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Original article

A new series of amodiaquine analogues modified in the basic side chain with in vitro antileishmanial and antiplasmodial activity

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ABSTRACT

The synthesis and the study of new amodiaquine derivatives bearing modified lateral basic chains as new agents with both antimalarial and antileishmanial activities are reported. The compounds were tested in vitro against *Leishmania donovani* MHOM/ET/67/HU3 and 2 strains of *Plasmodium falciparum*, 3D7 and K1. All the compounds show complex ionisation profiles. At physiological pH the ionised form(s) are in equilibrium with the uncharged form, while at acid pH all the products exist largely as protonated forms. The antiprotozoal profile indicates that all derivatives are endowed with both antimalarial and antileishmanial activity. Interestingly amodiaquine, together with some synthesised derivatives (**11**, **12**, **15**, **27**, **34**), displayed antileishmanial activity in the low micromolar range, although these compounds were also cytotoxic and have a narrow therapeutic window, most of the synthesised compounds proved to be potent antimalarials, a few of them showing a good activity against the chloroquine resistant K1 strain.

1. Introduction

Infections caused by protozoa parasites are widespread in developing regions of the world with an enormous impact on public health. Generally speaking the development of agents useful for the treatment of these diseases has been limited by the lack of interest of the pharmaceutical sector to develop drugs without a market. Amodiaquine (**AQ**, Chart 1) is an established antimalarial drug recently reintroduced in the World Health Organisation Model List of Essential Medicines [1,2]. Its clinical indication is the treatment of chloroquine resistant *P. falciparum* infection in association with artesunate [3]. In addition, it has been recently reported that a series of pyrazolo[3,4-b]pyridine derivatives (general structure **A**, Chart 1), structurally related to AQ and AQ itself are active against promastigote forms of *Leishmania amazonensis* at μM concentration [4]. These findings open new interesting potential perspectives for AQ derivatives not only as antimalarial but also as antileishmanial agents. A number of structural analogues of the lead AQ have been described. They are principally connected with the modification of the substitution pattern at the quinoline ring and with variations in the Mannich side chain [5]. In this paper we report the synthesis and the study of the dissociation constants (pK_as) of some new AQ derivatives bearing modified lateral basic chains. The synthesised compounds can formally be grouped in two series. The first series consists of compounds **7**–**10**, **13** and **16** bearing a third basic centre in the side chain, and of compound **6**, **11**–**12**, **15** where the third basic centre was replaced by bulky polar groups. It is generally admitted that a stronger basicity of the molecule increases the antimalarial activity due to a better uptake in the vacuole owing to the pH gradient between the cytosol and the acidic vacuole, while the use of bulky substituents was suggested as one possible factor contributing to the improvement of the activity against CQ-resistant *P. falciparum* strains [6]. The second series consists of compounds **26**–**35** which were designed by joining the AQ scaffold to a substituted piperazinyl moiety. The use of piperazine as a linker proved of some success in antimalarial drug design [6,7]. Recent work showed that compounds bearing a piperazinyl linker, a basic centre and heteroaromatic rings were able to inhibit *P. falciparum* growth at nM concentrations as well as to inhibit β-hematin formation at μM concentrations [8]. Moreover the use of heteroaromatic rings as substituents in a series of 2′-deoxyuridine derivatives led to antileishmanial compounds with improved potency [9]. According to these observations we decided to modify the properties of this series of compounds introducing differently substituted piperazine on the AQ scaffold. The synthesised compounds were tested against two strains of *Plasmodium falciparum* 3D7, a clone of NF54 sensitive to all antimalarial drugs (CQ-S) and K1, a strain originating from Thailand, resistant to

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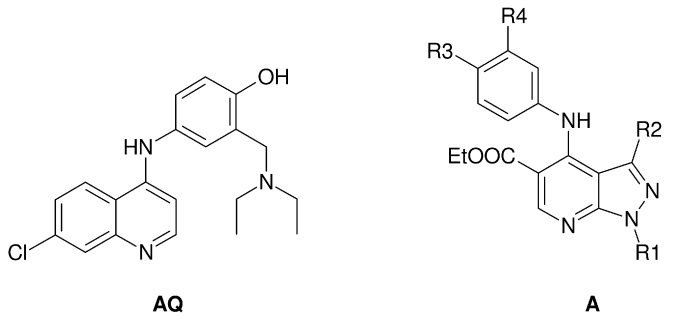


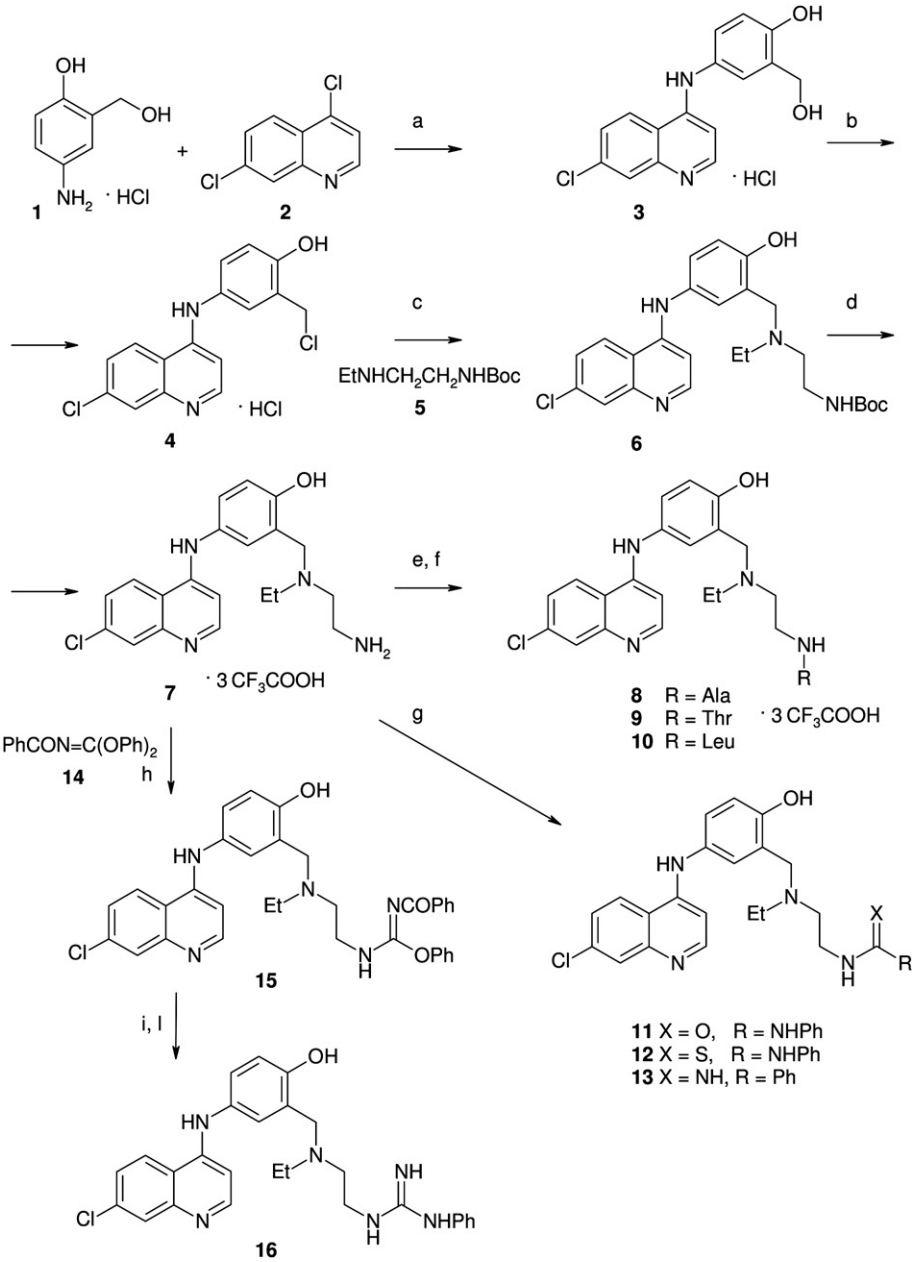
Chart 1.

chloroquine and pyrimethamine and against *Leishmania donovani* MHOM/ET/67/HU3, a reference strain causing visceral leishmaniasis.

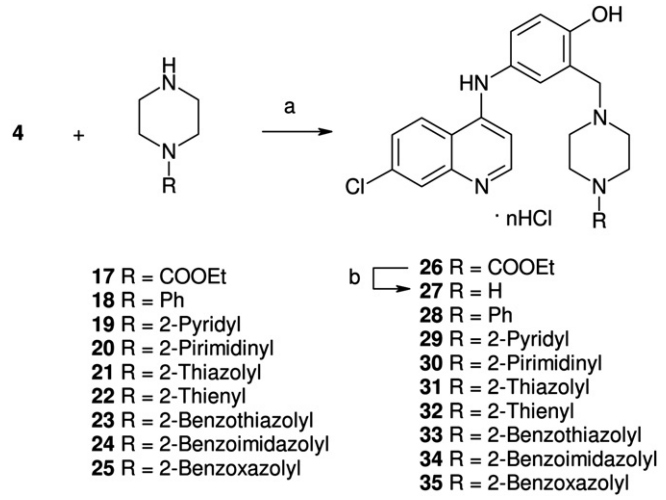
2. Results and discussion

2.1. Chemistry

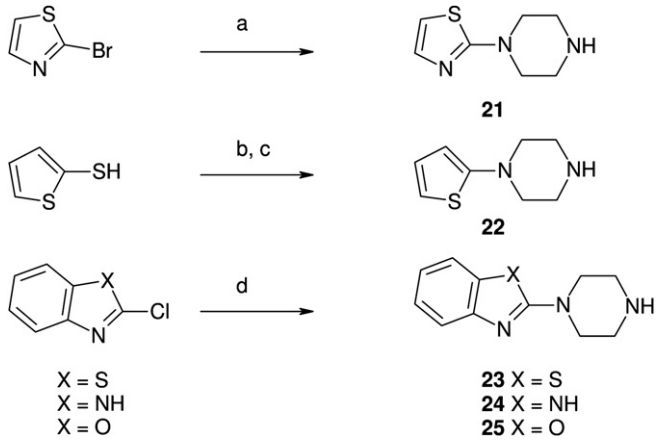
The products we designed can be grouped into two different classes on the basis of the synthetic pathway used for their preparation. The former class includes compounds **3**–**4**, **6**–**13**, **15**–**16**, (Scheme 1) the latter includes compounds **26**–**35** (Scheme 2). The synthesis of these products required the availability of the new chloromethyl substituted intermediate **4** that is a very flexible



Scheme 1. Preparation of key intermediate **4** and of final derivatives **3**, **6**–**13**, **15**–**16**. Reagents and conditions: (a) EtOH, reflux, 2.5 h; (b) conc. HCl, reflux, 18 h; (c) Et_3N, CH_3CN, RT, 18 h; (d) CF_3COOH, CH_2Cl_2, RT, 6 h; (e) DIPEA, HBTU, HOBt, N-Boc-AA(OH), RT, 16 h; (f) CF_3COOH, CH_2Cl_2, RT, 6 h; (g) Et_3N, PhNCO or PhNCS or PhC=NH(OCH_3), CH_3CN, RT or reflux; (h) Et_3N, **14**, CH_3CN, RT, 2 h; (i) $PhNH_2$, CH_3CN, reflux, 72 h; (l) 5 N HCl, reflux, 24 h.



Scheme 2. Preparation of final derivatives **26**–**35**. Reagents and conditions: (a) **17**–**25**, CH_3CN, RT, 18 h; (b) 5 N HCl, reflux, 18 h.



Scheme 3. Preparation of intermediates **21**–**25**. Reagents and conditions: (a) piperazine, n-BuOH, reflux 18 h; (b) **17**, toluene, reflux 1.5 h; (c) KOH, MeOH/H_2O, 80 °C, 12 h; (d) piperazine, iPrOH or n-BuOH, base, 0 °C-reflux.

scaffold to prepare analogues of AQ. To prepare **4**, 4-amino-2-(hydroxymethyl)phenol (**1**) was treated in refluxing ethanol with the chloro-substituted quinoline **2** to afford alcohol **3** in 95% yield. This intermediate was then refluxed in concentrated HCl for 18 h yielding desired **4** in high yield (91%). The intermediate **4** was treated with tert-butylcarbamate **5** to give the protected amine **6** that was easily hydrolised in CF_3COOH/CH_2Cl_2 to afford the amine intermediate **7**. O-Benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) mediated coupling of this compound with the appropriate N-Boc protected aminoacid in DMF in the presence of di-isopropylethylamine (DIPEA) yielded, after acid hydrolysis, the corresponding amino acid derivatives **8**–**10**. The target compounds **11**–**13** were obtained by reaction of **7** in basic medium (Et_3N) with phenylisocyanate, phenylisothiocyanate and benzenecarboxyimidoate respectively. Treatment of **7** with stoichiometric amount of diphenyl benzoylimidocarbonate (**14**) gave rise to **15** that was transformed into **16** by refluxing with aniline in CH_3CN and subsequent hydrolysis in boiling 5 N HCl.

The compounds **26**, **28**–**35**, bearing a piperazinyl moiety, were synthesised in good yields according to the procedure reported in Scheme 2. The intermediate **4** was treated with an excess of the appropriate piperazines **17**–**25** at room temperature in CH_3CN to afford the expected target compounds, which were characterised as hydrochloride salts with the exception of **32**, kept as free base. The monosubstituted piperazino-derivative **27** was obtained by acid hydrolysis of the protected precursor **26**.

The heteroaryl piperazines **21**, **23**–**25** were prepared by reaction of the suitable halo-substituted heterocycles with an excess of piperazine following, with marginal modifications, the procedures described in literature (see Section 4.1). The 1-thiophen-2-ylpiperazine (**22**) was synthesised by reaction of commercially available 2-mercaptothiophene with **17** followed by basic hydrolysis of the ethoxycarbonyl protection (Scheme 3).

2.2. Dissociation constants (pK_as)

Potentiometric titrations of the final target compounds were performed out with a Sirius GLpK_a automated potentiometric system. The titrations were carried out in water using methanol in different ratios as co-solvent. The aqueous pK_as were determined by extrapolation to 0% methanol according to Yasuda–Shedlovsky procedure. The pK_a values are listed in Table 1 and are referred to the basic ionisable centres of the products since the dissociation of the acid phenol OH is undetectable by the potentiometric method we used. Analysis of the data indicates that in many products there is not a well separated ionisation of the basic centres following the close pK_a values. However, using AQ as reference compound, we are induced to assign the pK_a values in the range 7.36–7.79 (pK_{a2}) to the prevalent ionisation of the 4-amino substituted quinoline centre. In the case of compounds **11**, **12** the pK_{a1} and pK_{a2} values are too close to do this attribution. At physiological pH the ionised form(s) are in equilibrium with the uncharged form. In the sole products **7**, **13**, **16** the percentage of the uncharged form is <1. At acid pH of the food vacuole (5.2) all the products exist largely as protonated forms and this should assure a good trapping of the products in parasite-infected erythrocytes, at least as far as the pH-driven mechanism is concerned [10].

2.3. Biological activities

A summary of the activity of the compounds is reported in Table 2.

2.3.1. Leishmania donovani

Generally speaking compounds **6**–**35** proved to be active against *L. donovani* intracellular amastigotes, their IC_{50} values lying in the 1.83–17.7 μM range. All of the synthesised compounds were less active than AQ (IC_{50} value of 1.4 μM). Compound **11**, with an IC_{50} value of 1.83 μM, showed the best activity comparable to that of the lead. The standard drug, sodium stibogluconate ($NaSb^V$) had an IC_{50} value of 188 μM Sb^V. However, at the higher concentrations used in

Table 1
Dissociations constants of the synthesised compounds and reference AQ.

No.	Dissociation constants[a]			No.	Dissociation constants[a]			No.	Dissociation constants[a]		
	pK_{a1}	pK_{a2}	pK_{a3}		pK_{a1}	pK_{a2}	pK_{a3}		pK_{a1}	pK_{a2}	pK_{a3}
AQ	8.50	7.07	–	**11**	7.65[b]	7.79[b]	–	**29**	6.49	7.59	5.54
3	–	7.71	–	**12**	7.53[b]	7.62[b]	–	**30**	6.38	7.60	2.83
4	–	7.78	–	**13**	5.85	7.62	10.0	**31**	5.56	7.52	2.86
6	7.30	7.57	–	**15**	6.66	7.62	–	**32**	6.42	7.58	2.66
7	5.87	7.55	9.05	**16**	5.94	7.61	10.1	**33**	6.11	7.45	5.09
8	6.25	7.59	7.99	**26**	6.08	7.40	–	**34**	6.72	7.62	5.12
9	5.98	7.36	8.05	**27**	3.87	7.50	8.88	**35**	6.25	7.60	5.14
10	6.11	7.42	8.05	**28**	6.34	7.68	2.86				

[a] Determined by potentiometry; S.D. ≤ 0.07.
[b] Uncertain attribution.

Table 2
Antiparasitic activity of synthesised compounds and AQ against *L. donovani*, *P. falciparum* 3D7 and K1 strains and cytotoxicity against KB cells.

No.	*L. donovani* MHOM/ET/67/HU3 IC_{50} (μM)[a]	*P. falciparum* 3D7 IC_{50} (nM)[a]	*P. falciparum* K1 IC_{50} (nM)[a]	*Toxicity* IC_{50} (μM)[b]
AQ	1.4	2.15	8.61	90.0
3	>89	712	1420	198
4	>83.5	695	557	25.6
6	8.77	2.12	2.12	8.81
7	5.65	5.47	5.47	25.6
8	5.15	12.2	14100	10.4
9	15.3	47.0	14100	54.4
10	4.69	22.6	14300	22.3
11	1.83	8.22	238	5.33
12	2.05	1.63	6.50	8.46
13	7.10	48.4	290	5.34
15	2.86	6.73	10.1	10.2
16	11.7	126	12100	9.03
26	14.9	5.28	7.04	11.4
27	2.29	18.8	225	11.8
28	9.68	3.52	1.80	8.63
29	6.71	0.323	1.40	16.1
30	7.21	3.48	10.4	8.03
31	17.7	0.670	0.420	5.02
32	9.14	2.22	Unable to fit curve	50.4
33	12.9	14.6	1480	45.2
34	1.87	3.08	1.54	2.44
35	14.6	1.68	387	8.02

[a] The IC_{50} represents the concentrations of the test compounds required to inhibit parasite growth by 50%; IC_{50} values are the mean of at least three determinations; standard errors were all within 10% of the mean; IC_{50} values of control drugs: Sb^V 188 μM; Chloroquine (3D7) 7.75 nM; Artesunate (K1) 2.46 nM.
[b] Toxicity determined on KB cells; The IC_{50} represents the concentrations of test compounds required to kill 50% of the test cells.

the assay, namely 30 and 10 μg/mL, toxicity to the host cell macrophage was seen for the majority of compounds as reflected in the cytotoxicity IC_{50} values. Sodium stibogluconate showed no toxicity at the top concentrations tested. The highest Therapeutic Index (TI), the IC_{50} cytotoxicity/IC_{50} antileishmanial activity, showed a narrow margin between in vitro efficacy and cytotoxicity (Table 2). At this stage the structural basis for selective toxicity against *L. donovani* amastigotes is difficult to determine.

2.3.2. *Plasmodium falciparum 3D7 and K1*

As expected compounds **3** and **4**, lacking the basic side chain, showed no activity against both *Plasmodium* strains. When the tertiary amine in benzylic position was re-introduced (compound **6**) the antiplasmodial activity was restored (IC_{50} 2.12 nM against both CQ-S and CQ-R strains). Addition of a primary amino group as the third basic centre (compound **7**) maintained the antiplasmodial activity with a slight decrease in the toxicity against KB cells. When the amino group was incorporated into an aminoacidic moiety (compounds **8**–**10**) or a stronger basic functionality was used (compounds **13**, **16**), the antiplasmodial activity against the CQ-S strain was lowered and that against CQ-R strain was completely abolished. The replacement of the third basic centre by non-basic bulky polar residues as in compounds **11**–**12**, **15** led to a drop in the antiplasmodial activity with the exception of derivative **12** which maintained the same activity with respect to the lead compound.

In the piperazine series the best results were obtained with 2-pyridyl, 2-thiazolyl, 2-benzoimidazolyl N^4-substituted piperazine derivatives **29**, **31** and **34** which showed IC_{50} values within the 0.32–3.08 nM range against CQ-S strain and 0.42–1.54 nM range against CQ-R strain, this might be attributable to an additional effect exerted by the heteroaromatic ring used. In general the compounds **4**, **6**, **7**, **12**, **15**, **26**, **28**, **29**, **30**, **31** and **34**, were equally potent against both *Plasmodium* strains, the remainder displaying strain sensitivity.

3. Conclusion

The introduction on AQ scaffold of lateral chains containing basic centres and/or hydrogen bonding moieties has produced derivatives that exhibit both antimalarial and antileishmanial actions. This has been reported previously for other quinoline-based compounds, for example chloroquine derivatives and propylquinoline derivatives [11]. A few compounds show highly potent antimalarial activity against the chloroquine resistant K1 strain. This activity is comparable to that showed by isoquine, an improved amodiaquine analogues recently reported [12]. To the best of our knowledge, this is the first report where the antileishmanial action of AQ and of some newly synthesised analogues against intracellular amastigote *L. donovani* is described. Although these compounds show a narrow therapeutic margin against *L. donovani*, there is chemical space to explore the design of more selective compounds. Further studies are necessary to address toxicity issues, in particular the synthesis of isoquine-like models with improved metabolic profile is being considered.

4. Experimental

4.1. Instrumentation and chemicals

All compounds were purified by recrystallisation before characterisation. Melting points were determined with a capillary apparatus Büchi B-540. Melting points with decomposition were determined after introduction of the sample at a temperature 10 °C lower than the melting point. A heating rate of 2 °C min^{-1} was used. Compounds **7**–**10** were highly hygroscopic amorphous foams, the determination of their melting point was affected by the complex thermal behaviour of these compounds; consequently the melting point was not reported. ^1H and ^{13}C NMR spectra were obtained on a Bruker Avance 300, at 300 and 75 MHz respectively; δ in ppm rel. to $SiMe_4$ as the internal standard; coupling constants J in Hz. ^{13}C NMR spectra were fully decoupled. The following abbreviations are used: s: singlet, d: doublet, dd: doublet doublet, t: triplet, qt: quartet, m: multiplet, br: broad, AQ: amodiaquine structure, pip: piperazine, pyr: pyridine, pyrim: pyrimidine, Tz: thiazole, Tph: thiophene, Bzt: benzothiazole, Bzm: benzoimidazole, Bzo: benzooxazole. Mass spectra were recorded on a Finnigan-Mat TSQ-700. Flash chromatography (FC) was performed on BDH silica gel (particle size 40–63 μm). HPLC measurements were carried out on a Varian Pro-Star 210 chromatograph equipped with a variable wavelength detector (iPro-Star 325). The chromatography was performed using a 5 μm particle size prepacked column Licrospher 250–25 C_{18} with a flow rate of 39 mL/min, λ 254 nm. When not otherwise specified, anhydrous magnesium sulphate ($MgSO_4$) was used as the drying agent of organic phases. Analysis (C, H, N) of the new compounds was performed by REDOX (Monza); the analytical results were within ±0.4% of the theoretical value. Structures **1** [13], **5** [14], **14** [15], **21** [16], **23** [17], **24** [18], **25** [19] were synthesised according to reported methods.

4.2. Chemistry

4.2.1. *4-[(7-Chloroquinolin-4-yl)amino]-2-(hydroxymethyl)phenol hydrochloride (**3**)*

To a solution of 4-amino-2-(hydroxymethyl)phenol hydrochloride **1** (11.4 g; 64.9 mmol) in EtOH (540 mL) 4,7-dichloroquinoline **2** (12.8 g; 64.9 mmol) was added and the mixture was refluxed for 2.5 h. The reaction was cooled and the solid collected on a buchner

funnel and dried in a dessiccator (over P_2O_5) to give the desired product (20.8 g; 95%) as yellow solid. An analytical sample was obtained by recrystallisation from MeOH/H_2O. Mp: 323 °C (dec.) ^{1}H NMR (DMSO-d_6): δ, 11.07 (s, 1H, exch. signal); 10.12 (s, 1H, exch. signal); 8.83 (d, 1H, $J = 9$ Hz, AQ-H_5); 8.46 (d, 1H, $J = 6.9$ Hz, AQ-H_2); 8.17 (s, 1H, AQ-H_8); 7.84 (d, 1H, $J = 9$ Hz, AQ-H_6), 7.35 (s, 1H, AQ-$H_{3'}$); 7.14 (d, 1H, $J = 8.4$ Hz, AQ-$H_{5'}$); 6.99 (d, 1H, $J = 8.4$ Hz, AQ-$H_{6'}$); 6.65 (d, 1H, $J = 6.9$ Hz, AQ-H_3); 5.21 (br s, 1H, exch. signal); 4.53 (s, 2H, CH_2OH). ^{13}C NMR (DMSO-d_6): δ, 155.3; 153.6; 143.1; 139.2; 138.3; 130.6; 127.7; 127.2; 126.1; 124.7; 124.3; 119.3; 115.7; 115.6; 100.0; 57.9. Anal. Calc. For $C_{16}H_{13}ClN_2O_2 \cdot HCl$ C% 56.99, H% 4.18, N% 8.31; found C% 56.85, H% 4.23, N% 8.36.

4.2.2. 2-Chloromethyl-4-[(7-chloroquinolin-4-yl)amino]phenol hydrochloride (**4**)

Derivative **3** (7.5 g; 22.2 mmol) was suspended in conc. HCl (400 mL) and the mixture was refluxed for 18 h. The solvent was evaporated under reduced pressure and the residue was triturated with Et_2O and filtered to give the desired product (7.2 g; 91%) as yellow solid. The product was used without further purification. Mp: 239.3–241.1 °C (dec.) ^{1}H NMR (DMSO-d_6): δ, 11.12 (s, 1H, exch. signal), 10.56 (s, 1H, exch. signal); 8.87 (d, 1H, $J = 9$ Hz, AQ-H_5); 8.50 (d, 1H, $J = 6.9$ Hz, AQ-H_2); 8.20 (s, 1H, AQ-H_8); 7.85 (d, 1H, $J = 9$ Hz, AQ-H_6); 7.36 (s, 1H, AQ-$H_{3'}$); 7.13 (d, 1H, $J = 8.5$ Hz, AQ-$H_{5'}$); 7.01 (d, 1H, $J = 8.5$ Hz, AQ-$H_{6'}$); 6.64 (d, 1H, $J = 6.9$ Hz, AQ-H_3); 4.77 (s, 2H, CH_2); 3.62 (br s, 1H, exch. signal). ^{13}C NMR (DMSO-d_6): δ, 156.2; 156.1; 143.9; 139.8; 139.1; 129.0; 128.5; 128.4; 128.1; 126.9; 125.9; 119.9; 117.5; 116.5; 100.8; 42.3. Anal. Calc. For $C_{16}H_{12}Cl_2N_2O \cdot HCl \cdot 0.2\ H_2O$ C% 53.49, H% 3.77, N% 7.80; found C% 53.42, H% 3.71, N% 7.44.

4.2.3. (2-{[5-(7-Chloroquinolin-4-ylamino)2-hydroxybenzyl]-ethylamino}ethyl)carbamic acid, tert-butyl ester (**6**)

To a stirred suspension of **4** (1.25 g; 2.81 mmol) in CH_3CN (10 mL) triethylamine (1.56 mL; 1.12 mmol) was added. After 10 min (2-ethylamino-ethyl)carbamic acid tert-butyl ester (**5**) (1.59 g; 8.43 mmol) was added and the reaction mixture was stirred at room temperature (r.t.) for 18 h. The solvent was removed under reduced pressure and the residue taken up with water (20 mL) and extracted with EtOAc (3 × 25 mL). The organic phase was dried (Na_2SO_4), the solvent removed under reduced pressure to leave a dark oil. The crude product was purified by flash chromatography eluting with CH_2Cl_2/MeOH 5% to obtain **7** (0.64 g; 48) as a light brown oil that became solid on standing. The product was recrystallized from iPr_2O/EtOAc to obtain a white amorphous solid. Mp: 161.4–161.8 °C (dec). ^{1}H NMR (DMSO-d_6): δ, 10.73 (s, 1H, exch. signal), 8.91 (s, 1H, exch. signal); 8.43 (d, 1H, $J = 9$ Hz, AQ-H_5); 8.38 (d, 1H, $J = 5.4$ Hz, AQ-H_2); 7.86 (d, 1H, $J = 2$ Hz, AQ-H_8); 7.53 (dd, 1H, $J = 9$, 2 Hz, AQ-H_6); 7.11 (m, 2H, AQ-$H_{3'}$, AQ-$H_{5'}$); 6.81 (m, 2H, NHBoc, AQ-$H_{6'}$); 6.60 (d, 1H, $J = 5.4$ Hz, AQ-H_3); 3.75 (s, 2H, CH_2Ph); 3.09 (m, 2H, CH_2NH); 2.60 (m, 4H, CH_2NCH_2); 1.37 (s, 9H, t-Bu); 1.04 (t, 3H, $J = 6.9$ Hz, CH_3). ^{13}C NMR (DMSO-d_6): δ, 155.5; 154.5; 152.7; 151.8; 149.4; 133.6; 130.4; 127.5; 125.4, 124.5; 124.4; 124.2; 124.1; 117.6; 115.9; 100.4; 77.5; 54.7; 51.9; 46.4; 37.3; 28.1; 10.8. MS (CI/isobutane) *m/z*: 471/473 [MH^+]. Anal. Calc. For $C_{25}H_{31}ClN_4O_3$ C% 63.75, H% 6.63, N% 11.90; found C% 63.60, H% 6.62, N% 12.01.

4.2.4. 2-{[(2-Aminoethyl)(ethyl)amino]methyl}-4-[(7-chloroquinolin-4-yl)amino]phenol trifluoroacetate (**7**)

To a stirred solution of **6** (1.00 g; 2.12 mmol) in CH_2Cl_2 (10 mL), CF_3COOH 2.38 mL was added, the mixture was stirred at r.t. for 6 h, then the solvent was evaporated under reduced pressure. The obtained residue was triturated with dry Et_2O (2 x 25 mL) at −15 °C until it was solid enough to be filtered and immediately stored in a dessiccator over P_2O_5/paraffin under vacuum. With this procedure **7** (1.48 g; 98%) was obtained as a white amorphous solid which dissolves if left in open air. ^{1}H NMR (DMSO-d_6): δ, 10.95 (s, 1H,exch. sign.); 8.85 (d, 1H, $J = 9.1$ Hz, AQ-H_5); 8.52 (d, 1H, $J = 7.0$ Hz, AQ-H_2); 8.25 (s, br, 2H, exch. sign.); 8.10 (d, 1H, $J = 2.0$ Hz, AQ-H_8); 7.89 (dd, 1H, $J_1 = 9.1$ Hz, $J_2 = 2.0$ Hz, AQ-H_6); 7.52 (d, 1H, $J = 2.5$, AQ-$H_{3'}$); 7.39 (dd, 1H, $J_1 = 8.6$ Hz, $J_2 = 2.5$ Hz AQ-$H_{5'}$); 7.18 (d, 1H, $J = 8.6$ Hz, AQ-$H_{6'}$); 6.72 (d, 1H, $J = 7.0$ Hz, AQ-$H_{3'}$); 4.21 (s, 2H, PhCH_2N); 3.28 (m, 6H, $J = 5.8$ Hz, $CH_2NCH_2CH_2$N); 1.27 (t, 3H, $J = 6.8$ Hz, CH_3). ^{13}C NMR (DMSO-d_6): δ, 156.3; 155.3; 143.4; 139.0; 138.5; 129.9; 128.8; 127.9; 127.4; 125.6; 122.5; 119.34; 118.6; 117.6; 116.9; 115.6; 114.6; 110.7; 100.2; 51.0; 47.9; 47.7; 33.4; 8.4. Anal. Calc. For $C_{20}H_{23}ClN_4O \cdot 3\ CF_3COOH \cdot H_2O$ C% 42.72, H% 3.86, N% 7.66; found C% 42.51, H% 3.81, N% 7.40.

4.2.5. General procedure for the synthesis of derivatives **8**–**10**

To a solution of **7** (0.4 g; 0.85 mmol) in DMF (10 mL), DIPEA (0.74 mL; 4.24 mmol), HBTU (0.64 g; 1.70 mmol), HOBt (0.23 g; 1.70 mmol) and the appropriate Boc-protected aminoacid (1.70 mmol) were added. After 16 h the reaction mixture was evaporated, the residue was dissolved in CH_2Cl_2 (25 mL) and washed with 10% $NaHCO_3$ (3 × 10 mL), brine (10 mL), dried and evaporated to yield a yellow oil. The oil was purified by flash chromatography eluting with EtOAc to obtain a yellow solid (94–96% yield). The product was dissolved in 30% CF_3COOH in CH_2Cl_2 (15 mL); after 6 h under stirring the mixture was evaporated and the residue was recrystallised from MeOH/Et_2O, then purified by RP18-HPLC (eluent: H_2O/gradient from 5 to 40% of CH_3CN).

4.2.5.1. N^1-{2-[{5-[(7-Chloroquinolin-4-yl)amino]-2-hydroxybenzyl}-(ethyl)amino]ethyl} alaninamide (**8**).

After evaporation and freeze-drying the product was obtained as a yellow solid. ^{1}H NMR (DMSO-d_6): δ, 10.89 (s, 1H), 9.72 (s br, 1H), 8.80 (m, 1H, AQ-H_5), 8.71 (d, 1H, $J = 9.2$ Hz, CONH), 8.53 (d, 1H, $J = 7.0$ Hz, AQ-H_2), 8.19 (s br, 2H), 8.08 (d, 1H, $J = 1.9$ Hz, AQ-H_8), 7.89 (dd, 1H, $J = 9.0$ Hz, 1.9 Hz, AQ-H_6), 7.51 (d, 1H, $J = 2.2$ Hz, AQ-$H_{3'}$), 7.39 (dd, 1H, $J = 8.6$ Hz, 2.2 Hz, AQ-$H_{5'}$), 7.16 (d, 1H, $J = 8.6$ Hz, AQ-$H_{6'}$), 6.71 (d, 1H, $J = 7.0$ Hz, AQ-H_3), 4.33 (s, 2H, PhCH_2N), 3.85 (m, 1H, CHAla), 3.57 (m, 2H, NHCH_2CH_2), 3.17 (s br, 4H, CH_2NCH_2), 1.35–1.26 (m, 6H, 2 CH_3). ^{13}C NMR (DMSO-d_6): δ, 170.2, 156.3, 155.3, 143.8, 139.4, 138.5, 129.8, 128.8, 128.2, 127.5, 125.7, 119.7, 118.0, 117.0, 115.8, 100.3, 53.6, 51.2, 50.4, 47.6, 34.0, 17.0, 8.6. Anal. Calc. For $C_{23}H_{28}ClN_5O_2 \cdot 3\ CF_3COOH \cdot 2\ H_2O$ C% 42.47, H% 4.30, N% 8.54; found C% 42.72, H% 4.12, N% 8.64.

4.2.5.2. N^1-{2-[{5-[(7-Chloroquinolin-4-yl)amino]-2-hydroxybenzyl}-(ethyl)amino]ethyl} threoninamide (**9**).

After evaporation and freeze-drying the product was obtained as a yellow solid. ^{1}H NMR (DMSO-d_6): δ, 10.97 (s, 1H), 9.83 (s br, 1H), 9.02 (m, 1H, AQ-H_5), 8.73 (d, 1H, $J = 9.1$ Hz, CONH), 8.53 (d, 1H, $J = 7.0$ Hz, AQ-H_2), 8.22 (s br, 3H), 8.11 (d,1H, $J = 1.3$ Hz, AQ-H_8), 7.88 (d, 1H, $J = 8.9$ Hz, AQ-H_6), 7.54 (s, 1H, AQ-$H_{3'}$), 7.40 (dd, 1H, $J = 8.6$ Hz, 1.9 Hz, AQ-$H_{5'}$), 7.19 (d, 1H, $J = 8.6$ Hz, AQ-$H_{6'}$), 6.72 (d, 1H, $J = 7.0$ Hz, AQ-H_3), 4.37 (s, 2H, PhCH_2N), 3.76 (s br, 1H, CHNH$_2$), 3.60–3.58 (m, 2H, AQ-CH_2), 3.23–3.21 (m, 4H, 2 CH_2), 1.67–1.55 (m, 3H, $CH_2 + CH$-Leu) 1.31 (t, 3H, $J = 6.9$ Hz CH_3CH_2), 0.88 (d, 6H, $J = 3.1$ Hz, 2 CH_3 Leu); ^{13}C NMR (DMSO-d_6): δ, 167.61, 156.2, 155.2, 143.5, 139.1, 138.4, 129.8, 128.7, 127.9, 127.3, 125.5, 119.4, 117.8, 116.9, 115.6, 100.2, 65.4, 58.1, 50.8, 50.0, 47.5, 33.8, 19.7, 8.4. Anal. Calc. For $C_{24}H_{30}ClN_5O_3 \cdot 3\ CF_3COOH \cdot 2\ H_2O$ C% 42.39, H% 4.39, N% 8.24; found C% 42.49, H% 4.10, N% 8.35.

4.2.5.3. N^1-{2-[{5-[(7-Chloroquinolin-4-yl)amino]-2-hydroxybenzyl}-(ethyl)amino]ethyl} leucinamide (**10**).

After evaporation and freeze-drying the product was obtained as a yellow solid. ^{1}H NMR (DMSO-d_6): δ, 10.97 (s, 1H), 9.83 (s, 1H), 9.02 (m, 1H), 8.73 (d, 1H, $J = 9.1$ Hz), 8.53 (d, 1H, $J = 7.0$ Hz), 8.28 (br s, 2H), 8.11 (d, 1H, $J = 1.3$ Hz), 7.88 (d, 1H, $J = 8.9$ Hz), 7.54 (s, 1H), 7.40 (dd, 1H,

$J_1 = 8.6$ Hz, $J_2 = 1.9$ Hz), 7.19 (d, 1H, $J = 8.6$ Hz), 6.72 (d, 1H, $J = 7.0$ Hz), 4.37 (s, 2H), 3.76 (br s, 1H), 3.60–3.58 (m, 2H), 3.23–3.21 (m, 4H), 1.67–1.55 (m, 3H), 1.31 (t, 3H, $J = 6.9$ Hz), 0.88 (d, 3H, $J = 3.1$ Hz); ^{13}C NMR (DMSO-d_6): δ, 169.7, 156.3, 155.3, 143.4, 139.0, 138.4, 129.9, 128.8, 127.9, 127.4, 125.6, 122.6, 119.3, 118.6, 117.8, 116.9, 115.6, 114.7, 110.8, 100.2, 50.9, 49.9, 47.5, 33.7, 23.5, 22.4, 21.7, 8.4. Anal. Calc. For $C_{26}H_{34}ClN_5O_2 \cdot 3.2\ CF_3COOH \cdot 2\ H_2O$ C% 43.98, H% 4.69, N% 7.91; found C% 43.95, H% 4.37, N% 7.80.

4.2.6. *N-[2-({[5-(7-Chloroquinolin-4-yl)amino]-2-hydroxybenzyl}-ethylamino)ethyl]-N′-phenyl-urea dihydrochloride (**11**)*

To a solution of **7** (0.40 g; 0.56 mmol) and Et_3N (0.23 mL; 1.68 mmol) in CH_3CN (10 ml), a solution of phenylisocyanate (0.07 g; 1.68 mmol) in CH_3CN (5 ml) was added dropwise under stirring. After the addition was over the mixture was stirred at r.t. for 24 h. The solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography eluting with CH_2Cl_2/MeOH 5% affording a pale yellow oil. The pure product (0.28 g; 84%) was dissolved in MeOH and converted into the corresponding hydrochloride by treatment with HCl–saturated Et_2O. The solvent was decanted off and the yellow semisolid material was recrystallized from dry MeOH/Et_2O. The yellow solid thus obtained was dissolved in water and freeze-dried to afford the desired product. Mp: 183.8–184.1 °C. ^{1}H NMR (DMSO-d_6): δ, 11.09 (s, 1H, exch. sign.); 11.00 (s, br, 1H, exch. sign.); 10.11 (s, br, 1H, exch. sign.); 9.22 (s, 1H, exch. sign); 8.85 (d, 1H, $J = 9.1$ Hz, AQ-H_5); 8.50 (d, 1H, $J = 7.0$ Hz, AQ-H_2); 8.13 (d, 1H, $J = 2.0$ Hz, AQ-H_8); 7.84 (dd, 1H, $J_1 = 9.1$ Hz, $J_2 = 2.0$ Hz, AQ-H_6); 7.65 (d, 1H, $J = 2.5$, AQ-$H_{3'}$); 7.35–7.11 (m, 6H, 5Ph*H*, AQ-$H_{5'}$); 6.81–6.76 (m, 2H, CH_2*NH*CO, AQ-$H_{6'}$); 6.75 (d, 2H, $J = 7.0$ Hz AQ-H_3); 4.35 (s, 2H, Ph*C*H_2N); 3.52 (d, 2H, $J = 5.8$ Hz, *C*H_2NH); 3.29–3.18 (m, 4H, *C*H_2N*C*H_2); 1.36 (t, 3H, $J = 7.1$ Hz, *C*H_3). ^{13}C NMR (DMSO-d_6): δ, 169.2; 156.0; 155.9; 155.0; 143.1; 140.0; 138.9; 138.1; 130.0; 128.6; 128.3; 127.9; 127.1; 125.9; 121.0; 119.1; 117.6; 116.8; 115.5; 100.2; 51.7; 50.4; 47.9; 34.4; 8.6. Anal. Calc. For $C_{27}H_{28}ClN_5O_2 \cdot$ 2 $HCl \cdot 2.5\ H_2O$ C% 53.34, H% 5.80, N% 11.52; found C% 53.18, H% 5.54, N% 11.66.

4.2.7. *N-[2-({[5-(7-Chloroquinolin-4-yl)amino]-2-hydroxybenzyl}-ethylamino)ethyl]-N′-phenyl-thiourea dihydrochloride (**12**)*

To a solution of **7** (0.40 g; 0.56 mmol) and Et_3N (0.23 mL; 1.68 mmol) in CH_3CN (15 ml), a solution of phenylisothiocyanate (0.08 g; 0.56 mmol) in CH_3CN (5 ml) was added dropwise under stirring. After the addition was over the mixture was stirred at r.t. for 24 h. The solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography eluting with CH_2Cl_2/MeOH 5% affording a pale yellow oil. The pure product (0.31 g, 91%) was dissolved in MeOH and converted into the corresponding hydrochloride by treatment with HCl–saturated Et_2O. The solvent was decanted off and the yellow semisolid material was recrystallized from dry MeOH/Et_2O. The yellow solid thus obtained was dissolved in water and freeze-dried to afford the desired product. Mp: 167.4–167.7 °C. ^{1}H NMR (DMSO-d_6): δ, 11.18 (s, 1H, exch. sign.); 10.96 (s, br, 1H, exch. sign.); 10.27 (s, br, 2H, exch. sign.); 8.91 (d, 1H, $J = 9.1$ Hz, AQ-H_5); 8.47 (d, 1H, $J = 7.1$ Hz, AQ-H_2); 8.39 (m, 1H, *NH*CS); 8.18 (*d*, 1H, $J = 2.0$ Hz, AQ-H_8); 7.84 (dd, 1H, $J_1 = 9.1$ Hz, $J_2 = 2.0$ Hz, AQ-H_6); 7.70 (*d*, 1H, $J = 2.3$ Hz, AQ-$H_{3'}$); 7.42–7.20 (m, 6H, 4Ph*H*, AQ-$H_{5'}$, AQ-$H_{6'}$); 7.08 (m, 1H, PhH_p); 6.83 (d, 1H, $J = 7.1$ Hz, AQ-H_3); 4.35 (s, 2H, Ph*C*H_2N); 3.96 (d, 2H, $J = 5.0$ Hz, *C*H_2NH); 3.27 (m, 4H, *C*H_2N*C*H_2); 1.37 (t, 3H, $J = 7.0$ Hz, *C*H_3). ^{13}C NMR (DMSO-d_6): δ, 180.9; 156.3; 155.1; 143.2; 139.1; 139.0; 138.4; 130.3; 128.8; 128.6; 128.0; 127.4; 126.2; 124.4; 123.2; 119.2; 117.6; 117.0; 115.8; 100.6; 51.7; 50.0; 48.0; 38.6; 8.7. Anal. Calc. For $C_{27}H_{28}ClN_5OS \cdot 2\ HCl \cdot 2\ H_2O$ C% 52.73, H% 5.57, N% 11.39; found C% 52.62, H% 5.22, N% 11.49.

4.2.8. *N-{2-[{5-[(7-Chloroquinolin-4-yl)amino]-2-hydroxybenzyl}-ethylamino}ethyl]-benzencarboximidamide trihydrochloride (**13**)*

To a solution of **7** (0.40 g; 0.56 mmol) and Et_3N (0.23 mL; 1.68 mmol) in CH_3CN (10 mL), a solution of methyl-benzenecarboximidoate [20] (0.08 g; 0.56 mmol) in CH_3CN (5 mL) was added dropwise under stirring. After the addition was over the mixture was stirred under reflux for 72 h. After the mixture reached r.t. the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography eluting with CH_2Cl_2/MeOH 5% affording a pale yellow oil. The pure product (0.28 g, 80%) was dissolved in MeOH and converted into the corresponding hydrochloride by treatment with HCl–saturated Et_2O. The solvent was decanted off and the yellow semisolid material was recrystallized from dry MeOH/Et_2O. The yellow solid thus obtained was dissolved in water and freeze-dried to afford the desired product. Mp: 201.3 °C (dec.)^{1}H NMR (CD_3OD): δ, 8.64 (d, 1H, $J = 9.1$ Hz, AQ-H_5); 8.36 (d, 1H, $J = 7.1$ Hz, AQ-H_2); 7.96 (d, 1H, $J = 2.0$ Hz, AQ-H_8); 7.84–7.57 (*m*, 7H, 5Ph*H*, AQ-$H_{3'}$, AQ-H_6); 7.44 (dd, 1H, $J_1 = 8.7$ Hz, $J_2 = 2.3$ Hz, AQ-$H_{5'}$); 7.14 (d, 1H, $J = 8.7$ Hz, AQ-$H_{6'}$); 6.93 (d, 1H, $J = 7.1$ Hz, AQ-H_3); 4.58 (*s*, 2H, Ph*C*H_2); 4.10 (*m*, 2H, *C*H_2NH); 3.71 (*m*, 2H, *C*H_2N); 3.30 (*m*, 2H, N*C*H_2CH$_3$); 1.52 (*t*, 3H, $J = 7.11$ Hz, *C*H_3). ^{13}C NMR (CD_3OD): δ, 166.5; 157.8; 157.3; 144.2; 141.4; 140.4; 135.1; 131.6; 130.6; 130.5; 130.0; 129.3: 129.1; 126.5; 120.4; 118.7; 118.2; 117.2; 101.9; 52.9; 51.1; 50.4; 39.4; 25.3; 9.3. Anal. Calc. For $C_{27}H_{28}ClN_5O \cdot 3\ HCl \cdot 2\ H_2O$ C% 52.36, H% 5.70, N% 11.31; found C% 52.41, H% 5.41, N% 11.05.

4.2.9. *N′-benzoyl-N-{2-[({5-[(7-Chloroquinolin-4-yl)amino]-2-hydroxybenzyl}ethylamino]ethyl)carbamimidic acid, phenyl ester (**15**)*

To a solution of **7** (0.40 g; 0.56 mmol) and Et_3N (0.23 mL; 1.68 mmol) in CH_3CN (15 ml), a solution of **14** (0.18 g; 0.56 mmol) in CH_3CN (5 ml) was added dropwise under stirring. After the addition was over the mixture was stirred at r.t. for 2 h. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography eluting with CH_2Cl_2/MeOH 5% affording a pale yellow oil. The pure product (0.27 g, 80%) was recrystallized from dichloromethane/n-hexane to afford a yellow solid. Mp: 144.5–144.9 °C. ^{1}H NMR ($CDCl_3$): δ, 10.21 (*t*, 1H, exch. sign.) 8.43 (d, 1H, $J = 5.4$ Hz, AQ-H_2); 7.97 (d, 1H, $J = 1.5$ Hz, AQ-H_8); 7.89(*m*, 2H, COPhH_o); 7.78 (d, 1H, $J = 8.9$ Hz, AQ-H_5); 7.44–7.11 (*m*, 10H, 7Ph*H*, AQ-H_6, AQ-$H_{5'}$, *NH*CON); 6.94 (*s*, 1H, AQ-$H_{3'}$); 6.84 (d, 1H, $J = 8.5$ Hz, AQ-$H_{6'}$); 6.77 (*s*, 1H, OPhH_p); 6.61 (d, 1H, $J = 5.4$ Hz, AQ-H_3); 3.85 (*s*, 2H, Ph*C*H_2); 3.68 (*m*, 2H, *C*H_2CH$_3$); 2.89 (*t*, 2H, $J = 6.6$ Hz, N*C*H_2); 2.77 (*m*, 2H, HN*C*H_2); 1.82 (*t*, 3H, $J = 7.1$ Hz, *C*H_3). ^{13}C NMR ($CDCl_3$): δ, 177.9; 162.6; 155.9; 151.8; 151.6, 149.4; 149.2; 137.0; 135.1; 131.9; 130.4; 129.3; 129.1; 128.7; 127.9; 125.8; 125.7; 125.6; 125.4; 122.9; 122.0; 121.3; 117.5; 117.4; 101.3; 57.4; 52.1; 47.9; 38.9; 11.4. Anal. Calc. For $C_{34}H_{32}ClN_5O_3$ C% 68.74, H% 5.43, N% 11.79; found C% 68.35, H% 5.55, N% 11.61.

4.2.10. *N-[2-({5-[(7-Chloroquinolin-4-yl)amino]-2-hydroxybenzyl}ethylamino)ethyl]-N′-phenylguanidine trihydrochloride (**16**)*

To a solution of **15** (0.89 g, 1.5 mmol) in CH_3CN (15 ml) aniline (1.4 g; 15 mmol) was added under stirring. The mixture was refluxed for 72 h and evaporated under reduced pressure. The residue was taken up with water (100 ml) and extracted with EtOAc (3 × 30 ml). The organic phase was washed with brine (100 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to leave a dark-yellow semisolid material. The crude product was partially purified by flash chromatography eluting with CH_2Cl_2/MeOH 5% and immediately dissolved in 5 N HCl (30 ml) and refluxed for 24 h. After cooling the mixture was evaporated under reduced pressure to leave a yellow oil which

was purified by flash chromatography with gradient elution CH_2Cl_2 with 10 to 30% NH_3-saturated MeOH. The pure product (0.40 g, 55%) was dissolved in MeOH/conc. HCl 1/1 (20 ml) and the mixture was stirred at r.t. for 3 h. The solvent was evaporated under reduced pressure and the yellow semisolid material was recrystallized from iPrOH/iPr_2O. The yellow solid obtained was freeze-dried to afford the desired product. Mp: 180.4–180.7 °C (dec.). ^{1}H NMR (CD_3OD): δ, 8.30 (d, 1H, $J = 9.4$ Hz, AQ-H_5); 8.26 (d, 1H, $J = 5.5$ Hz, AQ-H_2); 7.79 (d, 1H, $J = 1.8$ Hz, AQ-H_8); 7.44–7.12 (*m*, 8H, 5Ph*H*, AQ-$H_{3'}$, AQ-H_6, AQ-$H_{5'}$); 6.87 (d, 1H, $J = 9.6$ Hz, AQ-$H_{6'}$); 6.58 (d, 1H, $J = 5.5$ Hz, AQ-H_3); 3.75 (*s*, 2H, PhCH_2); 3.47 (*m*, 2H, CH_2CH_2NH); 2.79 (*m*, 2H, NCH_2CH_2); 2.69 (*m*, 2H, CH_2CH_3); 1.14 (*t*, 3H, $J = 7.0$ Hz, CH_3). ^{13}C NMR (CD_3OD): δ, 156.4; 155.2; 151.5; 151.1; 148.7; 135.8; 135.5; 131.1; 130.0; 127.4, 127.3; 126.4; 125.9; 125.6; 125.3; 124.7; 124.0; 118.0; 116.6; 100.7; 54.7; 49.0; 48.0; 40.4; 10.7. Anal. Calc. For $C_{27}H_{29}ClN_6O \cdot 3\ HCl \cdot 2.5\ H_2O$ C% 50.33, H% 5.80, N% 13.04; found C% 50.01, H% 5.45, N% 12.60.

4.2.11. 1-(Thiophen-2-yl)piperazine (**22**)

To a stirred solution of 2-mercaptothiophene (2.0 g; 17.5 mmol) in toluene (14 mL) ethoxycarbonyl piperazine (2.7 g; 17.5 mmol) was added and the mixture was refluxed for 1.5 h. The reaction mixture was purified by flash chromatography eluting with PE/CH_2Cl_2 50% then CH_2Cl_2 100% to yield 2.7 g of a yellow oil. The obtained product was dissolved in MeOH/H_2O 4/1 (110 mL) and KOH (7.45 g; 133 mmol) was slowly added under stirring at r.t. After the addition was over the mixture was heated at 80 °C for 12 h. The solvent was evaporated under reduced pressure and the residue taken up with water (30 mL) and extracted with CH_2Cl_2 (3×25 mL). The organic phase was dried and evaporated under reduced pressure to leave an oil which was purified by flash chromatography eluting with CH_2Cl_2/MeOH 10% to give 1.45 g (49%) of a colorless oil, pure enough to be used in the next step. ^{1}H NMR (DMSO-d_6): δ, 6.77–6.74 (*m*, 1H, H_4); 6.69 (*m*, 1H, H_5); 6.12 (*m*, 1H, H_3); 2.96 (*m*, 4H, 2CH_2-pip); 2.82 (*m*, 4H, 2CH_2-pip). ^{13}C NMR (DMSO-d_6): δ, 139.7; 126.3; 111.6; 104.5; 52.1; 45.1. MS (CI/isobutane) (*m*/*z*): 169 [MH^+]. Spectral data are in keeping with those of an authentic sample [21].

4.2.12. 4-{5-[(7-Chloroquinolin-4-yl)amino]-2-hydroxybenzyl}-piperazine-1-carboxylic acid, ethyl ester dihydrochloride (**26**)

To a stirred solution of **4** (1.2 g; 3.38 mmol) in CH_3CN (30 mL), 1-ethoxycarbonylpiperazine **17** (2.2 mL; 15 mmol) was added and the mixture was stirred at r.t. for 3 h. The solvent was evaporated under reduced pressure, the residue treated with water (40 mL) and stirred for 20 min until a pale yellow precipitate was formed. The solid, consisting of crude product, was collected, washed with water, dried and then purified by flash chromatography eluting with CH_2Cl_2/MeOH 5% to yield the desired product as a yellow solid (1.27 g; 86%). The product was converted into the corresponding hydrochloride by treatment with HCl–saturated MeOH, recrystallized from MeOH/Et_2O and freeze-dried to afford a yellow solid. Mp: 242.4–244.7 °C. ^{1}H NMR (DMSO-d_6): δ, 11.21 (*s*, br, 1H, AQ-*OH*); 8.95 (d, 1H, $J = 9.0$ Hz, AQ-H_5); 8.48 (d, 1H, $J = 7.0$ Hz. AQ-H_2); 8.20 (d, 1H, $J = 2.0$ Hz, AQ-H_8); 7.85 (dd, 1H, $J = 2.0$, 9.0 Hz, AQ-H_6); 7.70 (d, 1H, $J = 2.4$ Hz, AQ-$H_{3'}$); 7.40 (dd, 1H, $J = 2.5$, 8.7 Hz, AQ-$H_{5'}$); 7.23 (d, 1H, $J = 8.7$ Hz, AQ-$H_{6'}$); 7.01 (d, 1H, $J = 7.0$ Hz, AQ-H_3); 4.31 (*s*, 2H, CH_2-Ph); 4.10 (*q*, 2H, $J = 7.0$ Hz, O–CH_2); 3.40 (*s*, 8H, 4CH_2-Pip); 1.21 (*t*, 3H, $J = 7.0$ Hz, CH_3). ^{13}C NMR (DMSO-d_6): δ, 156.3; 154.9; 154.4; 143.2; 139.0; 138.4; 130.5; 128.6; 127.9; 127.3; 119.2; 116.9; 116.8; 115.7; 100.7; 66.7; 61.4; 53.3; 50.3; 14.6. MS (CI/isobutane) (*m*/*z*): 441/443 [MH^+]. Anal. Calc. For $C_{23}H_{25}ClN_4O_3 \cdot 2\ HCl \cdot 3\ H_2O$ C% 48.64, H% 5.86, N% 9.86; found C% 48.69, H% 5.48, N% 9.98.

4.2.13. 4-[(7-Chloroquinolin-4-yl)amino]-2-[(piperazin-1-yl)methyl]-phenol trihydrochloride (**27**)

A solution of **26** (1.27 g; 2.47 mmol) in 6 N HCl (20 mL) was refluxed for 18 h, after evaporation of the solvent under reduced pressure the obtained product was recrystallized from iPrOH to yield **27** (1.1 g; 94%) as a yellow solid that was freeze-dried. Mp: 271.6–272.8 °C. ^{1}H NMR (DMSO-d_6): δ, 12.22 (*s*, br, 1H, NH^+); 11.25 (*s*. br, 1H, AQ-*OH*); 10.94 (*s*, br, 1H, NH^+); 10.09 (*s*, br, 2H, NH_2^+); 8.96 (d, 1H, $J = 9.0$ Hz, AQ-H_5); 8.46 (d, 1H, $J = 6.9$ Hz, AQ-H_2); 8.19 (*s*, 1H, AQ-H_8); 7.82 (d, 1H, $J = 9.0$ Hz, AQ-H_6); 7.65 (s, 1H, AQ-$H_{3'}$); 7.40 (d, 1H, $J = 8.5$ Hz, AQ-$H_{5'}$); 7.25 (d, 1H, $J = 8.5$ Hz, AQ-$H_{6'}$); 7.04 (d, 1H, $J = 6.9$ Hz, AQ-H_3); 4.35 (*s*, 2H, CH_2-Ph); 3.51 (*s*, br, 8H, 4CH_2-Pip). ^{13}C NMR (DMSO-d_6): δ, 156.5; 155.0; 154.8; 143.1; 139.0; 138.3; 130.5; 128.6; 127.9; 127.2; 119.1; 117.1; 116.7; 115.8; 101.0; 61.4; 47.1. Anal. Calc. For $C_{20}H_{21}ClN_4O_3 \cdot 3\ HCl \cdot 3\ H_2O$ C% 45.13, H% 5.60, N% 10.52; found C% 44.81, H% 5.23, N% 10.32.

4.2.14. General procedure for the synthesis of derivatives **28**–**35**

To a stirred solution of **4** (0.5 g; 1.4 mmol) in CH_3CN (15 mL) the appropriate piperazine **18**–**25** (6.3 mmol) was added and the mixture was stirred at r.t. for 18 h. The solvent was evaporated under reduced pressure, the residue treated with water (40 mL) and stirred for 20 min until a yellow precipitate was formed. The solid, consisting of crude product, was collected, washed with water, dried and then purified by flash chromatography eluting with CH_2Cl_2/MeOH 5–10% to yield the desired product.

4.2.14.1. 4-[(7-Chloroquinolin-4-yl)amino]-2-[(4-phenyl-piperazin-1-yl)methyl]-phenol trihydrochloride (**28**).

The product was converted into the corresponding hydrochloride by treatment with HCl saturated MeOH, recrystallized from MeOH/Et_2O and freeze-dried to afford a yellow solid (66%). Mp: 256.5–258.3 ^{1}H NMR (DMSO-d_6): δ, 11.39 (*s*, br, 1H, NH^+); 11.24 (*s*, br, 1H, AQ-*OH*); 10.90 (*s*, br, 1H, NH^+); 8.95 (d, 1H, $J = 9.2$ Hz, AQ-H_5); 8.47 (d, 1H, $J = 6.8$ Hz, AQ-H_2); 8.20 (d, 1H, $J = 2.0$ Hz, AQ-H_8); 7.84 (d, 1H, $J = 9.2$ Hz, AQ-H_6); 7.73 (d, 1H, $J = 2.4$ Hz, AQ-$H_{3'}$); 7.42–7.38 (dd, 1H, $J = 2.4$, 8.7 Hz, AQ-$H_{5'}$); 7.28–7.23 (*m*, 3H, AQ-$H_{6'}$, Ph-$H_{3,5}$); 7.0–6.95 (*m*, 3H, AQ-H_3, Ph-$H_{2,6}$); 6.87 (*t*, 1H, Ph-H_4); 4.37 (*s*, 2H, ArCH_2-N); 4.28 (*s*, br, 2H, 2NH^+-Pip); 3.82 (*m*, 2H, CH_2-Pip); 3.49 (*m*, 2H, CH_2-Pip); 3.30–3.23 (*m*, 4H, 2CH_2-Pip). ^{13}C NMR (DMSO-d_6): δ, 157.2; 155.7; 150.4; 143.9; 139.8; 139.2; 131.5; 130.0; 129.5; 128.8; 128.1; 127.1; 120.9; 119.9; 117.9; 117.6; 116.8; 116.6; 101.5; 53.7; 51.2; 46.1. MS (CI/isobutane) (*m*/*z*): 445/447 [MH^+]. Anal. Calc. For $C_{26}H_{25}ClN_4O \cdot 3\ HCl \cdot 0.75\ H_2O$ C% 54.99, H% 5.24, N% 9.87; found C% 55.02, H% 5.27, N% 9.89.

4.2.14.2. 4-[(7-Chloroquinolin-4-yl)amino]-2-{[4-(pyridin-2-yl)piperazin-1-yl]methyl}-phenol trihydrochloride (**29**).

The product was converted into the corresponding hydrochloride by treatment with HCl–saturated MeOH, recrystallized from MeOH/Et_2O and freeze-dried to afford a yellow solid (85%). M.p.: 243.1–245.3 °C. ^{1}H NMR (DMSO-d_6): δ, 11.68 (*s*, br, 1H, NH^+); 11.21 (*s*, br, 1H, AQ-*OH*); 10.89 (*s*, br, 1H, NH^+); 8.94 (d, 1H, $J = 9.2$ Hz, AQ-H_5); 8.50 (d, 1H, $J = 7.0$ Hz, AQ-H_2); 8.20 (d, 1H, $J = 2.0$ Hz, AQ-H_8); 8.11 (dd, 1H, $J = 1.4$, 5.7 Hz, Pyr-H_6); 7.97 (*t*, 1H, Pyr-H_4); 7.85 (dd, 1H, $J = 2.0$, 9.1 Hz, AQ-H_6); 7.71 (d, 1H, $J = 2.5$ Hz, AQ-$H_{3'}$); 7.4 (d, 2H, $J = 2.5$, 8.7 Hz, AQ-$H_{5'}$); 7.35 (d, 1H, Pyr-H_3); 7.25 (d, 1H, $J = 8.7$ Hz, AQ-$H_{6'}$); 7.02–6.94 (*m*, 2H, AQ-H_3, Pyr-H_5); 4.36 (*s*, 2H, PhCH_2); 3.70 (*m*, 2H, CH_2-Pip); 3.52 (d, 2H, CH_2-Pip); 3.30 (*s*, 2H, CH_2-Pip).

^{13}C NMR (Free base) (DMSO-d_6): δ,159.2; 154.3; 152.2; 149.8; 149.8; 147.9; 137.9; 134.1; 131.0; 127.9; 126.4; 125.0; 124.9; 124.6; 123.9; 118.1; 116.4; 113.4; 107.4; 100.9; 58.2; 52.4; 44.9.

MS (CI/isobutane) (*m*/*z*): 446/448 [MH^+]. Anal. Calc. For. $C_{25}H_{24}ClN_5O \cdot 3\ HCl \cdot 3.5\ H_2O$ C% 48.56, H% 5.54, N% 11.32; found C% 48.33, H% 5.14, N% 11.38.

*4.2.14.3. 4-[(7-Chloroquinolin-4-yl)amino]-2-{[4-(pyrimidin-2-yl)piperazin-1-yl]methyl}-phenol trihydrochloride (**30**).* The product was converted into the corresponding hydrochloride by treatment with HCl–saturated MeOH, recrystallized from MeOH and freeze-dried to afford a yellow solid (86%). Mp: 255.4–256.7 °C. ^{1}H NMR (DMSO-d_6): δ, 11.52 (*s*, br, 1H, *NH*$^+$); 11.26 (*s*, br, 1H, AQ-*OH*); 10.9 (*s*, br, 1H, *NH*$^+$); 8.97 (d, 1H, *J* = 9.1 Hz, AQ-*H*$_5$); 8.46 (*m*, 3H, AQ-*H*$_2$, Pyrim-*H*$_{4/6}$); 8.20 (d, 1H, *J* = 2.0 Hz, AQ-*H*$_8$); 7.82 (dd, 1H, *J* = 2.0, 9.1 Hz, AQ-*H*$_6$); 7.73 (d, 1H, *J* = 2.5 Hz, AQ-*H*$_{3'}$); 7.39 (dd, 1H, *J* = 2.5, 8.7 Hz, AQ-*H*$_{5'}$); 7.25 (d, 1H, *J* = 8.7 Hz, AQ-*H*$_{6'}$); 6.99 (d, 1H, *J* = 7.0 Hz, AQ-*H*$_3$); 6.78 (*t*, 1H, *J* = 4.8 Hz, Pyrim-*H*$_5$); 4.68 (*m*, 2H, *CH*$_2$-Pip); 4.39 (*s*, 2H, *CH*$_2$-Ph); 3-58-3.47 (*m*, 4H, 2*CH*$_2$-Pip); 3.17 (*m*, 2H, *CH*$_2$-Pip). ^{13}C NMR (DMSO-d_6): δ, 160.7; 158.5; 156.7; 155.1; 143.4; 139.3; 138.6; 130.9; 128.9; 128.3; 127.6; 126.9; 126.6; 119.4; 117.3; 117.1; 116.0; 115.4; 111.6; 101.1; 100.9; 98.7; 53.5; 50.6; 40.7. MS (CI/isobutane) (*m*/*z*): 447/449 [MH$^+$]. Anal. Calc. For $C_{24}H_{23}ClN_6O\cdot 3HCl\cdot H_2O$ C% 50.19, H% 4.91, N% 14.63; found C% 50.09, H% 5.04, N% 14.67.

*4.2.14.4. 4-[(7-Chloroquinolin-4-yl)amino]-2-{[4-(1,3-thiazol-2-yl)piperazin-1-yl]methyl}-phenol trihydrochloride (**31**).* The product was converted into the corresponding hydrochloride by treatment with HCl–saturated MeOH, recrystallized from MeOH/Et_2O and freeze-dried to afford a yellow solid (85%). Mp: 255.5–257.3 °C. ^{1}H NMR (DMSO-d_6): δ, 11.76 (*s*, br, 1H, *NH*$^+$); 11.25 (*s*, br, 1H, AQ-*OH*); 10.92 (*s*, br, 1H, *NH*$^+$); 8.96 (d, 1H, *J* = 9.1 Hz, AQ-*H*$_5$); 8.45 (d, 1H, *J* = 6.9 Hz, AQ-*H*$_2$); 8.19 (d, 1H, *J* = 1.2 Hz, AQ-*H*$_8$); 7.8 (d, 1H, *J* = 9.1 Hz, AQ-*H*$_6$); 7.7 (d, 1H, *J* = 1.9 Hz, AQ-*H*$_{3'}$); 7.38 (dd, 1H, *J* = 1.9, 8.7 Hz, AQ-*H*$_{6'}$); 7.32 (d, 1H, *J* = 3.8 Hz, Tz-*H*$_4$); 7.24 (d, 1H, *J* = 8.7 Hz, AQ-*H*$_{5'}$); 7.05 (d, 1H, *J* = 3.8 Hz, Tz-*H*$_5$); 7.0 (d, 1H, *J* = 6.9 Hz, AQ-*H*$_3$); 4.35 (*s*, 2H, *CH*$_2$-Ph); 4.13 (*m*, 2H, *CH*$_2$-Pip); 3.76 (*m*, 2H, *CH*$_2$-Pip); 3.5 (*m*, 2H, *CH*$_2$-Pip); 3.34 (*m*, 2H, *CH*$_2$-Pip). ^{13}C NMR (DMSO-d_6): δ, 170.2; 156.5; 154.9; 143.2; 139.1; 138.5; 134.8; 130.7; 128.9; 128.2; 127.4; 126.5; 119.3; 117.2; 116.6; 115.9; 110; 100.9; 53.1; 49.5; 48.9. MS (CI/isobutane) (*m*/*z*): 452/454 [MH$^+$]. Anal. Calc. For $C_{23}H_{22}ClN_5O\cdot 3\ HCl\cdot 2\ H_2O$ C% 46.24, H% 4.89, N% 11.72; found C% 45.86, H% 4.86, N% 11.63.

*4.2.14.5. 4-[(7-Chloroquinolin-4-yl)amino]-2-{[4-(thiophen-2-yl)piperazin-1-yl]methyl}-phenol (**32**).* The product was recrystallised from EtOH and freeze-dried to afford a yellow solid (83%). Mp: 181.9–182.7 °C. ^{1}H NMR ($CDCl_3 + CD_3OD$): δ, 8.31 (d, 1H, *J* = 5.4 Hz, AQ-*H*$_2$); 8.12 (d, 1H, *J* = 8.0 Hz, AQ-*H*$_5$); 7.87 (s, 1H, AQ-*H*$_8$); 7.48–7.42 (m, 1H, AQ-*H*$_6$); 7.16 (m, 1H, AQ-*H*$_{6'}$); 7.4 (s, 1H, AQ-*H*$_{3'}$); 6.90 (m, 1H, AQ-*H*$_3$); 6.79 (m, 2H, AQ-*H*$_{5'}$, Tph-*H*$_4$); 6.68–6.64 (m, 1H, Tph-*H*$_5$); 6.20 (d, *J* = 2.5, 1H, Tph-*H*$_3$); 3.81 (s, 2H, *CH*$_2$-Ph); 3.25 (s, 4H, 2 *CH*$_2$-Pip); 2.78 (s, 4H, 2 *CH*$_2$-Pip). ^{13}C NMR ($CDCl_3 + CD_3OD$): δ, 158.3; 155.4; 151.4; 151.0; 149.1; 138.4; 135.9; 127.3; 126.3; 126.1; 123.1; 122.5; 118.1; 117.3; 117.2; 116.8; 113.7; 107.1; 101.2; 61.5; 52.6; 52.5. MS (CI/isobutane) (*m*/*z*): 451/453 [MH$^+$]. Anal. Calc. For $C_{24}H_{23}ClN_4OS$ C% 63.92, H% 5.14, N% 12.42; found C% 63.67, H% 5.14, N% 12.42.

*4.2.14.6. 2-{[4-(Benzothiazol-2-yl)piperazin-1-yl]methyl}-4-[(7-Chloroquinolin-4-yl)amino]-phenol trihydrochloride (**33**).* The product was converted into the corresponding hydrochloride by treatment with HCl–saturated MeOH, recrystallised from MeOH and freeze-dried to afford a yellow solid (86%). Mp: 250.4–252.2 °C. ^{1}H NMR (DMSO-d_6): δ, 1.72 (*s*, br, 1H, *NH*$^+$); 11.23 (*s*, 1H, AQ-*OH*); 10.91 (*s*, br, 1H, *NH*$^+$); 8.96 (d, 1H, *J* = 9.27 Hz, AQ-*H*$_5$); 8.47 (d, 1H, *J* = 6.9 Hz, AQ-*H*$_2$); 8.21 (d, 1H, *J* = 2.0 Hz, AQ-*H*$_8$); 7.88–7.81 (*m*, 2H, AQ-*H*$_6$, Bzt-*H*$_4$); 7.73 (d, 1H, *J* = 2.3 z, AQ-*H*$_{3'}$); 7.55 (d, 1H, *J* = 8.0 Hz, Bzt-*H*$_4$); 7.39–7.35 (*m*, 2H, AQ-*H*$_{5'}$, Bzt-*H*$_{5/6}$); 7.25 (d, 1H, *J* = 8.7 Hz, AQ-*H*$_{6'}$); 7.17 (*t*, 1H, *J* = 8.0 Hz, Bzt-*H*$_{5/6}$); 7.02 (d, 1H, *J* = 7.0 Hz, AQ-*H*$_3$); 4.39 (*s*, 2H, *CH*$_2$-Ph); 4.23 (*m*, 2H, *CH*$_2$-Pip); 3.82 (*m*, 2H, *CH*$_2$-Pip); 3.36 (*m*, 2H, *CH*$_2$-Pip); 2.51 (*m*, 2H, *CH*$_2$-Pip). ^{13}C NMR (DMSO-d_6): δ, 167.6; 162.2; 154.6; 142.9; 138.8; 138.1; 130.3; 129.8; 128.5; 127.8; 127.1; 126.2; 126.1; 122.1; 121.5; 118.9; 118.4; 116.8; 116.4; 115.6; 100.6; 52.9; 49.4; 44.9. MS (CI/isobutane) (*m*/*z*): 502/504 [MH$^+$]. Anal. Calc. For $C_{27}H_{24}ClN_5OS\cdot 3\ HCl\cdot 4\ H_2O$ C% 47.45, H% 5.16, N% 10.25; found C% 47.65, H% 4.77, N% 10.41.

*4.2.14.7. 2-{[4-(1H-Benzoimidazol-2-yl)piperazin-1-yl]methyl}-4-[(7-Chloroquinolin-4-yl)amino]-phenol trihydrochloride (**34**).* The product was converted into the corresponding hydrochloride by treatment with conc. HCl (15 mL) in MeOH (20 mL), treated with abs. EtOH (3 × 40 mL), recrystallised from MeOH and freeze-dried to afford a yellow solid (75%). Mp: 279.1–281 °C. ^{1}H NMR (DMSO-d_6): δ, 11.23 (s, br, 1H, AQ-*OH*); 8.93 (d, 1H, *J* = 9.1 Hz, AQ-*H*$_5$); 8.54 (d, 1H, *J* = 7.0 Hz, AQ-*H*$_2$); 8.19 (d, 1H, *J* = 2.0 Hz, AQ-*H*$_8$); 7.82 (dd, 1H, *J* = 2.0, 9.1 Hz, AQ-*H*$_6$); 7.69 (*s*, 1H, AQ-*H*$_{3'}$); 7.46–7.38 (m, 3H, Bzm-*H*$_{4,\ 7}$, AQ-*H*$_{6'}$); 7.28–7.23 (m, 3H, AQ-*H*$_{5'}$, Bzm-*H*$_{5,\ 6}$); 7.0 (d, 1H, *J* = 7.0 Hz, AQ-*H*$_3$); 4.34 (s, 2H, *CH*$_2$-Ph); 3.49 (m, 8H, 4 *CH*$_2$-Pip). ^{13}C NMR (DMSO-d_6): δ, 156.2; 154.7; 149.7; 143.0; 138.8; 138.1; 130.2; 130.1; 128.5; 127.8; 127.1; 126.0; 123.2; 118.9; 116.8; 115.5; 111.3; 111.2; 100.6; 93.2; 59.7; 53.0; 49.0; 43.9. MS (CI/isobutane) (*m*/*z*): 485/487 [MH$^+$]. Anal. Calc. For. $C_{27}H_{25}ClN_6O\cdot 3\ HCl\cdot 3\ H_2O$ C% 50.01, H% 5.28, N% 12.96; found C% 50.18, H% 4.97, N% 13.06.

*4.2.14.8. 2-{[4-(Benzooxazol-2-yl)piperazin-1-yl]methyl}-4-[(7-Chloroquinolin-4-yl)amino]-phenol dihydrochloride (**35**).* The product was converted into the corresponding hydrochloride by treatment with conc. HCl (15 mL) in MeOH (20 mL), treated with abs. EtOH (3 × 40 mL), recrystallised from MeOH and freeze-dried to afford a yellow solid (94%). Mp: 253.5–255.2 °C. ^{1}H NMR (DMSO-d_6): δ, 11.61 (s, br, 1H, *NH*$^+$); 11.20 (s, 1H, AQ-*OH*); 10.88 (s, br, 1H, *NH*\); 8.94 (d, 1H, *J* = 9.0 Hz, AQ-*H*$_5$); 8.48 (d, 1H, *J* = 6.9 Hz, AQ-*H*$_2$); 8.20 (d, 1H, *J* = 2.0, AQ-*H*$_8$); 7.84 (d, 1H, *J* = 2.0, 9.0 Hz, AQ-*H*$_6$); 7.73 (d, 1H, *J* = 2.4 Hz, AQ-*H*$_{3'}$); 7.47 (d, 1H, *J* = 7.8 Hz, AQ-*H*$_{6'}$), 7.39–7.35 (m, 2H, Bzo-*H*$_{4,\ 7}$); 7.26–7.21 (m, 2H, AQ-*H*$_{5'}$, Bzo-*H*$_{5/6}$); 7.10 (t, 1H, *J* = 7.8 Hz, Bzo-*H*$_{5/6}$); 7.02 (d, 1H, *J* = 6.9 Hz, AQ-*H*$_3$); 4.36 (s, 2H, Ph*CH*$_2$); 4.30–4.25 (m, 2H, *CH*$_2$-Pip); 3.82–3.74 (m, 2H, *CH*$_2$-Pip); 3.54–3.50 (m, 2H, *CH*$_2$-Pip); 3.37–3.20 (*m*, 2H, *CH*$_2$-Pip). ^{13}C NMR (DMSO-d_6): δ, 160.9; 156.2; 154.7; 148.3; 143.1; 141.9; 138.9; 138.2; 130.4; 128.5; 127.8; 127.2; 126.0; 124.1; 121.2; 119.0; 116.8; 116.5; 116.0; 115.6; 109.2; 100.6; 53.0; 49.4; 42.4. Anal. Calc. For $C_{27}H_{24}ClN_5O_2\cdot 2\ HCl\cdot 2\ H_2O$ C% 54.51, H% 5.08, N% 11.77; found C% 54.55, H% 4.83, N% 11.60.

4.3. Dissociation constants (pK_as)

The ionisation constants of compounds were determined by potentiometric titration with the GLpK_a apparatus (Sirius Analytical Instruments Ltd, Forest Row, East Sussex, UK). Apparent ionisation constants (p$_s K_a$) were obtained in co-solvent mixtures because of the low aqueous solubility of compounds according to the following procedure. At least five separate 20 mL semiaqueous solutions of the compounds (about 1 mM in 40–65 wt% methanol) were initially acidified to pH 1.8 with 0.5 N HCl. The solutions were then titrated with standardised 0.5 N KOH to pH 12.2. The titrations were performed under argon at 25.0 ± 0.1 °C. The initial estimates of the apparent ionisation constants (p$_s K_a$) were obtained by Bjerrum plots; these values were finally refined by a weighted non-linear least-squares procedure. Aqueous pK_a values were obtained by extrapolation using the Yasuda–Shedlovsky procedure [22]. The molar % of species was calculated using the experimental pK_a values from the Henderson–Hasselbalch equation.

4.4. Biological studies

4.4.1. Leishmania donovani

Briefly, peritoneal exudate macrophages were harvested from CD1 mice (Charles Rivers Ltd, UK), 24 h after starch induction. After washing the macrophages were dispensed into Lab-tek™16-well tissue culture slides and maintained in RPMI 1640 (Sigma, UK) + 10% heat-inactivated foetal calf serum (HIFCS, Harlan, UK) at 37 °C, 5% CO_2/air mixture for 24 h. *Leishmania donovani* MHOM/ET/67/HU3 amastigotes were harvested from an infected golden hamster spleen and were used to infect the macrophages at a ratio of 5 parasites:1 macrophage. Infected cells were left for a further 24 h and then exposed to drug for a total of 5 days, with the overlay being replaced on day 3 [23]. The top concentration for the test compounds was 30 μg/mL and all concentrations were carried out in quadruplicate. On day 5 the overlay was removed, the slides fixed (100% methanol) and stained (10% Giemsa, 10 min) before being evaluated microscopically. IC_{50} (IC_{90}) values were calculated using MS*xl*fit and Prism™ (Sigmoidal regression).

4.4.2. Plasmodium falciparum 3D7 and K1

Plasmodium falciparum cultures were mantained in RPMI 1640 medium (Sigma, UK) 37 °C, 5% CO_2 in 5% hematocrit. Synchronised ring stage cultures were prepared at 1% parasitemia and 50 μl added per well, the top test drug final concentration being 30 μg/mL. After 24 h incubation, 37 °C, 5% CO_2 5 μl (^{3}H)-hypoxanthine was added (0.2 μCi/well) [24,25] and plates were shaken for 1 min and then incubated for 48 h. The plates were freeze/thawed rapidly, harvested and dried. Radioactive hypoxanthine uptake was measured by scintillation counter. IC_{50} values were calculated (Sigmoidal regression, Prism™).

4.4.3. Toxicity on KB cells

KB cells, derived from a human carcinoma of the nasopharynx and typically used in assays for antineoplastic agents, were maintained in RPMI 1640 medium (Sigma, UK), 10% heat-inactivated foetal calf serum, 37 °C, 5% CO_2. KB cell monolayers, prepared in 96-well plates, were exposed to the test compounds for 72 h [26]. Podophyllotoxin was used as a positive control. 20 μl of Alamar Blue™ was added to each well. After a further 2–4 h incubation the plates were read (Molecular Devices Gemini™) at EX/EX 530/580, cut-off 550 nm. The IC_{50} values were calculated by sigmoidal regression analysis (Prism™)

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