2-a]azepine-3-carboxylate (**4b**), which were washed with petroleum ether and dried. The compounds (**4a**) or (**4b**) were solved in ethanol, heated to reflux and exposed to gaseous ammonia for 5 hours. The solvent was evaporated and a solution of 10% sodium hydroxide was added under stirring. After 30 minutes, the alkaline solution was neutralized with acetic acid. The resultant precipitate of *4-oxo-3,4,6,7,8,9-hexahydropyrimido[4,5-b]indolizine-10-carbonitrile* (**5a**) or *4-oxo-4,6,7,8,9,10-hexahydro-3H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile* (**5b**) was filtered off and rinsed with water. The dried compounds (**5a**) or (**5b**) were added to 20 mL of phosphoryl chloride and heated to reflux overnight. After cooling to room temperature, the reaction mixture was given to ice cold water under stirring. The resultant precipitate of compounds (**6a**) or (**6b**) was filtered off and washed with water before drying.

*2-(piperidin-2-ylidene)malononitrile (***2a***)*. White needles; yield 77%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.01 (s, 1H), 3.23 (t, J = 6.0 Hz, 2H), 2.55 (t, J = 6.0 Hz, 2H), 1.74-159 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.5, 117.2, 116.0, 45.3, 42.3, 26.8, 20.7, 18.0.

*2-(azepan-2-ylidene)malononitrile (***2b***)*. White needles; yield 55%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.93 (s, 1H), 3.41-3.38 (m, 2H), 2.67-2.64 (m, 2H), 1.70-1.64 (m, 2H), 1.58-1.55 (m, 2H), 1.52-1.46 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 177.1, 117.4, 115.9, 46.9, 44.8, 30.9, 29.2, 27.8, 24.2.

*Ethyl-2-amino-1-cyano-5,6,7,8-tetrahydroindolizine-3-carboxylate (***3a***)***.** White powder; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.75 (s, 2H), 4.19 (q, J = 7.1 Hz, 2H), 4.05 (t, J = 6.1 Hz, 2H), 2.70 (t, J = 6.4 Hz, 2H), 1.88-1.83 (m, 2H), 1.74-1.69 (m, 2H), 1.25 (t, J = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 160.7, 146.0, 142.5, 115.2, 104.0, 79.3, 59.0, 45.7, 22.4, 22.3, 18.3, 14.6.

*Ethyl-2-amino-1-cyano-6,7,8,9-tetrahydro-5H-pyrrolo[1,2-a]azepine-3-carboxylate (***3b***)*. Pale yellow powder; yield 55%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.67 (s, 2H), 4.49-4.40 (m, 2H), 4.21 (q, J = 7.1 Hz, 2H), 2.77-2.73 (m, 2H), 1.76-1.70 (m, 2H), 1.60-1.54 (m, 4H), 1.25 (t, J = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 160.7, 148.6, 145.3, 115.3, 104.1, 80.7, 59.2, 46.1, 29.8, 27.5, 26.1, 25.6, 14.6.

*Ethyl-(E)-1-cyano-2-(((dimethylamino)methylene)amino)-5,6,7,8-tetrahydroindolizine-3-carboxylate (***4a***)*. Yellow needles; yield: 75%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.52 (s, 1H), 4.12 (t, J = 6.1 Hz, 2H), 4.07 (q, J = 7.1 Hz, 2H), 2.97 (s, 3H), 2.91 (s, 3H), 2.75 (t, J = 6.4 Hz, 2H), 1.91-1.85 (m, 2H), 1.77-1.71 (m, 2H), 1.17 (t, J = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 160.5, 156.2 (2C), 148.7, 141.6, 115.7, 110.3, 86.5, 59.2, 46.0, 33.8, 22.5, 22.5, 18.4, 14.0.

*Ethyl-(E)-1-cyano-2-(((dimethylamino)methylene)amino)-6,7,8,9-tetrahydro-5H-pyrrolo[1,2-a]azepine-3-carboxylate (***4b***)*. Yellow powder; yield: 40%. Used without further purification and characterization.

*4-oxo-3,4,6,7,8,9-hexahydropyrimido[4,5-b]indolizine-10-carbonitrile (***5a***).* White powder; yield: 68%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.21 (s, 1H), 7.90 (s, 1H), 4.35 (t, J = 6.4 Hz, 2H), 2.95 (t, J = 6.4 Hz, 2H), 1.99-1.93 (m, 2H), 1.87-1.80 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 153.2, 146.3, 145.5, 144.8, 116.7, 114.3, 84.0, 45.6, 22.4, 21.8, 18.3.

*4-oxo-4,6,7,8,9,10-hexahydro-3H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (***5b***)*. White powder; yield 12%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.30 (s, 1H), 4.82-4.69 (m, 2H), 2.01-2.95 (m, 2H), 1.85-1.79 (m, 2H), 1.72-1.63 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 153.5, 152.2, 144.8, 144.5, 117.0, 114.5, 85.5, 47.1, 30.0, 27.8, 26.4, 25.7.

*4-chloro-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**6a**).* White needles; yield 42%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.71 (s, 1H), 4.52 (t, J = 6.2 Hz, 2H), 3.15 (t, J = 6.4 Hz, 2H), 2.09-2.03 (m, 2H), 1.91-1.85 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.8, 151.3, 150.8, 142.1, 123.5, 113.4, 83.8, 46.0, 23.6, 21.8, 17.8.

*4-chloro-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (**6b**). Brown* powder; yield 71%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.73 (s, 1H), 4.80-4.75 (m, 2H), 3.20-3.16 (m, 2H), 1.90-1.71 (m, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 160.1, 150.8, 150.8, 141.5, 123.6, 113.6, 85.4, 47.00, 29.3, 27.1, 26.9, 25.2.

General procedure for the preparation of compounds **11a-i**. 1.1 equivalents of the amine were added to a saturated solution of 2-(ethoxymethylene)malononitrile in ethanol under stirring. After 5-60 minutes the resulting precipitate was rinsed with ethanol and diethyl ether yielding compounds (**7a**) or (**7b**). After filtration and drying, the compounds (**7a**) or (**7b**) were solved in dimethyl formamide and heated to 100 °C. An equimolar amount of ethyl bromoacetate was added dropwise, followed by reactions and procedures as described above, resulting in the title compounds.

*2-((methylamino)methylene)malononitrile (**7a**)*. Orange needles; yield: 63%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.91 (s, 1H), 7.86 (s, 1H), 2.96 (s, 3H).

*2-((ethylamino)methylene)malononitrile (**7b**)*. Yellow needles; yield: 54%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.12 (s, 1H), 7.89 (d, J = 14.8 Hz, 1H), 3.28-3.24 (m, 2H), 1.11 (t, J = 7.2 Hz, 3H).

*2-((propylamino)methylene)malononitrile (**7c**)*. Yellow needles; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.10 (s, 1H), 7.89 (d, J = 14.3 Hz, 1H), 3.21 (q, J = 7.2 Hz, 2H), 1.50 (dt, J = 14.6, 7.3 Hz, 2H), 0.82 (t, J = 7.4 Hz, 3H).

*2-((isopropylamino)methylene)malononitrile (**7d**)*. Orange needles; yield: 88%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.12 (s, 1H), 7.88 (d, J = 14.6 Hz, 1H), 3.67-3.57 (m, 1H), 1.16 (d, J = 6.6 Hz, 6H).

*2-((cyclopropylamino)methylene)malononitrile (**7e**)*. Yellow needles; yield: 60%. Used without further purification and characterization.

*2-((phenylamino)methylene)malononitrile (**7f**)* Yellow needles; yield: 78%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.09 (s, 1H), 8.49 (s, 1H), 7.42 (d, J = 7.7 Hz, 2H), 7.37 (t, J = 7.4 Hz, 2H), 7.17 (t, J = 7.3 Hz, 1H).

*2-((benzylamino)methylene)malononitrile (**7g**)*. Yellow needles; yield: 61%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.58 (s, 1H), 8.08 (s, 1H), 7.40-7.35 (m, 2H), 7.34-7.28 (m, 3H), 4.44 (s, 2H).

*2-((phenethylamino)methylene)malononitrile (**7h**)*. White needles; yield: 61%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.16 (s, 1H), 7.76 (s, 1H), 7.33-7.29 (m, 2H), 7.24-7.21 (m, 1H), 7.21-7.18 (m, 2H), 3.49 (t, J = 7.1 Hz, 2H), 2.82 (t, J = 7.3 Hz, 2H).

*2-(((3-phenylpropyl)amino)methylene)malononitrile (**7i**)*. Yellow powder; yield: 70%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.10 (s, 1H), 7.88 (s, 1H), 7.30-7.25 (m, 2H), 7.21-7.16 (m, 3H), 3.26 (t, J = 7.1 Hz, 2H), 2.56 (t, J = 7.5 Hz, 2H), 1.86-1.79 (m, 2H).

*Ethyl-3-amino-4-cyano-1-methyl-1H-pyrrole-2-carboxylate (**8a**)*. Brown powder; yield: 53%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.50 (s, 1H), 5.71 (s, 2H), 4.21 (q, J = 7.1 Hz, 2H), 3.69 (s, 3H), 1.26 (t, J = 7.1 Hz, 3H).

*Ethyl-3-amino-4-cyano-1-ethyl-1H-pyrrole-2-carboxylate (***8b***)*. Brown powder; yield: 23%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.59 (s, 1H), 5.74 (s, 2H), 4.23 (q, J = 7.1 Hz, 2H), 4.13 (q, J = 7.1 Hz, 2H), 1.27 (t, J = 7.1 Hz, 3H), 1.23 (t, J = 7.1 Hz, 3H).

*Ethyl-3-amino-4-cyano-1-propyl-1H-pyrrole-2-carboxylate (***8c***)*. Orange powder; yield: 66%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.58 (s, 1H), 5.76 (s, 2H), 4.22 (q, J = 7.1 Hz, 2H), 4.06 (t, J = 7.1 Hz, 2H), 1.66-1.58 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H), 0.77 (t, J = 7.4 Hz, 3H).

*Ethyl-3-amino-4-cyano-1-isopropyl-1H-pyrrole-2-carboxylate (***8d***)*. White powder; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.73 (s, 1H), 5.73 (s, 2H), 5.09 (m, 1H), 4.23 (q, J = 7.1 Hz, 2H), 1.31 (d, J = 6.7 Hz, 6H), 1.27 (t, J = 7.1 Hz, 3H).

*Ethyl-3-amino-4-cyano-1-cyclopropyl-1H-pyrrole-2-carboxylate (***8e***)*. White powder; yield: 85%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.55 (s, 1H), 5.77 (s, 2H), 4.23 (q, J = 7.1 Hz, 2H), 3.62-3.56 (m, 1H), 1.27 (t, J = 7.1 Hz, 3H), 0.91-0.87 (m, 4H).

*Ethyl-3-amino-4-cyano-1-phenyl-1H-pyrrole-2-carboxylate (***8f***)*. Brown powder; yield: 77%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.72 (s, 1H), 7.45-7.38 (m, 3H), 7.33-7.30 (m, 2H), 5.96 (s, 2H), 3.99 (q, J = 7.1 Hz, 2H), 0.95 (t, J = 7.1 Hz, 3H).

*Ethyl-3-amino-1-benzyl-4-cyano-1H-pyrrole-2-carboxylate (***8g***)*. Brown powder; yield: 71%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.78 (s, 1H), 7.33-7.29 (m, 2H), 7.26-7.22 (m, 1H), 7.09-7.05 (m, 2H), 5.82 (s, 2H), 5.36 (s, 2H), 4.12 (q, J = 7.1 Hz, 2H), 1.13 (t, J = 7.1 Hz, 3H).

*Ethyl-3-amino-4-cyano-1-phenethyl-1H-pyrrole-2-carboxylate (***8h***)*. Brown powder; yield: 90%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.39 (s, 1H), 7.30-7.26 (m, 2H), 7.23-7.18 (m, 1H), 7.15-7.12 (m, 2H), 5.78 (s, 2H), 4.33 (t, J = 7.4 Hz, 2H), 4.26 (q, J = 7.1 Hz, 2H), 2.91 (t, J = 7.2 Hz, 2H), 1.29 (t, J = 7.1 Hz, 3H).

*Ethyl-3-amino-4-cyano-1-(3-phenylpropyl)-1H-pyrrole-2-carboxylate (***8i***)*. Brown powder; yield: 92%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.59 (s, 1H), 7.28-7.24 (m, 2H), 7.19-7.15 (m, 3H), 5.78 (s, 2H), 4.19 (q, J = 7.1, 2H), 4.13 (t, J = 7.2 Hz, 2H), 2.52 (t, J = 7.6 Hz, 2H), 1.97-1.89 (m, 2H), 1.17 (t, J = 7.1 Hz, 3H).

*Ethyl-(E)-4-cyano-3-(((dimethylamino)methylene)amino)-1-methyl-1H-pyrrole-2-carboxylate (***9a***)*. Brown oil; quantitative yield. Used without further purification and characterization.

*Ethyl-(E)-4-cyano-3-(((dimethylamino)methylene)amino)-1-ethyl-1H-pyrrole-2-carboxylate (***9b***)*. Brown oil; quantitative yield. Used without further purification and characterization.

*Ethyl-(E)-4-cyano-3-(((dimethylamino)methylene)amino)-1-propyl-1H-pyrrole-2-carboxylate (***9c***)*. Orange powder; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.68 (s, 1H), 7.55 (s, 1H), 4.14 (t, J = 7.2 Hz, 2H), 4.11 (q, J = 7.1 Hz, 2H), 2.98 (s, 3H), 2.91 (s, 3H), 1.68-1.59 (m, 2H), 1.18 (t, J = 7.1 Hz, 3H), 0.78 (t, J = 7.4 Hz, 3H).

*Ethyl-(E)-4-cyano-3-(((dimethylamino)methylene)amino)-1-isopropyl-1H-pyrrole-2-carboxylate (***9d***)*. Brown oil; quantitative yield. Used without further purification and characterization.

*Ethyl-(E)-4-cyano-1-cyclopropyl-3-(((dimethylamino)methylene)amino)-1H-pyrrole-2-carboxylate (***9e***)*. Brown oil; quantitative yield. Used without further purification and characterization.

*Ethyl-(E)-4-cyano-3-(((dimethylamino)methylene)amino)-1-phenyl-1H-pyrrole-2-carboxylate (***9f***)*. Yellow needles; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.82 (s, 1H), 7.74 (s, 1H), 7.47-7.38 (m, 3H), 7.32-7.28 (m, 2H), 3.92 (q, J = 7.1 Hz), 3.01 (s, 3H), 2.94 (s, 3H), 0.94 (t, J = 7.1 Hz, 3H).

*Ethyl-(E)-1-benzyl-4-cyano-3-(((dimethylamino)methylene)amino)-1H-pyrrole-2-carboxylate (***9g***)*. Brown powder; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.87 (s, 1H), 7.56 (s, 1H), 7.33-7.29 (m, 2H), 7.27-7.22 (m, 1H), 7.10-7.06 (m, 2H), 5.46 (s, 2H), 4.03 (q, J = 7.1 Hz, 2H), 2.97 (s, 3H), 2.91 (s, 3H), 1.10 (t, J = 7.1 Hz, 3H).

*Ethyl-(E)-4-cyano-3-(((dimethylamino)methylene)amino)-1-phenethyl-1H-pyrrole-2-carboxylate (***9h***)*. Orange powder; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.55 (s, 1H), 7.49 (s, 1H), 7.30-7.26 (m, 2H), 7.23-7.19 (m, 1H), 7.16-7.13 (m, 2H), 4.40 (t, J = 7.5 Hz, 2H), 4.14 (q, J = 7.1 Hz), 2.99 (s, 3H), 2.94 (t, J = 7.7 Hz, 2H), 2.92 (s, 3H), 1.19 (t, J = 7.1 Hz, 3H).

*Ethyl-(E)-4-cyano-3-(((dimethylamino)methylene)amino)-1-(3-phenylpropyl)-1H-pyrrole-2-carboxylate (***9i***)*. Orange powder; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.69 (s, 1H), 7.54 (s, 1H), 7.29-7.24 (m, 2H), 7.19-7.15 (m, 3H), 4.23 (t, J = 7.1 Hz, 2H), 4.10 (q, J = 7.1 Hz, 2H), 2.98 (s, 3H), 2.91 (s, 3H), 2.53 (t, J = 7.6 Hz, 2H), 1.99-1.92 (m, 2H), 1.17 (t, J = 7.1 Hz, 3H).

*5-methyl-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***10a***)*. White powder; yield: 49%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.31 (s, 1H), 8.14 (s, 1H), 7.94 (s, 1H), 4.01 (s, 3H).

*5-ethyl-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***10b***)*. Brown powder; yield: 45%.^{1}H NMR (500 MHz, DMSO-d_6) δ 12.34 (s, 1H), 8.25 (s, 1H), 7.95 (s, 1H), 4.40 (q, J = 7.2 Hz, 2H), 1.37 (t, J = 7.2 Hz, 3H).

*4-oxo-5-propyl-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***10c***)*. Brown powder; yield: 24%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 8.24 (s, 1H), 7.95 (s, 1H), 4.34 (t, J = 7.0 Hz, 2H), 1.81-1.74 (m, 2H), 0.80 (t, J = 7.4 Hz, 3H).

*5-isopropyl-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***10d***)*. Brown powder; yield: 56%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.26 (s, 1H), 8.41 (s, 1H), 7.96 (s, 1H), 5.30 (m, 1H), 1.44 (d, J = 6.7 Hz, 6H).

*5-cyclopropyl-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***10e***)*. Brown powder; yield: 6%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.13 (s, 1H), 7.94 (s, 1H), 4.10-4.04 (m, 1H), 1.09-0.98 (m, 4H).

*4-oxo-5-phenyl-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***10f***)*. White powder; yield: 21%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.44 (s, 1H), 8.48 (s, 1H), 8.05 (s, 1H), 7.56-7.45 (m, 5H).

5-benzyl-4-oxo-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile *(***10g***)*. Yellow powder; yield 47%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.31 (s, 1H), 7.97 (s, 1H), 7.35-7.24 (m, 5H), 5.63 (s, 2H).

*4-oxo-5-phenethyl-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***10h***)*. Yellow powder; yield: 43%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.97 (s, 1H), 7.96 (s, 1H), 7.28-7.24 (m, 2H), 7.21-7.17 (m, 1H), 7.15-7.12 (m, 2H), 4.61 (t, J = 7.2), 3.08 (t, J = 7.3, 2H).

*4-oxo-5-(3-phenylpropyl)-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***10i***)*. White powder; yield: 21%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.09 (s, 1H), 7.93 (s, 1H), 7.26-7.22 (m, 2H), 7.18-7.14 (m, 3H), 4.43 (t, J = 7.0, 2H, CH_2), 2.54 (t, J = 7.7, 2H), 2.13-2.05 (m, 2H).

*4-chloro-5-methyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***11a***)*. Brown powder; yield: 66%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.79 (s, 1H), 8.76 (s, 1H), 4.14 (s, 3H).

*4-chloro-5-ethyl-4,5-dihydro-3H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***11b***)*. Brown powder; yield: 79%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.86 (s, 1H), 8.80 (s, 1H), 4.56 (q, J = 7.2 Hz, 2H), 1.45 (t, J = 7.2 Hz, 3H).

*4-chloro-5-propyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***11c***)*. Brown powder; yield: 94%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.86 (s, 1H), 8.81 (s, 1H), 4.48 (t, J = 7.2 Hz, 2H), 1.88-1.81 (m, 2H), 0.87 (t, J = 7.4 Hz, 3H).

*4-chloro-5-isopropyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***11d***)*. Brown powder; yield 32%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.05 (s, 1H), 8.80 (s, 1H), 5.40 (m, 1H), 1.54 (d, J = 6.6 Hz, 6H).

*4-chloro-5-cyclopropyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***11e***)*. Brown powder; quantitative yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.83 (s, 1H), 8.80 (s, 1H), 3.93-3.88 (m, 1H), 1.28-1.14 (m, 4H).

*4-chloro-5-phenyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***11f***)*. Brown powder; yield 94%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.01 (s, 1H), 8.91 (s, 1H), 7.68-7.65 (m, 2H), 7.63-7.57 (m, 3H).

*5-benzyl-4-chloro-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***11g***)*. Brown powder; yield 83%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.98 (s, 1H), 8.83 (s, 1H), 7.36-7.32 (m, 2H), 7.31-7.27 (m, 1H), 7.16- 7.12 (m, 2H), 5.81 (s, 2H).

*4-chloro-5-phenethyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***11h***)*. Brown powder; yield 88%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.81 (s, 1H), 8.65 (s, 1H), 7.28-7.24 (m, 2H), 7.21-7.19 (m, 1H), 7.15-7.11 (m, 2H), 4.77 (t, J = 7.4, 2H), 3.15 (t, J = 7.4, 2H).

*4-chloro-5-(3-phenylpropyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***11i***)*. Brown powder; yield 69%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.84 (s, 1H), 8.79 (s, 1H), 7.26-

7.22 (m, 2H), 7.20-7.13 (m, 3H), 4.55 (t, *J* = 7.3, 2H), 2.66 (t, *J* = 7.5, 2H), 2.20-2.13 (m, 2H).

*General Procedure for the Preparation of Compounds **12-38***. The title compounds were prepared as described in the literature[77] with minor modifications. To a solution of compounds **6a-b** or **11a-i** in 0.5 mL of dimethyl formamide a solution of the corresponding primary or secondary amine in 0.5 mL of dimethyl formamide was added. The reaction mixture was heated in the microwave (200 W, 110 °C, 20-60 minutes). The resulting product was purified by column chromatography with gradient elution as described above. The products were crystallized in a mixture of ethanol and petroleum ether (1:1).

*4-(4-phenethylpiperazin-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**12**)*. Synthesized from **6a** and phenylethylpiperazine; yield 66%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.46 (s, 1H), 7.29-7.22 (m, 4H), 7.19-7.15 (m, *J* = 7.1, 1.6 Hz, 1H), 3.34-3.29 (m, 4H), 4.35 (t, *J* = 5.6 Hz, 2H), 3.11 (t, *J* = 6.5 Hz, 2H), 2.78 (t, *J* = 7.2 Hz, 2H), 2.61-2.68 (m, 4H), 2.58 (t, *J* = 7.4 Hz, 2H), 1.98-1.87 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.4, 151.2, 151.0, 150.2, 140.5, 128.8 (2C), 128.4 (2C), 126.0, 118.2, 114.4, 83.6, 59.8, 52.2 (2C), 50.4 (2C), 46.1, 32.84, 22.9, 22.6, 18.4. LC-MS: (*m*/*z*) calc.: 386.5; found: 387.4 $[M+H]^+$. Purity: 95%.

*4-(4-benzylpiperazin-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**13**)*. Synthesized from **6a** and benzylpiperazine; yield 48%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.45 (s, 1H), 7.34-7.31 (m, 4H), 7.28-7.23 (m, 1H), 4.33 (t, *J* = 5.6 Hz, 2H), 3.36-3.30 (m, 4H), 3.54 (s, 2H), 3.10 (t, *J* = 6.5 Hz, 2H), 2.66-2.51 (m, 4H), 2.03-1.81 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.3, 151.2, 150.9, 150.2, 138.1, 129.1 (2C), 128.3 (2C), 127.1, 118.2, 114.4, 83.6, 62.2, 52.3 (2C), 50.3 (2C), 46.1, 22.9, 22.6, 18.4. LC-MS: (*m*/*z*) calc.: 373.2; found: 372.5 $[M+H]^+$. Purity: 94%.

*4-(4-benzhydrylpiperazin-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**14**)*. Synthesized from **6a** and diphenylmethylpiperazine, yield 70%, white needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.45 (s, 1H), 7.49-7.47 (m, 4H), 7.33-7.27 (m, 4H), 7.21-7.16 (m, 2H), 4.37 (s, 1H, CH), 4.31 (t, 5.5 Hz, 2H), 3.30-3.40 (m, 4H), 3.09 (t, J = 6.5 Hz, 2H), 2.49-2.60 (m, 4H), 1.82-1.95 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.2, 151.3, 150.9, 150.13, 142.9 (2C), 128.7 (4C), 127.7 (4C), 127.1 (2C), 118.2, 114.4, 83.6, 75.4, 51.3 (2C), 50.4 (2C), 46,0, 22.9, 22.6, 18.4. LC-MS: (m/z) calc.: 448.6; found: 449.4 $[M+H]^+$. Purity: 92%..

*4-(4-phenylpiperazin-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**15**)*. Synthesized from **6a** and phenylpiperazine; yield 13%, white needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.50 (s, 1H), 7.24 (dd, J = 8.7, 7.3 Hz, 2H), 7.00 (d, J = 8.6 Hz, 2H), 6.81 (t, J = 7.3 Hz 1H), 4.40 (t, J = 5.6 Hz, 2H), 3.50-3.43 (m, 4H), 3.36-3.31 (m, 4H), 3.13 (t, J = 6.4 Hz, 2H), 2.00-1.88 (m, 4H). ^{13}C NMR (126 MHz DMSO-d_6) δ 154.3, 151.3, 150.1, 150.1, 150.3, 129.1 (2C), 119.4, 118.4, 115.8 (2C), 114.4, 83.6, 50.3 (2C), 48.1 (2C), 46.1, 23.0, 22.6, 18.4. LC-MS: (m/z) calc.: 358.5; found: 359.4 $[M+H]^+$. Purity: 92%.

*4-(4-methylpiperazin-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**16**)*. Synthesized from **6a** and methylpiperazine; yield 45%, white powder. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.45 (s, 1H), 4.34 (t, J = 5.6 Hz, 2H), 3.31-3.25 (m, 4H), 3.10 (t, J = 6.5 Hz, 2H), 2.56-2.51 (m, 4H), 2.23 (s, 3H), 1.99-1.88 (m, 4H). ^{13}C NMR (126 MHz DMSO-d_6) δ 154.4, 151.2, 151.0, 150.2, 118.2, 114.5, 83.6, 54.2 (2C), 50.2 (2C), 46.1, 45.8, 22.9, 22.6, 18.4. LC-MS: (m/z) calc.: 296.4; found: 297.2 $[M+H]^+$. Purity: 100%..

*4-(piperazin-1-yl)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (**17**)*. Synthesized from **6a** and piperazine; yield 59%, white powder. ^{1}H NMR (500 MHz,

DMSO-d_6) δ 8.45 (s, 1H), 4.34 (t, J = 5.6 Hz, 2H), 3.23 (t, J = 4.6 Hz, 4H), 3.10 (t, J 6.5 Hz, 2H), 2.86 (J = 4.8 Hz, 4H), 1.98-1.86 (m, 4H). ^{13}C NMR (126 MHz DMSO-d_6) δ 154.7, 151.3, 150.8, 150.2, 118.2, 114.5, 83.6, 51.6 (2C), 46.1, 45.2 (2C), 22.9, 22.6, 18.4. LC-MS: (m/z) calc.: 282.4; found: 283.2 $[M+H]^+$. Purity: 100%

*4-((3-phenylpropyl)amino)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (***18***)*. Synthesized from **6a** and phenylpropylamine; yield 40%, yellow neeldes. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.20 (s, 1H), 7.25 (t, J = 7.5 Hz, 2H), 7.20 (d, J = 7.2 Hz, 2H), 7.15 (t, J = 7.2 Hz, 1H), 6.80 (t, J 5.4 Hz, 1H), 4.37 (t, J = 6.1 Hz, 2H), 3.52-3.47 (m, 2H), 2.99 (t, J = 6.3 Hz, 2H), 2.65 (t, J = 7.6 Hz, 2H), 2.03-1.98 (m, 2H), 1.95-1.89 (m, 2H), 1.86-1.80 (m, 2H). ^{13}C NMR (126 MHz DMSO-d_6) δ 152.2, 150.0, 147.7, 147.7, 142.1, 128.5 (2C), 128.5 (2C), 125.9, 115.1, 114.0, 82.8, 45.7, 33.0, 30.7, 23.1, 22.1, 18.2. LC-MS: (m/z) calc.: 331.4; found: 332.1 $[M+H]^+$. Purity: 99%.

*4-(phenethylamino)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (***19***)*. Synthesized from **6a** and phenylethylamine; yield 18%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.24 (s, 1H), 7.29 (t, J = 7.4 Hz, 2H), 7.25 (d, J = 6.9 Hz, 2H), 7.20 (t, J = 7.3 Hz, 1H), 6.92 (t, J = 5.5 Hz, 1H), 4.34 (t, J = 6.1 Hz, 2H), 3.73-3.63 (m, 2H), 3.00 (t, J = 6.3 Hz, 2H), 2.90 (t, J = 7.4, 2H), 2.04-1.97 (m, 2H), 1.97-1.80 (m, 2H). ^{13}C NMR (126 MHz DMSO-d_6) δ 152.2, 149.7, 147.7, 147.6, 139.8, 128.8 (2C), 128.5 (2C), 126.2, 114.9, 114.0, 82.9, 45.6, 42.1, 35.1, 23.0, 22.0, 18.1. LC-MS: (m/z) calc.: 317.4; found: 318.1 $[M+H]^+$. Purity: 100%

4-(benzylamino)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile ***20****)*. Synthesized from **6a** and benzylamine; yield 26%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.17 (s, 1H), 7.46 (d, J = 6.4 Hz, 1H), 7.36 (d, J = 7.3 Hz, 2H), 7.28 (t, J = 7.6 Hz, 2H), 7.20 (t, J = 7.3 Hz, 1H), 4.71 (d, J = 5.8 Hz, 2H), 4.47 (t, J = 6.1 Hz, 2H), 3.01 (t, J = 6.3 Hz, 2H), 2.06-2.00 (m, 2H), 1.88-1.82 (m, 2H). ^{13}C NMR

(126 MHz DMSO-d_6) δ 152.1, 149.6, 148.8, 147.8, 140.1, 128.3 (2C), 127.3 (2C), 126.7, 114.9, 113.9, 82.9, 45.8, 43.5, 23.1, 22.1, 18.2. LC-MS: (*m*/*z*) calc.: 303.4; found: 304.1 $[M+H]^+$. Purity: 100%

*4-(phenylamino)-6,7,8,9-tetrahydropyrimido[4,5-b]indolizine-10-carbonitrile (***21***)*. Synthesized from **6a** and aniline; yield 19%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.49 (s, 1H), 8.31 (s, 1H), 7.55 (d, *J* = 7.7 Hz, 2H), 7.33 (t, *J* = 7.9 Hz, 2H), 7.07 (t, *J* = 7.4 Hz, 1H), 4.57 (t, *J* = 6.1 Hz, 2H), 3.08 (t, *J* = 6.4 Hz, 2H), 2.07-2.02 (m, 2H), 1.90-1.85 (m, 2H). ^{13}C NMR (126 MHz DMSO-d_6) δ 151.5, 149.6, 149.2, 147.8, 139.6, 128.6 (2C), 123.4, 122.4 (2C), 114.9, 114.7, 83.22, 45.7, 23.2, 22.1, 18.3. LC-MS: (*m*/*z*) calc.: 289.3; found: 290.1 $[M+H]^+$. Purity: 98%

*4-(4-phenethylpiperazin-1-yl)-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (***22***)*. Synthesized from **6b** and phenylethylpiperazine; yield 16%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.47 (s, 1H), 7.29-7.22 (m, 4H), 7.19-7.15 (m, 1H), 4.59-4.39 (m, 2H), 3.40-3.28 (m, 4H), 3.14-3.03 (m, 2H), 2.77 (t, *J* = 7.2 Hz, 2H), 2.69-2.62 (m, 4H), 2.59 (t, *J* = 7.2 Hz, 2H), 1.90-1.81 (m, 2H), 1.81-1.70 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.6, 153.7, 151.0, 149.6, 140.5, 128.8 (2C), 128.3 (2C), 126.0, 117.9, 114.6, 85.4, 59.7, 52.1 (2C), 49.8 (2C), 47.4, 32.8, 29.9, 27.9, 27.1, 25.6. LC-MS: (*m*/*z*) calc.: 400.5; found: 401.3 $[M+H]^+$. Purity: 96%

*4-(4-benzylpiperazin-1-yl)-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (***23***)*. Synthesized from **6b** and benzylpiperazine; yield 36%, white needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.46, (s, 1H), 7.35-7.31 (m, 4H), 7.28-7.22 (m, 1H), 4.52-4.40 (m, 2H), 3.36-3.28 (m, 4H), 3.54 (s, 2H), 3.12-3.04 (m, 2H), 2.69-2.51 (m, 4H), 1.87-1.79 (m, 2H), 1.80-1.67 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.6, 153.7, 151.1, 149.6, 138.1, 129.0 (2C), 128.4 (2C), 127.2, 117.9,

114.6, 85.3, 62.1, 52.1 (2C), 49.7 (2C), 47.4, 27.9, 27.9, 27.1, 25.6. LC-MS: (*m*/*z*) calc.: 386.5; found: 387.4 $[M+H]^+$. Purity: 99%

*4-(4-benzhydrylpiperazin-1-yl)-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (***24***)*. Synthesized from **6b** and diphenylmethylpiperazine; yield 51%, white needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.46 (s, 1H), 7.48-7.43 (m, 4H), 7.32-7.27 (m, 4H), 7.21-7.16 (m, 2H), 4.49-4.39 (m, 2H), 4.38 (s, 1H), 3.40-3.29 (m, 4H), 3.11-2.99 (m, 2H), 2.56-2.50 (m, 4H), 1.84-1.76 (m, 2H), 1.76-1.63 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.5, 153.6, 151.1, 149.8, 142.8 (2C), 128.7 (4C), 127.7 (4C), 127.1 (2C), 114.6, 117.9, 85.2, 75.2, 51.1 (2C), 49.8 (2C), 47.4, 29.9, 27.8, 27.1, 25.6. LC-MS: (*m*/*z*) calc.: 462.6; found: 463.3 $[M+H]^+$. Purity: 98%

*4-(4-benzylpiperazin-1-yl)-7,8,9,10-tetrahydro-6H-pyrimido[4',5':4,5]pyrrolo[1,2-a]azepine-11-carbonitrile (***25***)*. Synthesized from **6b** and diphenylmethylpiperazine; yield 52%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.51 (s, 1H), 7.27-7.20 (m, 2H), 7.03-6.98 (m, 2H), 6.84-6.78 (m, 1H), 4.56-4.49 (m, 2H), 3.47-3.41 (m, 4H), 3.38-3.31 (m, 4H), 3.14-3.09 (m, 2H), 1.90 -1.72 (m, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.8, 153.7, 151.1, 151.0, 149.7, 129.1 (2C), 119.4, 118.0, 115.9 (2C), 114.6, 85.3, 49.7 (2C), 47.9 (2C), 47.5, 29,9, 27.8, 27.1, 25.6. LC-MS: (*m*/*z*) calc.: 372.5; found: 373.4 $[M+H]^+$. Purity: 99%

*5-methyl-4-(4-phenethylpiperazin-1-yl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***26***)*. Synthesized from **11a** and phenethylpiperazine; yield: 72%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.50 (s, 1H), 8.44 (s, 1H), 7.29-7.25 (m, 2H), 7.25-7.22 (m, 2H), 7.19-7.15 (m, 1H), 4.01 (s, 3H), 3.41-3.34 (m, 4H), 2.77 (t, J = 7.3 Hz, 2H), 2.68-2.61 (m, 4H), 2.58 (t, J = 7.4 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.9, 151.3, 150.1, 141.5, 140.5, 128.8 (2C), 128.4 (2C), 126.0, 118.6, 114.4, 85.4, 59.8, 52.2

(2C), 50.1 (2C), 36.8, 32.8. LC-MS: (*m*/*z*) calc.: 346.4; found: 347.2 $[M+H]^+$. Purity: 100%

*5-ethyl-4-(4-phenethylpiperazin-1-yl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***27***)*. Synthesized from **11b** and phenethylpiperazine; yield: 64%, white needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.61 (s, 1H), 8.54 (s, 1H), 7.29-7.22 (m, 4H), 7.19-7.15 (m, 1H), 4.34 (q, *J* = 7.2 Hz, 2H), 3.36-3.30 (m, 4H), 2.78 (t, *J* = 7.2 Hz, 2H), 2.70-2.62 (m, 4H), 2.59 (t, *J* = 7.3 Hz, 2H), 1.37 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.9, 151.4, 150.6, 140.6, 140.5, 128.8 (2C), 128.4 (2C), 126.0, 117.8, 114.3, 86.1, 59.7, 52.2 (2C), 50.1 (2C), 44.1, 32.8, 16.1. LC-MS: (*m*/*z*) calc.: 360.5; found: 361.2 $[M+H]^+$. Purity: 100%

*4-(4-phenethylpiperazin-1-yl)-5-propyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***28***)*. Synthesized from **11c** and phenethylpiperazine; yield: 72%, white needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.59 (s, 1H), 8.55 (s, 1H), 7.29-7.22 (m, 4H), 7.20-7.15 (m, 1H), 4.26 (t, *J* = 7.4 Hz, 2H), 3.33-3.30 (m, 4H), 2.78 (t, *J* = 7.2 Hz, 2H), 2.70-2.62 (m, 4H), 2.59 (t, *J* = 7.3), 1.79-1.71 (m, 2H), 0.72 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.1, 141.4, 150.6, 141.2, 140.5, 128.8 (2C), 128.4 (2C), 126.0, 118.0, 114.3, 85.9, 59.7, 52.4, 50.8 (2C), 50.1 (2C), 32.8, 24.2, 10.7. LC-MS: (*m*/*z*) calc.: 374.5; found: 375.2 $[M+H]^+$. Purity: 100%

*5-isopropyl-4-(4-phenethylpiperazin-1-yl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***29***)*. Synthesized from **11d** and phenethylpiperazine; yield: 18%, pale yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.81 (s, 1H), 8.55 (s, 1H), 7.29-7.22 (m, 4H), 7.19-7.15 (m, 1H), 4.99 (hept, *J* = 6.7 Hz, 1H), 3.32-3.30 (m, 4H), 2.78 (t, *J* = 7.2 Hz, 2H), 2.70-2.61 (m, 4H), 2.59 (t, *J* = 7.2 Hz, 2H), 1.44 (d, *J* = 6.6 Hz, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.9, 151.3, 150.3, 140.5, 138.3, 128.8 (2C), 128.4 (2C), 126.0, 117.3, 114.4, 87.0, 59.7, 52.1 (2C), 50.0, 49.9 (2C), 32.8, 23.1 (2C).

*5-cyclopropyl-4-(4-phenethylpiperazin-1-yl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**30**)*. Synthesized from **11e** and phenethylpiperazine; yield: 47%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.46 (s, 1H), 8.45 (s, 1H), 7.29-7.21 (m, 4H), 7.19-7.15 (m,1H), 3.97 (quint, J = 3.8 Hz, 1H), 3.56-3.48 (m, 4H), 2.77 (t, J = 7.2 Hz, 2H), 2.64-2.60 (m, 4H), 2.57 (t, J = 7.4 Hz, 2H), 1.14-1.03 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.4, 151.3, 150.3, 140.5, 140.2, 128.8 (2C), 128.4 (2C), 126.0, 118.6, 114.4, 85.7, 59.8, 52.4 (2C), 49.7 (2C), 32.8, 32.2, 8.5 (2C). LC-MS: (m/z) calc.: 372.5; found: 373.3 $[M+H]^+$. Purity: 100%

*4-(4-phenethylpiperazin-1-yl)-5-phenyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**31**)*. Synthesized from **11f** and phenethylpiperazine; yield: 50%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.74 (s, 1H), 8.58 (s, 1H), 7.61-7.57 (m, 2H), 7.54-7.48 (m, 3H), 7.25-7.21 (m, 2H), 7.17-7.12 (m, 3H), 3.12-3.00 (m, 4H), 2.61 (t, J = 7.4 Hz, 2H) 2.34 (t, J = 7.5 Hz, 2H), 2.12-2.02 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 153.2, 151.8, 151.2, 141.4, 140.4, 137.9, 129.6 (2C), 128.7 (2C), 128.5, 128.3 (2C), 125.9, 125.3 (2C), 115.3, 114.1, 88.2, 59.6, 51.4 (2C), 48.7 (2C), 32.5. LC-MS: (m/z) calc.: 408.5; found: 409.4 $[M+H]^+$. Purity: 100%

*4-(4-benzylpiperazin-1-yl)-5-phenyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (**32**)*. Synthesized from **11f** and benzylpiperazine; yield: 15%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.73 (s, 1H), 8.57 (s, 1H), 7.58-7.49 (m, 3H), 7.49-7.45 (m, 2H), 7.30-7.25 (m, 2H), 7.25-7.21 (m, 1H), 719-7.16 (m, 2H), 3.31 (s, 2H), 312-3.00 (m, 4H), 2.04-1.91 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 153.1, 151.8, 151.2, 141.4, 137.9, 137.7, 129.6 (2C), 128.9 (2C), 128.5, 128.3 (2C), 127.1, 125.3 (2C), 115.3, 114.1, 88.1, 61.9, 51.3 (2C), 48.7 (2C). LC-MS: (m/z) calc.: 394.5; found: 395.3 $[M+H]^+$. Purity: 100%

*5-benzyl-4-(4-phenethylpiperazin-1-yl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***33***)*. Synthesized from **11g** and phenethylpiperazine; yield: 41%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.65 (s, 1H), 8.56 (1H), 7.33-7.11 (m, 10H), 5.52 (s, 2H), 3.39-3.30 (m, 4H), 2.76 (t, J = 7.2 Hz, 2H), 2.68-2.60 (m, 4H), 2.58 (t, J = 7.2 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.2, 151.7, 151.0, 141.7, 140.5, 137.0, 128.9 (2C), 128.8 (2C), 128.4 (2C), 128.1, 127.1 (2C), 126.0, 117.9, 114.1, 87.0, 59.7, 52.2 (2C), 52.1, 50.0 (2C), 32.8. LC-MS: (m/z) calc.: 422.5; found: 423.3 $[M+H]^+$. Purity: 100%

*5-benzyl-4-(4-benzylpiperazin-1-yl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***34***)*. Synthesized from **11g** and benzylpiperazine; yield: 13%, yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.64 (s, 1H), 8.55 (s, 1H), 7.35-7.22 (m, 8H), 7.13-7.09 (m, 2H), 5.51 (s, 2H), 3.54 (m, 2H), 3.36-3.30 (m, 4H), 2.57-2.52 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.1, 151.7, 151.0, 141.7, 138.0, 136.9, 129.1 (2C), 128.9 (2C), 128.3 (2C), 128.1, 127.2, 127.1 (2C), 117.9, 114.0, 86.9, 62.1, 52.1 (2C), 52.1, 49.9. LC-MS: (m/z) calc.: 408.5; found: 409.4 $[M+H]^+$. Purity: 98%

*5-phenethyl-4-(4-phenethylpiperazin-1-yl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***35***)*. Synthesized from **11h** and phenethylpiperazine; yield: 60%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.55 (s, 1H), 8.49 (s, 1H), 730-7.21 (m, 4H), 7.20-7.09 (m, 4H), 7.00-6.96 (m, 2H), 4.55 (t, J = 7.1 Hz, 2H), 3.27-3.18 (m, 4H), 3.00 (t, J = 7.1 Hz, 2H), 2.77 (t, J = 7.2 Hz, 2H), 2.69-2.60 (m, 4H), 2.58 (t, J = 7.3 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.9, 151.3, 150.4, 141.1, 140.5, 137.3, 128.8 (2C), 128.7 (2C), 128.4 (2C), 128.2 (2C), 126.7, 126.0, 118.1, 114.3, 86.2, 59.8, 52.2 (2C), 50.3, 50.0 (2C), 37.2, 32.7. LC-MS: (m/z) calc.: 436.6; found: 437.5 $[M+H]^+$. Purity: 100%.

*4-(4-benzylpiperazin-1-yl)-5-phenethyl-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***36***)*. Synthesized from **11h** and benzylpiperazine; yield: 25%, white needles. ^{1}H NMR (500

MHz, DMSO-d_6) δ 8.54 (s, 1H), 8.48 (s, 1H), 7.36-7.30 (m, 4H), 7.29-7.24 (m, 1H), 7.18-7.10 (m, 3H), 6.99-6.95 (m, 2H), 4.52 (t, J = 7.2 Hz, 2H), 3.54 (s, 2H), 3.27-3.20 (m, 4H), 3.00 (t, J = 7.1 Hz, 2H), 2.61-2.52 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ = 154.8, 151.3, 150.4, 141.0, 137.9, 137.2, 129.0 (2C), 128.7 (2C), 128.3 (2C). 128,2 (2C), 127.1, 126.7, 118.0, 117.2, 86.1, 62.2, 52.1 (2C), 50.2, 49.9 (2C), 37.1. LC-MS: (m/z) calc.: 422.5; found: 423.3 $[M+H]^+$. Purity: 100%

*4-(4-phenethylpiperazin-1-yl)-5-(3-phenylpropyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***37***)*. Synthesized from **11i** and phenethylpiperazine; yield: 37%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.62 (s, 1H), 8.53 (s, 1H), 7.30-7.25 (m, 2H), 7.25-7.21 (m, 4H), 7.20-7.16 (m, 1H), 7.16-7.12 (m, 1H), 7.10-7.07 (m, 2H), 4.31 (t, J = 7.5 Hz, 2H), 3.25-3.15 (m, 4H), 2.75 (t, J = 7.2 Hz, 2H), 2.54 (t, J = 7.4 Hz, 2H), 2.53-2.50 (m, 4H), 2.48-2.45 (m, 2H), 2.13-2.05 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.0, 151.4, 150.4, 140.9, 140.5, 128.8 (2C), 128.5 (2C), 128.4 (2C), 128.3 (2C), 126.2, 126.0, 118.0, 114.3, 86.2, 59.6, 52.1 (2C), 50.0 (2C), 48.6, 32.8, 32.0, 32.0. LC-MS: (m/z) calc.: 450.6; found: 451.5 $[M+H]^+$. Purity: 99%

*4-(4-benzylpiperazin-1-yl)-5-(3-phenylpropyl)-5H-pyrrolo[3,2-d]pyrimidine-7-carbonitrile (***38***)*. Synthesized from **11i** and benzylpiperazine; yield: 29%, yellow needles. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.61 (s, 1H), 8.52 (s, 1H), 7.35-7.30 (m, 4H), 7.27-7.22 (m, 3H), 7.20-7.16 (m, 1H), 7.10-7.06 (m, 2H), 4.30 (t, J = 7.4 Hz, 2H), 3.50 (s, 2H), 3.25-3.14 (m, 4H), 2.46 (t, J = 7.3 Hz, 2H), 2.45-2.37 (m, 4H), 2.11-2.04 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 154.9, 151.4, 150.4, 140.9, 140.5, 138.0, 129.0 (2C), 128.5 (2C), 128.3 (4C), 127.2, 126.2, 118.0, 114.3, 86.2, 62.0, 52.0 (2C), 50.0 (2C), 48.6, 32.0, 32.0. LC-MS: (m/z) calc.: 436.6; found: 437.5 $[M+H]^+$. Purity: 100%.

Biological Investigation. *Chemicals.* The reference compounds cyclosporine A and Ko143 ((3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1′,2′:1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester) were purchased from Tocris bioscience (Bristol, IO, USA). Indometacin was supplied by Sigma-Aldrich (St. Louis, MO, USA). The fluorescence dyes calcein AM and pheophorbide A were delivered by Calbiochem (EMD Chemicals (San Diego, CA, USA), supply by Merck KGaA (Damstadt, Germany)) and Frontier Scientific Inc. (Logan, UT, USA), respectively. Daunorubicin was provided by Sigma (Oakville, ON, Canada). All other chemicals were purchased from Carl Roth GmbH (Karlsruhe, Germany), Merck KgaA (Darmstadt, Germany), Th. Greyer GmbH Co KG (Renningen, Germany) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All references and compounds were stored as 10 mM stock solutions in DMSO at -20 °C. Dilutions and dilution series were prepared in Krebs-HEPES buffer (KHB, consisting of 1.3 mM $CaCl_2$, 11.7 mM $_D$-Glucose monohydrate, 10.0 mM HEPES (N-2-hydroxyethylpiperazin-N'-2-ethansulfonic acid), 4.7 mM KCl, 1.2 mM KH_2PO_3, 1.2 mM $MgSO_4$, 118.6 mM NaCl, and 4.2 mM $NaHCO_3$, adjusted to pH 7.4 with sodium hydroxide solution and sterilized by filtration with membrane filters (Whatman FP 30/0.2 µM CA-S filter units, GE Healthcare UK limited, Buckinghamshire, UK) with Braun Injekt 29 mL syringe (ALMO-Erzeugnisse, Erwin Busch GmbH, Bad Arolsen, Germany), stored in cellstar 50 mL tubes (Greiner bio one, Frickenhausen, Germany).

Calcein AM Accumulation Assay to Evaluate the Inhibitory Effect toward MRP1. The calcein AM accumulation assay was performed as depicted in the literature[78,79] and described earlier with minor modifications.[66,80,81,82] 20 µL of a solution of KHB and the test compound in concentrations between 100 nM and 100 µM were added into clear

F bottom 96-well microplates (Greiner bio one, Frickenhausen, Germany). The cells were harvested and counted as described above. 160 µL containing approximately 60,000 cells were seeded into each well and incubated with the test compounds for 20 minutes in 5% CO_2-humidified atmosphere at 37 °C. 20 µL of a 3.125 µM solution of calcein AM (protected from light) were added followed by instant measurement of the fluorescence increase in constant time intervals of 60 seconds with a Fluostar Optima or Fluostar Polarstar microplate reader (BMG-Labtech, Software versions 2.00R2, 2.20 and 4.11-0, respectively) tempered at 37 °C using an excitation wave length of 485 nm and an emission wave length of 520 nm. Cyclosporine A, indometacin and compound **12** were used as standard inhibitors. The slope of the linear part of the fluorescence-time curve was calculated between minutes 5 and 30 and plotted against the logarithmic concentration of the test compounds. Concentration−response curves were generated by nonlinear regression using the four-parameter logistic equation with variable Hill slope with GraphPad Prism version 5.03 for Windows (San Diego, CA, USA).

Calcein AM Accumulation Assay to Screen for P-gp Inhibition. All compounds were screened for P-gp inhibition as described earlier with minor modifications.[66,82,83,84,85,86,87,88,89,90,91] Dilution series, cell harvesting and counting was performed as stated above. To 20 µL of the 100 µM test compound solution were added 160 µL of a cell suspension containing approximately 30.000 cells per well. The microplates were incubated for 30 minutes in 5% CO_2-humidified atmosphere at 37 °C. 20 µL of a 3.125 µM solution of calcein AM (protected from light) were added and the fluorescence increase was measured instantly as described above. The slope of the fluorescence-time curve was calculated between minutes 5 and 30. The effect value of the 10 µM concentration was compared to the effect of 10 µM

cyclosporine A and expressed as percentage inhibition in comparison to the standard compound. Compounds with an inhibition level below 25% were not further characterized. For compounds with an inhibition level above 25% full dose-response curves were generated to determine the IC_{50}-value using the four-parameter logistic equation with variable Hill slope (GraphPad Prism). If necessary the top value was constraint to the standard compound.

Daunorubicin Accumulation Assay. For further analysis and for confirmation of existing results the daunorubicin accumulation assay was performed as described before with minor modifications.[92] H69 AR cells were prepared as described in the calcein AM accumulation assay. The obtained cell pellet was resuspended in fresh culture medium and 160 µL were seeded into colourless 96 well plates at a density of approximately 60,000 cells per well. 20 µL of the test compound in various concentrations prepared in cell culture medium without further supplements were preincubated with the cells. After 15 min, 20 µL of a 30 µM daunorubicin solution were added to each well. To achieve steady state conditions the 96 well plate was kept protected from light under 5% CO_2-humidified atmosphere and 37 °C for 180 min. Before starting measurement the cells were resuspended to get a homogeneous suspension and to remove adherent cells from the bottom. Fluorescence was measured by flow cytometry (FACScalibur, Becton Dickinson Biosciences, Heidelberg, Germany). An argon laser with an excitation wavelength of 488 nm excited dauorubicin which could be detected in the FL3 channel (≥ 670 nm). Concentration response curves were generated by nonlinear regression based on the 4-parameter logistic equation with variable Hill slope (GraphPad Prism) as stated above.

Pheophorbide A Accumulation Assay to Determine the Inhibitory Activity toward BCRP. The pheophorbide A accumulation assay was conducted with the MDCK II BCRP cell line as described earlier.[73,86,93,94] Cells were harvested and prepared as described above. 20 µL of various test compounds in different concentrations between 1 µM and 100 µM were added to an U-shaped clear 96 well plate (Greiner, Frickenhausen, Germany). 160 µL of the cell suspension containing approximately 45,000 cells were added to each well and preincubated for 20 min in 5% CO_2-huminidified atmosphere at 37 °C. 20 µL of a 5 µM pheophorbide A solution (protected from light) were added to each well and the microplate was maintained under 5% CO_2 and 37 °C for an incubation time of 120 min to reach steady state conditions. Before starting measurement the cells were resuspended to get a homogeneous suspension and to remove adherent cells from the bottom. Fluorescence was measured by flow cytometry. An argon laser with an excitation wavelength of 488 nm excited pheophorbide A which could be detected in the FL3 channel (≥ 670 nm). BCRP expression was measured with GFP detection in the FL1 channel (530/15). The effect value of the 10 µM concentration was compared to the effect of 10 µM Ko143 that was used as standard inhibitor for BCRP. Compounds showing more than 25% inhibition were further evaluated by generating full dose-response curves. The top value of the four-parameter logistic curve was constrained to the fluorescence obtained with Ko143.

MTT Assay to Determine Cytotoxicity of Selected Compounds. The determination of intrinsic toxicity of selected compounds was conducted as described in the literature with minor modifications.[6767,76,80,82,89,94,95,] 20 µL of a 100 nM to 100 µM solution of the test compound in cell culture medium was pipetted into 96-well tissue-culture treated plates (Starlab GmbH, Hamburg, Germany) before adding 180 µL of a cell suspension of H69 AR cells containing approximately 20,000 cells per well. Pure

medium without other ingredients was used as negative control and 10% DMSO was used as positive control, defining 100% and 0% viability, respectively. The microplate was kept under CO_2-humidified atmosphere at 37 °C. After an incubation time of 72 hours, 20 µL of a 5 mg/mL solution of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) were added followed by a second incubation time of 1 hour. The supernatant was removed and 100 µL of DMSO were added per well. Spectrophotometric measurement of the absorbance at 570 nm was performed using an Ex Multiscan microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA) with a background correction at 690 nm. The effect values were plotted against logarithmic concentrations of the test compounds and dose-response curves with variable Hill slope were calculated with non-linear regression using GraphPad Prism.

MTT-efficacy Assay to Determine Capability of Compounds to Restore Daunorubicin Sensitivity. The ability of selected compounds to reverse MRP1 mediated multidrug resistance was determined with a similar experiment. 20 µL of a 10 µM, 50 µM and 100 µM concentrated dilution of the test compound were added to a 96 well plate before adding 160 µL of the cell suspension containing approximately 20,000 cells. A dilution series of daunorubicin was added to yield a final concentration range between 100 nM and 100 µM. After an incubation period of 72 hours the experiment was conducted as stated above.

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Abbreviations used.

ABC, ATP-binding cassette; ABCB1 *synonymous for* P-gp; ABCC1, *synonymous for* MRP1; ABCG2, *synonymous for* BCRP; APT, attached proton test; ATP, adenosine 5'-triphosphate; BCRP, Breast Cancer Resistance Protein; calcein AM, calcein acetoxymethyl ester; δ chemical shift in ppm; DAD, diode array detector; DEPT, distortion less enhancement by polarization transfer; DMS, dimethyl sulfate; FACS, fluorescence activated cell sorting, GI_{50}, half-maximum growth inhibitory concentration; GSH, glutathione, reduced; GSSG, glutathione, oxidized; HEPES, 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonic acid; log P calc., predicted partition coefficient of the neutral molecule; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; $[M+H]^+$, ionization from protonation; MDR, multidrug resistance; MRP1, Multidrug Resistance-associated Protein 1; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromid; NSCLC, non-small cell lung cancer; P-gp, permeability (P) glycoprotein; SCLC, small cell lung cancer; TKI, tyrosine kinase inhibitors; *(Z), cis.*

Supporting Information.

Molecular formula strings and the associated biological data together with dose-response curves of standard and reference inhibitors are given.

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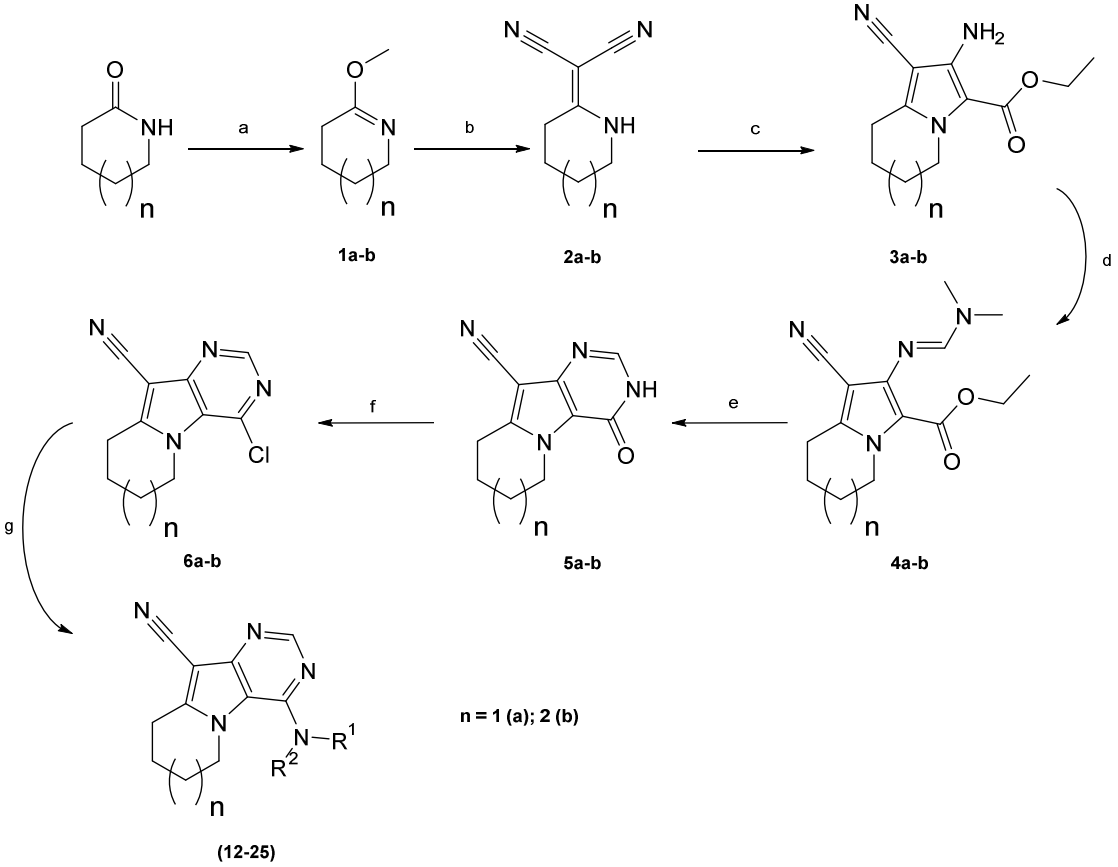

Scheme 1: Synthesis of pyrrolopyrimidines with variations at position 4.

(a) DMS, benzene, reflux, 3 h; (b) malononitrile, EtOH, rt, 1 h; (c) ethyl bromoacetate, DMF, 100 °C, 5 h; (d) DMF-DMA, DMF, 100 °C, 5 h; (e) NH_3; EtOH, reflux, 5 h; (f) $POCl_3$, TEA, reflux, 5 h; (g), amine, TEA, DMF, 200 W, 110 °C, 1 h.

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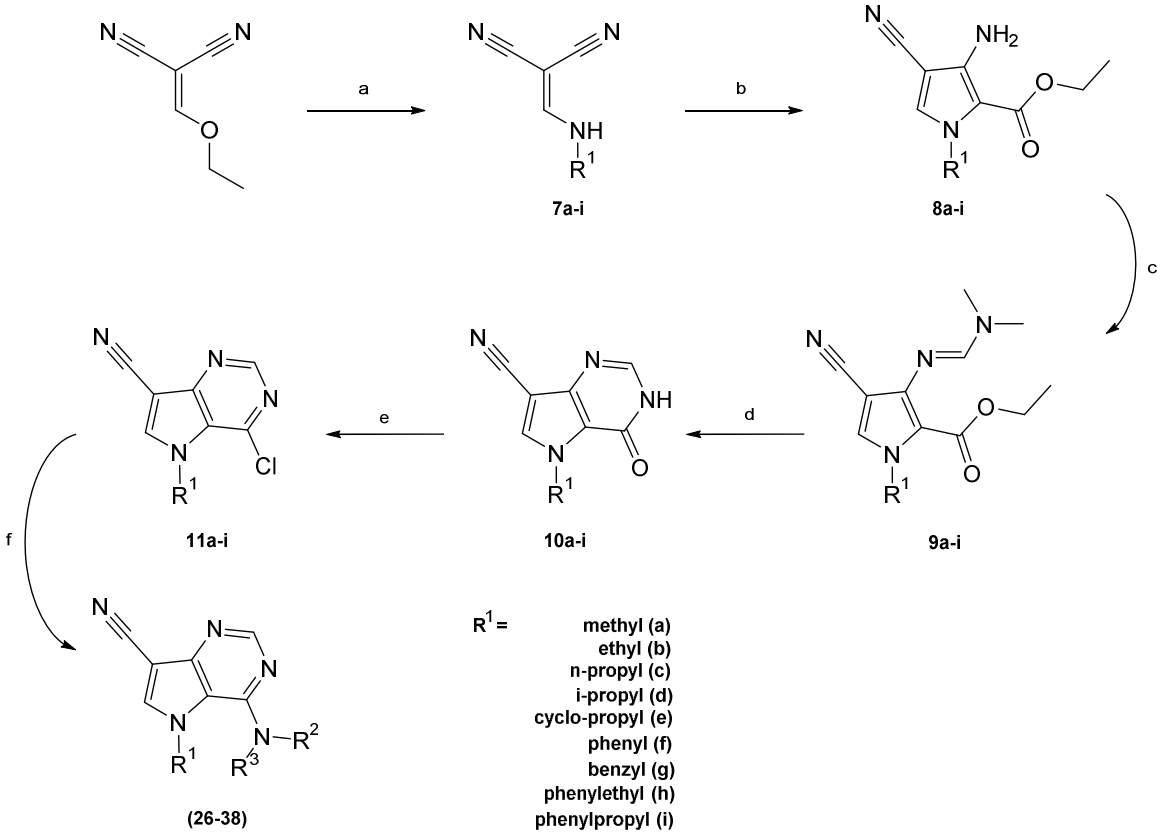
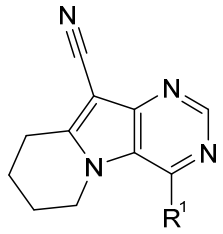


Scheme 2: Synthesis of pyrrolopyrimidines with variations at positions 5 and 6.

(a) H_2N-R^1, EtOH, rt, 1 h; (b) ethyl bromoacetate, DMF, 100 °C, 5 h; (c) DMF-DMA, DMF, 100 °C, 5 h; (d) NH_3, EtOH, reflux, 5 h; (e) $POCl_3$, TEA, reflux, 5 h; (f) amine, TEA, DMF, 200 W, 110 °C, 1 h.

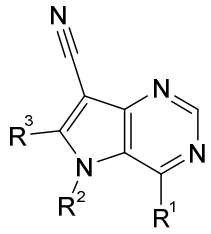
Table 1: Summary of calcein AM, daunorubicin and pheophorbide A assay results of condensed cyclohexyl pyrrolopyrimidines with variations at position 4 (compound sets one and two). Shown is mean ± standard deviation (SD) of at least 3 independent experiments of duplicate measurements. n. t. = not tested, due to low effect in screening.



Comp.	R^1	MRP1 Calcein AM IC_{50} ± SD [μM]	MRP1 Daunorubicin IC_{50} ± SD [μM]	P-gp Calcein AM IC_{50} ± SD [μM]	BCRP Pheophorbide A IC_{50} ± SD [μM]	log P (calc.)
12	Phenylethylpiperazinyl	0.370 ± 0.036	0.197 ± 0.025	18.8 ± 3.7	8.19 ± 1.72	2.81
13	Benzylpiperazinyl	0.671 ± 0.071	0.636 ± 0.057	32.3 ± 5.4	8.24 ± 1.69	2.36
14	Diphenylmethylpiperazinyl	1.62 ± 0.15	1.07 ± 0.06	n. t.	14.3 ± 1.4	3.82
15	Phenylpiperazinyl	2.56 ± 0.40	2.90 ± 0.12	n. t.	5.91 ± 1.28	2.02
16	4-Methylpiperazinyl	5.06 ± 0.27	2.87 ± 0.33	n. t.	4.25 ± 0.42	0.86
17	Piperazinyl	27.3 ± 7.1	10.8 ± 0.4	n. t.	n. t.	0.17
18	Phenylpropylamino	2.50 ± 0.50	2.84 ± 0.32	n. t.	6.07 ± 0.99	3.92
19	Phenylethylamino	8.37 ± 1.84	4.98 ± 0.47	n. t.	n. t.	3.45
20	Benzylamino	14.2 ± 2.9	8.80 ± 1.33	n. t.	15.8 ± 2.7	2.89

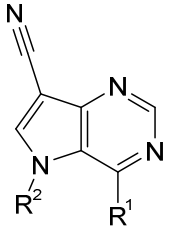
21	Anilino	14.2 ± 1.8	9.41 ± 0.61	n. a.	7.68 ± 0.92	2.73

Table 2: Summary of calcein AM, daunorubicin and pheophorbide A assay results of pyrrolopyrimidines with variations at positions 5 and 6 (compound sets three and four). Shown is mean ± SD of at least 3 independent experiments of duplicate measurements. n. t. = not tested, due to low effect in screening.



Comp.	R^1	R^2	R^3	MRP1 Calcein AM IC_{50} ± SD [μM]	MRP1 Daunorubicin IC_{50} ± SD [μM]	P-gp Calcein AM IC_{50} ± SD [μM]	BCRP Pheophorbide A IC_{50} ± SD [μM]	log P (calc.)
22	Phenylethylpiperazinyl	cycloheptyl		0.833 ± 0.055	0.643 ± 0.064	19.2 ± 2.5	9.94 ± 0.91	3.38
23	Benzylpiperazinyl	cycloheptyl		1.06 ± 0.04	0.981 ± 0.072	21.7 ± 2.6	4.68 ± 0.57	2.93
24	Diphenylmethylpiperazinyl	cycloheptyl		1.95 ± 0.17	1.01 ± 0.049	28.6 ± 6.8	8.71 ± 0.56	4.38
25	Phenylpiperazinyl	cycloheptyl		8.98 ± 1,48	15.1 ± 1.9	n. t.	n. t.	2.59
26	Phenylethylpiperazinyl	Methyl	H	0.714 ± 0.055	0.700 ± 0.099	n. t.	n. t.	1.75
27	Phenylethylpiperazinyl	Ethyl	H	1.06 ± 0.10	0.789 ± 0.093	n. t.	n. t.	2.28
28	Phenylethylpiperazinyl	Propyl	H	0.543 ± 0.071	0.418 ± 0.027	41.9± 17.3	n. t.	2.81
29	Phenylethylpiperazinyl	Isopropyl	H	0.821 ± 0.131	1.24 ± 0.13	n. t.	n. t.	2.63
30	Phenylethylpiperazinyl	Cyclopropyl	H	0.247 ± 0.045	0.200 ± 0.025	n. t.	n. t.	1.76

Table 3: Summary of calcein AM, daunorubicin and pheophorbide A assay results of pyrrolopyrimidines with aliphatic-aromatic variations at position 5 (compound set five). Shown is mean ± SD of at least 3 independent experiments of duplicate measurements. n. t. = not tested, due to low effect in screening.



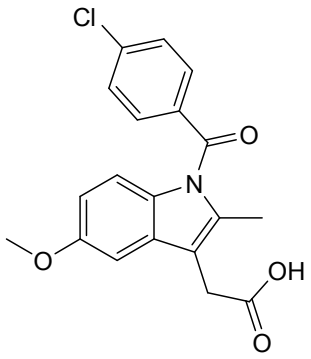
Comp.	R^1	R^2	MRP1 Calcein AM IC_{50} ± SD [μM]	MRP1 Daunorubicin IC_{50} ± SD [μM]	P-gp Calcein AM IC_{50} ± SD [μM]	BCRP Pheophorbide A IC_{50} ± SD [μM]	log P (calc.)
31	Phenylethylpiperazinyl	Phenyl	0.750 ± 0.118	0.340 ± 0.008	6.82 ± 0.50	n. t.	3.97
32	Benzylpiperazinyl	Phenyl	1.22 ± 0.26	0.866 ± 0.084	n. t.	n. t.	3.52
33	Phenylethylpiperazinyl	Benzyl	1.00 ± 0.08	0.623 ± 0.091	10.6 ± 1.1	n. t.	3.46
34	Benzylpiperazinyl	Benzyl	2.77 ± 0.43	0.876 ± 0.027	n. t.	n. t.	3.01
35	Phenylethylpiperazinyl	Phenylethyl	1.02 ± 0.07	1.12 ± 0.14	32.5± 4.8	n. t.	3.90
36	Benzylpiperazinyl	Phenylethyl	0.910 ± 0.234	0.983 ± 0.171	n. t.	n. t.	3.44
37	Phenylethylpiperazinyl	Phenylpropyl	0.956 ± 0.150	0.586 ± 0.030	n. t.	n. t.	4.34
38	Benzylpiperazinyl	Phenylpropyl	3.15 ± 0.20	2.36 ± 0.10	n. t.	n. t.	3.89

Table 4. Half-maximum growth inhibition values (GI_{50}) of selected pyrrolopyrimidine derivatives. Data obtained using the MRP1 over expressing cell line H69 AR. Shown is mean ± SD of at least 3 independent experiments of duplicate measurements.

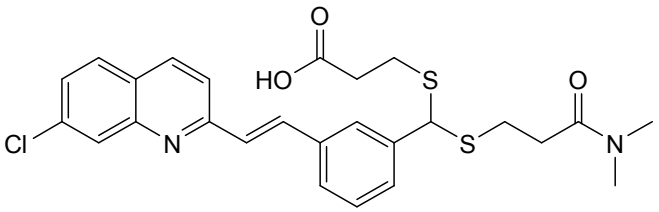
Comp.	GI_{50} ± SD [μM]
12	39.9 ± 18.6
17	60.6 ± 21.2
18	132 ± 45
30	36.0 ± 5.4
31	23.6 ± 10.8

Table 5: Shift of half-maximum growth inhibition values (GI_{50}) of daunorubicin in presence of selected pyrrolopyrimidine derivatives at concentrations of 1 μM, 5 μM and 10 μM. Data obtained using the MRP1 over expressing cell line H69 AR. Shown is mean ± SD of at least 3 independent experiments of duplicate measurements.

Comp.	GI_{50} (no comp) ± SD [μM]	GI_{50} (1 μM) ± SD [μM]	GI_{50} (5 μM) ± SD [μM]	GI_{50} (10 μM) ± SD [μM]
12	2.59 ± 0.47	0.934 ± 0.417	0.636 ± 0.227	0.375 ± 0.125
17	3.14 ± 0.75	2.94 ± 0.69	2.48 ± 0.52	1.83 ± 0.42
18	2.78 ± 0.51	2.41 ± 0.81	0.751 ± 0.097	0.450 ± 0.072
30	2.41 ± 0.22	0.827 ± 0.229	0.588 ± 0.203	0.395 ± 0.118
31	3.23 ± 1.24	0.782 ± 0.310	0.535 ± 0.215	0.389 ± 0.138



Indometacin



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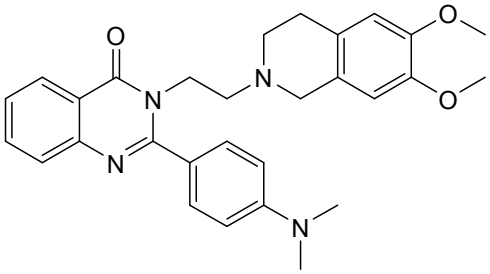

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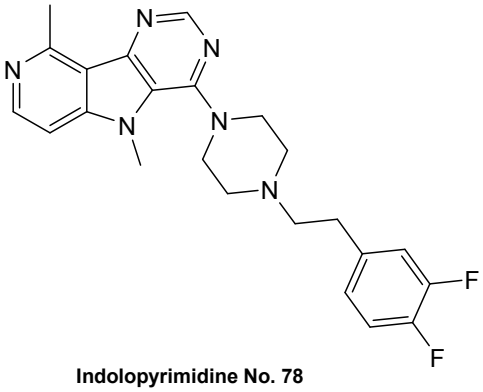

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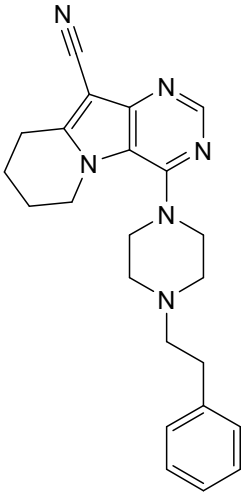
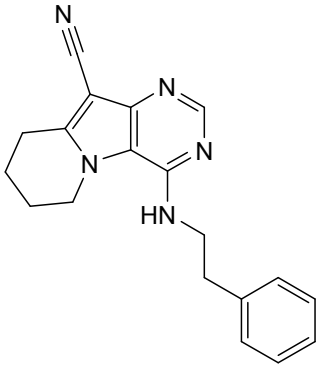


Compound 12



Compound 19

Figure 1. Depiction of common MRP1 inhibitors. Shown are quinazolinone[48] and indolopyrimidine derivatives[75] published by *Wang* et. al. as well as already described compounds **12** and **19** as representatives of pyrrolopyrimidines.[74]

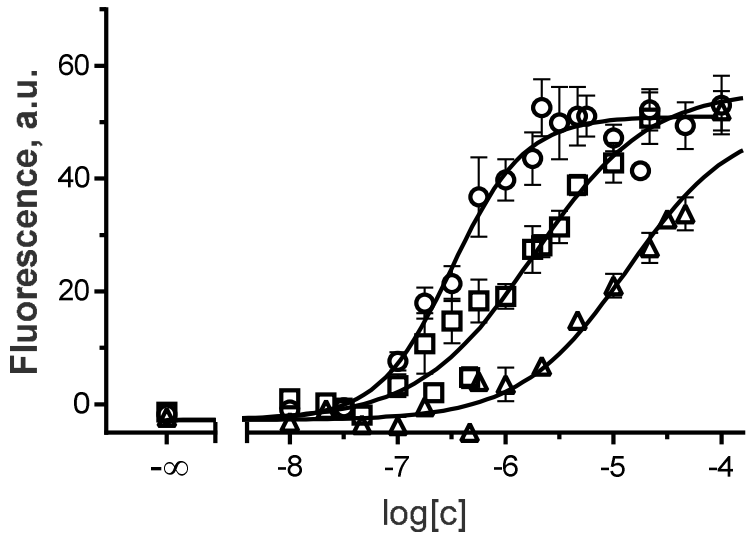


Figure 2. Dose-response curves of compounds **13** (open squares) and **15** (open triangles) in comparison to standard inhibitor compound **12** (open circles) measured in the calcein AM assay. Shown is mean ± SD of at least 3 independent experiments of duplicate measurements.

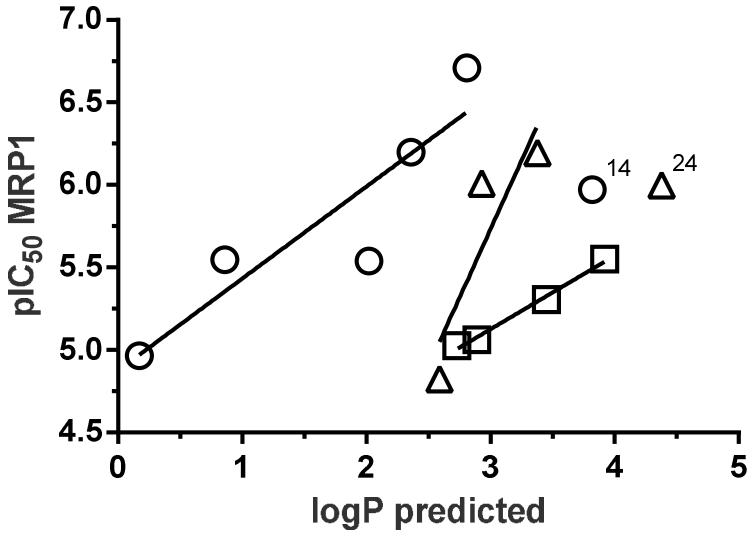


Figure 3: Plot of negative decadic logarithm of IC_{50} values obtained in the daunorubicin assay and predicted partition coefficients (log P) of compound sets one (open circle), two (open square) and three (open triangle). Compounds **14** and **24** were excluded from linear regression (right open circle and triangle, respectively).

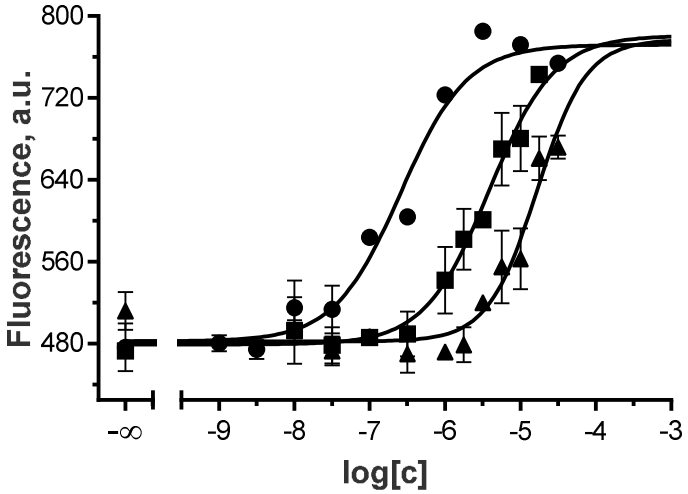


Figure 4. Comparison of dose-response curves of compounds **12** (circles), **16** (squares) and **17** (triangles) measured in the daunorubicin assay. Shown is a representative experiment out of three independent experiments performed with duplicate measurements.

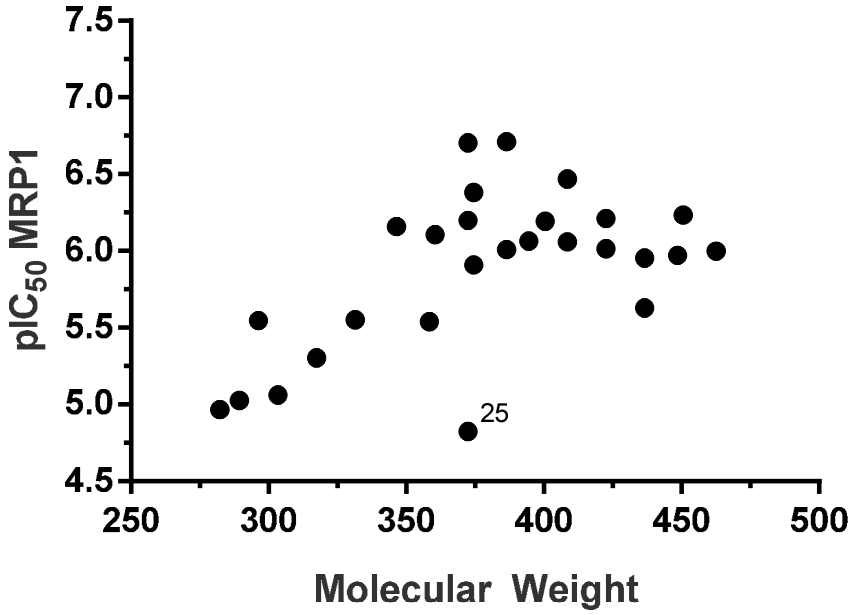


Figure 5. Plot of MRP1 activities of all compound sets obtained by the daunorubicin accumulation assay and molecular weight.

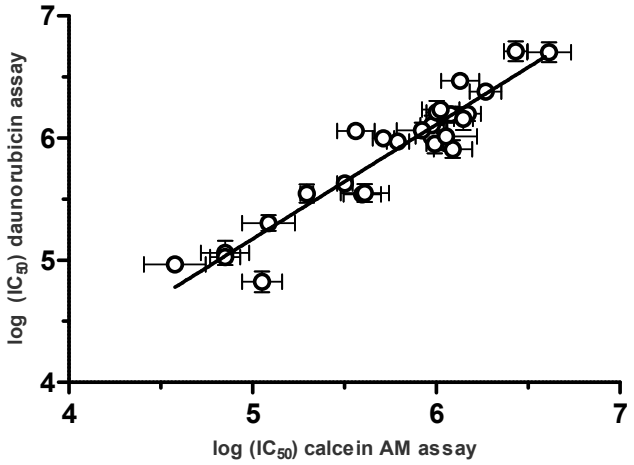


Figure 6. Comparison of activity values of all five datasets obtained by the calcein AM assay and daunorubicin accumulation assay, respectively. Squared correlation coefficient = 0.90.

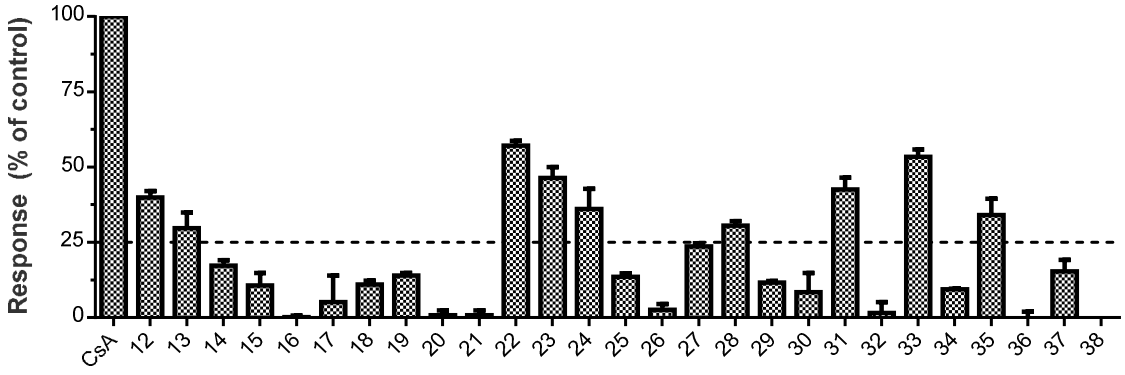


Figure 7. Summary of P-gp screening obtained by the calcein AM assay. Compounds with an inhibition level of more than 25% at 10 μM in comparison to the standard inhibitor cyclosporine A (10 μM) were further characterized by generating full dose-response curves to determine IC_{50} values (tables 1 to 3). Shown is mean ± SD of at least 3 independent experiments of duplicate measurements.

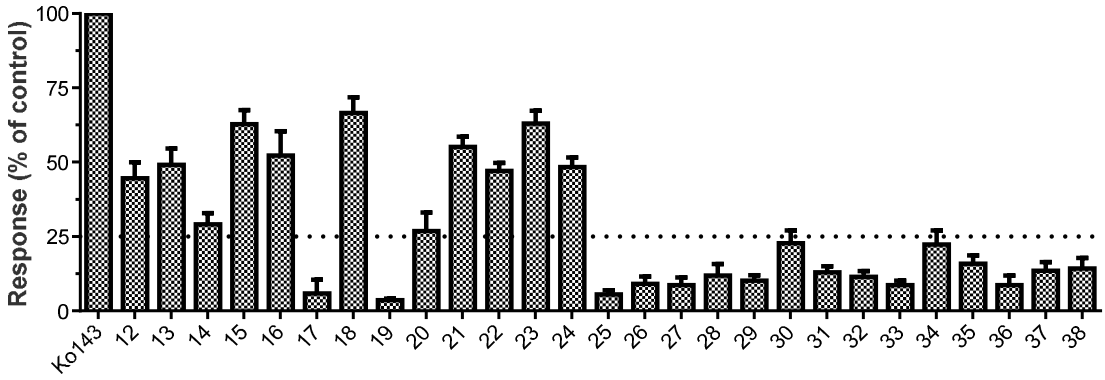


Figure 8. Summary of BCRP screening obtained by the pheophorbide A assay. Compounds with an inhibition level of more than 25% at a 10 μM concentration in comparison to the standard inhibitor Ko143 (10 μM) were further characterized by generating full dose-response curves to determine IC_{50} values (tables 1 to 3). Shown is mean ± SD of at least 3 independent experiments of duplicate measurements.

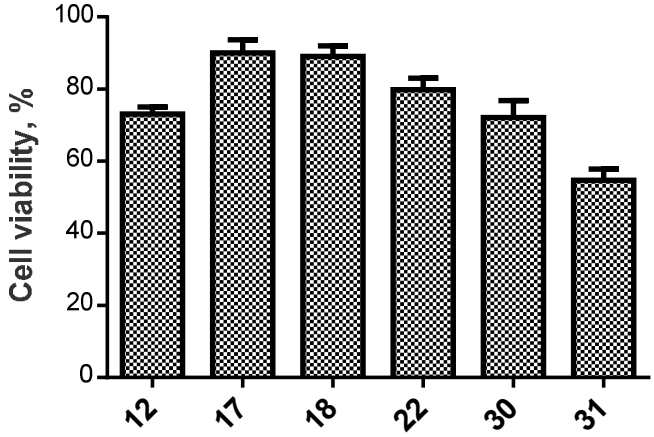


Figure 9. Summary of results of the MTT cytotoxicity assay at 10 μM compound concentration. Pure cell culture medium without further supplements was used as negative control, 10% DMSO as positive control, for defining 100% and 0% cell viability, respectively. Data obtained using the MRP1 over expressing cell line H69 AR. Shown is mean ± SD of at least 3 independent experiments of duplicate measurements.

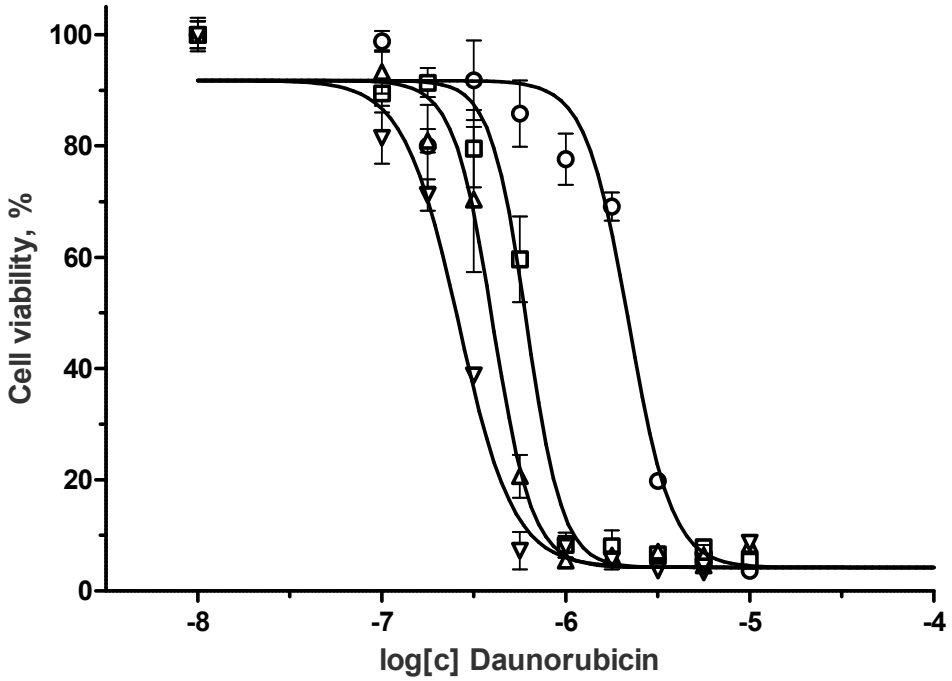


Figure 10. Sensitization of daunorubicin resistant H69 AR cells by compound **30**. Measurements were conducted in duplicate at concentrations of 1 μM (open squares), 5 μM (open triangle), 10 μM (open down triangle) and no supplementation of inhibitor (open circles). Shown is a representative experiment out of three independent experiments.

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