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Amodiaquine analogues containing NO-donor substructures: synthesis and their preliminary evaluation as potential tools in the treatment of cerebral malaria

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Abstract

The synthesis and physico-chemical properties of novel compounds obtained by conjugation of amodiaquine with moieties containing either furoxan or nitrooxy NO-donor substructures are described. The synthesised compounds were tested in vitro against both the chloroquine sensitive, D10 and the chloroquine resistant, W-2 strains of *Plasmodium falciparum* (*P. falciparum*). Most of the compounds showed an antiplasmodial activity comparable to that of the parent drug. By comparing the activities of simple related structures devoid of the ability to release NO, it appears that the contribution of NO to the antiplasmodial action in vitro is marginal. All the compounds were able to relax rat aorta strips with a NO-dependent mechanism, thus showing their capacity to release NO in the vessels. A preliminary in vivo study using *Plasmodium berghei* ANKA-infected mice showed a trend for prolonged survival of mice with cerebral malaria treated with compound

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40, which is potent and fast amodiaquine-derived NO donor, when compared with amodiaquine alone or with compound **31**, a milder NO-donor. The two compounds showed in vivo antiplasmodial activity similar to that of amodiaquine.

Keywords: Amodiaquine; Cerebral Malaria; Nitric Oxide; Furoxans; Nitrooxy Derivatives

1. Introduction

Malaria is one of the most important causes of parasitic infection and death in the world. A WHO report estimates in 189-327 millions the cases of malaria and in about 1 million the associated deaths, the major part of them occurring in children living in sub-Saharan Africa [1]. There is an increasing need both of new antimalarial drugs and of improvements of those already in use, in particular of agents active against the infection due to *Plasmodium falciparum*, which is the main agent of severe malaria.

Nitric oxide (NO) is a physiological messenger with multiple actions. In the cardiovascular system it is involved in maintaining the micro- and macro-vascular homeostasis [2]. In particular, it induces vasodilation and inhibits platelet aggregation through a cyclic GMP (cGMP) dependent mechanism and it modulates the expression of the cell-adhesion molecules (CAMs) on endothelial cells. In the central nervous system, it plays complex roles in learning, memory formation and neurotransmitters release, while in the peripheral nervous system behaves as a neurotransmitter at the ends of non-adrenergic, non-cholinergic nerves controlling a number of gastrointestinal, respiratory and genitourinary functions [3]. In the innate immune system NO is one of the final effector molecules against pathogens of different origin. It regulates other immunological functions, as well, including T- and B-cell proliferation, leukocytes rolling and cytokines production [4]. Finally, NO seems to play an important role in the pathogenesis of *P falciparum* malaria both as antiplasmodial agent and regulator of the immune response to the parasite. This suggested the hypothesis that derivatives of

NO could be effective antimalarial agents [5]. Indeed, it was found that some NO-donors display in vitro toxic action against *P. falciparum* [6-8].

Severe falciparum malaria, including cerebral malaria (CM), is associated with tissue ischemia related to cytoadherence of parasitized erythrocytes to microvascular endothelium. CM has been reported to be associated to low NO bioavailability in the vasculature [9,10]. This is due to both an increased scavenging of NO in the blood, caused by the high concentration of free oxyhaemoglobin (HbO^{2+}) from the haemolysis of parasitized red blood cells and to hypoargininemia. Administration of exogenous NO to the murine model of CM induced by *P.berghei* ANKA (PbA) restored signalling in the brain, decreased proinflammatory biomarkers in the blood, and markedly reduced vascular leak and petechial hemorrhages in the brain [10a]. Moreover, a significant improvement of CM has been observed in patients after infusion of l-arginine demonstrating that the NO pathway can be targeted for adjunctive treatment in falciparum malaria [10b]. Amodiaquine (AQ, Chart 1) is an established antimalarial drug recently reintroduced in the World Health Organisation Model List of Essential Medicines [11, 12].

As development of our previous work aimed at designing new NO-donor multifunctional drugs [13], we now report a study concerning the synthesis, the dissociation constants and the in vitro anti *P. falciparum* activity of new NO-donor amodiaquine derivatives (NO-AQ) containing either furoxan (1,2,5-oxadiazole 2-oxide) or nitrooxy ($-\text{ONO}_2$) NO-donor substructures. The capacity of NO-AQ of relaxing rat aorta strips with a NO-dependent mechanism, and in vivo preliminary data which seem to confirm their potential utility in the treatment of CM, are also discussed.

Insert Chart 1

2. Results and discussion

2.1 Chemistry

Derivatives **3**, **4**, **7**, **12-15**, bearing nitrooxy functionalities and a primary or a secondary amino group, used for building the final nitrooxy substituted compounds, were synthesised following the pathways described in Scheme 1. Compounds **3** and **4** were synthesised by nitration with fuming nitric acid of the related commercially available amino alcohols **1** and **2**. For the synthesis of 6-(ethylamino)hexan-1-ol (**6**) the commercially available 6-chlorohexanol (**5**) was treated with ethylamine and then with HCl to give **5a**·HCl. The hydrochloride was converted into the corresponding free base by ion-exchange chromatography on cationic resin (Amberlite IRA-400). This base was transformed into **7**·HNO₃ by treatment with fuming nitric acid in 34% overall yield. For the synthesis of piperidino- and piperazino-based nitrooxy derivatives, the commercially available piperidino or piperazino alcohols **8-11** were nitrated following the usual procedure to afford compounds **12-15** in 60-67% yields.

Insert Scheme 1

The synthesis of 6-(ethylamino)hexane-1,1-diyl dinitrate (**26**) was achieved through the route depicted in Scheme 2. The commercially available hex-5-en-1-ol (**16**) was converted into the corresponding phthalimido derivative **17**, by reaction of the intermediate methansulphonate **16a** with potassium phthalimide. Double bond oxidation with KMnO₄ in acetone/water afforded the diol **18**, which was then transformed into the dioxolane **19** with acetone and catalytic pyridinium *p*-toluenesulfonate (PPTS). Cleavage of the phthalimide moiety by refluxing with hydrazine hydrate in THF afforded the amine **20**. The Fukuyama procedure [14] was used in order to obtain the mono-alkylated amine **23**. 2-nitro-benzenesulphonamide derivative **21**, resulting from reaction of **20** with 2-nitrobenzenesulfonyl chloride in the presence of triethylamine, was alkylated with excess ethyl iodide in basic medium to afford **22**. Treatment of this product with thiophenol and KOH under nitrogen atmosphere gave the expected secondary amine **23**. The dioxolane ring of this intermediate was cleaved by treatment with refluxing 80 % CF₃COOH solution and subsequent conversion of the obtained aminodiols trifluoroacetate **24** to the corresponding free base by ion-exchange

chromatography; **25** was then treated with fuming nitric acid to afford the final double nitrated aminodiol **26** as HNO₃ salt.

Insert Scheme 2

The synthesis of final AQ derivatives **28-31**, **32-35** bearing nitrooxy groups (Scheme 3) was carried out by nucleophilic displacement of benzylic chlorine atom present on versatile intermediate **27** we have previously described [15], by the appropriate nitrooxy substituted primary and secondary amines. The reaction, was performed in a 1:1 mixture of DMF and CH₃CN, in the presence of triethylamine. Compounds **28-30**, **32-35** were converted to the corresponding hydrochlorides by treatment of their methanolic solution with HCl-saturated Et₂O, while compound **31** was kept as the free base.

Insert Scheme 3

The final AQ derivatives **37**, **40** bearing furoxan moieties were synthesised starting from **27** (Scheme 4). To obtain the phenylfuroxanyl substituted compound **37** the chloromethyl substituted intermediate **27** was transformed into hydroxyamodiaquine **36** by treatment with 2-ethylamino ethanol in acetonitrile. This last compound was treated with 3-phenyl-4-benzenesulfonyl furoxan and 50% (w/w) NaOH, under nitrogen atmosphere in distilled THF/DMF mixture, to give the desired product. Owing to extensive decomposition of the reaction mixture, it was impossible to obtain **40** in the same way. This product was obtained by reacting **27** with *N*-ethyl-2-((3-phenylsulfonyl)furoxan-4-yl)oxy)ethanamine **39** that, in its turn, was obtained by NaOH-mediated reaction of 2-(ethylamino)ethanol with bis-benzenesulfonyl furoxan **38**. The proposed structure of **39** was confirmed by its ¹³C-NMR spectrum, which is typical of a 4-alkoxy-3-phenylsulfonyl substituted furoxan [16].

Insert Scheme 4

2.2 Dissociation constants determination

Potentiometric titrations of the final amodiaquine derivatives were performed 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 (see experimental). The pK_a values are listed in Table 1. Using AQ as reference compound (pK_{a1}=8.47, lateral chain nitrogen; pK_{a2}=7.42, 4-aminoquinoline centre) [17] it is reasonable to assign in the analogues **28-35**, **37**, **40**, the higher pK_{a2} values to the basic centre of the lateral chains, and the lower pK_{a1} values to the 4-aminoquinoline moiety. In the case of the piperazino substituted compounds **34**, **35**, pK_{a3} values could be related with aminoquinoline scaffold, while pK_{a1} and pK_{a2} values with the piperazine substructure. From the data of the Table 1, it is immediately deducible that at pH (5.2) of parasite food vacuole all the products exist in the dicationic form in equilibrium with the monocationic one, while at physiological pH (7.4) as complex equilibrium between neutral, monocationic and dicationic species.

2.3 Biological activities

2.3.1 In vitro antimalarial activity against (CQS) D10 and (CQR) W-2 strains of *P. falciparum*

All the final products were screened in vitro against the chloroquine sensitive (CQ-S) D10 and the chloroquine resistant (CQ-R) W-2 strains of *P. falciparum*. The results, expressed as 50% inhibitory concentrations (IC₅₀) are shown in Table 2. All tested compounds including the NO-AQ

derivatives retained significant activity against both strains in the low nM range. The most active compounds are the piperidine and the piperazine derivatives **32** and **35**, which display an antiplasmodial potency near to that of the lead. Also the activity of this class against the W-2, CQ-R strain is similar to that of AQ, within the limits of the experimental errors, with the only exception of the furoxan and piperazine compounds **40** and **34**, respectively, which show signs of cross resistance with CQ.

No activity in the nM range was observed for the simple nitric acid esters **3**, **7**, **12-15**, used to prepare the nitrooxysubstituted NO-AQ, as well as for the two simple furoxans **A**, **B** (Table 2) structurally similar to the furoxan moieties present in **37** and **40** respectively. These results indicate that the contribution of NO to the antiplasmodial properties of NO-AQ is, if any, secondary to that of the lead structure.

2.3.2 Vasodilator activity

All the NO-AQ products described in this work were able to relax rat aorta strips precontracted with phenylephrine. The vasodilator potencies, expressed as EC₅₀, are reported in Table 2. Analysis of the data indicates that the most potent compounds appear to be the dinitrooxy- and the furoxan-substituted products **35** and **40**, respectively, which display their action in the nM range. The remaining products show EC₅₀ values all included in a very narrow range 0.10÷1.2 µM. When the experiments were repeated in the presence of 1 µM ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), a well known inhibitor of the soluble guanylate cyclase (sGC), a decrease in the potencies was observed. These results indicate the ability of these products to release NO in the vessels with consequent NO-dependent vasodilation, thus suggesting their potential use as adjunct therapy for CM.

2.3.3. Ability of compounds 31 and 40 to improve survival of mice from late-stage cerebral malaria (CM).

To test this hypothesis, preliminary in vivo experiments were conducted with compounds **31** and **40** using the murine model of CM caused by *P. berghei* ANKA (PbA). Infected mice with late stage CM (i.e., presenting neurological signs, hypothermia and low motor scores) treated with compound **40** (1.4mg per mouse) daily for 5 days presented a trend for delayed mortality and increased survival as compared to AQ (1mg per mouse) alone. Compound **40** slightly improved the survival to 23% and had an efficacy in parasite clearance (76% reduction of parasitaemia in 24 hours) similar to that of AQ (8% survival, 72% of parasite clearance in 24 hours) (Figures 1A and 1C). The same trend was not observed with compound **31** (1.15mg per mouse), a milder NO donor (Figures 1B and 1D). Total parasite clearance with both compounds and AQ was achieved after 72 hours and three doses of treatment in mice that survived and no recrudescence was observed after a 5-day treatment regimen (Figure 1C and 1D).

Conclusions

We have developed a new series of NO-AQ derivatives which are able to retain a high degree of activity in vitro against both CQ-S and CQ-R strains of *P. falciparum*. At least in vitro, the contribution of NO to the antiplasmodial properties of these products seems to be secondary to that of the lead. All the compounds are able, in different degree, to dilate pre-contracted rat aorta strips with a NO-dependent mechanism, and consequently they could be capable to help restoring the vascular homeostasis which is deeply compromised in CM. Indeed, low NO bioavailability plays a role in the pathogenesis of murine CM [10], which is associated with cerebral microcirculatory dysfunction and vasoconstriction [18]. Treatment with an NO donor, 1-[N-(3-Aminopropyl)-N-(3-ammoniopropyl)]diazene-1,2-diolate (DPTA-NO) largely prevents the neurological syndrome

and this is associated with improved cerebral vascular responses [19]. We asked whether a hybrid compound endowed with antimalarial and NO activities could improve survival in mice with late stage CM, in comparison with its lead compound presenting antimalarial activity only. Our preliminary results with compounds **40** and **31** in the murine model of CM by *P. berghei* ANKA indeed showed a trend for increased survival of the animals treated with compound **40**, which is a potent and fast NO-donor. It is remarkable that survival rates were increased three-fold, especially considering that mice left untreated at late stage of CM die in a few hours, and AQ by itself was highly ineffective at this stage. Earlier treatment and shorter intervals for drug administration may help to further improve the efficacy. The same trend for increased survival was not observed with compound **31**, a milder NO-donor. Further in vivo studies are in progress to disclose the interesting potentialities of these products, and of other NO-donor antimalarial drugs, in the treatment of CM.

3. Experimental

3.1. Instrumentation and chemicals

Melting points were determined with a capillary apparatus Büchi B-540 or Büchi B-530 (uncorrected). Melting point 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. ¹H and ¹³C NMR spectra were obtained on a Bruker Avance 300, at 300 and 75 MHz respectively or Bruker AC-200, at 200 and 50 MHz respectively; δ in ppm rel. to SiMe₄ as the internal standard; coupling constants *J* in Hz. ¹³C 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, Fx: furoxan, Q: quinoline ring, AQ: amodiaquine. Mass spectra were recorded on a Finnigan-Mat TSQ-700. Flash chromatography (FC) was performed on BDH silica gel (particle size 40-63 μ m). When not otherwise specified, anhydrous magnesium sulphate (MgSO₄) was used as the drying agent of organic phases. Analysis (C, H, N) of the target compounds was performed by Service de

Microanalyse, Université de Genève, Genève (CH) and REDOX (Monza) and the results were within $\pm 0.4\%$ of the theoretical. 3-phenyl-4-benzenesulfonyl furoxan [20], **3** [21], **27** [15], **38** [22], **A**, **B** [23], were synthesised according to reported methods.

3.2. Chemistry

3.2.1. 6-(ethylamino)hexan-1-ol (**6**)

6-chlorohexan-1-ol (**5**) (3.5 g; 26 mmol) was added to 70 ml of EtNH₂ 70% solution in water. The solution was kept under stirring at room temperature for 48 h. The solution was then evaporated under reduced pressure and the residue was taken up with 1N HCl and washed three times with CH₂Cl₂; the aqueous phase was filtered and dried under reduced pressure. The residue was eluted through an ion-exchange column over Amberlite IRA-400 resin to afford 1.76 g of the title product as a colourless oil (52% free base). ¹H-NMR (CD₃OD): δ , 3.58 – 3.53 (m, 2H, CH₂O); 3.02 – 2.89 (m, 4H, CH₂NHCH₂); 1.68 – 1.41 (m, 6H, 3 CH₂); 1.28 (t, $J = 7.2$ Hz, 3H, CH₃). ¹³C-NMR (CD₃OD): δ , 61.3; 47.4; 42.8; 32.0; 26.5; 26.2; 25.2; 10.8. MS CI (isobutane) (m/z): 146 [MH⁺].

3.2.2. N-ethyl-6-(nitrooxy)hexan-1-ammonium nitrate (**7**)

To 10 ml of fuming nitric acid at -15 °C, 0.55g (3.8 mmol) of **6** were added dropwise. The mixture was allowed to warm at room temperature and was kept under stirring for 24 h. The solution was then evaporated under reduced pressure and the residue was dropped in 100 ml of anhydrous Et₂O at -15 °C. After 2 h at 0 °C the solvent was decanted off and the semisolid residue was triturated several times with fresh diethyl ether and was used without further purification. The product was characterized as free base which was obtained by extraction of a slurry of the semisolid with Na₂CO₃, with EtOAc. The organic phase was then washed with brine, dried over Na₂SO₄ and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel eluting with DCM/MeOH 5 % to afford 0.62 g of the desired product as a colorless oil (65 %). ¹H-NMR (DMSO-*d*₆): δ , 4.51 (t, $J = 6.1$ Hz, 2H, CH₂ONO₂); 2.55 – 2.43 (m, 4H,

CH₂NCH₂); 1.70 – 1.61 (m, 2H, CH₂); 1.44 – 1.31 (m, 6H, 3CH₂); 0.99 (t, $J = 7.1$ Hz, 3H, CH₃).

¹³C-NMR (DMSO-*d*₆): δ , 74.2; 49.2; 43.8; 29.3; 26.7; 26.4; 25.4; 15.1.

3.2.3. General procedure for the synthesis of derivatives **4**, **12**–**15**.

The appropriate alcohol was added dropwise to 15 ml of fuming nitric acid at – 15 °C. The mixture was allowed to warm at room temperature and was kept under stirring for 24 h. The solution was then evaporated under reduced pressure and the residue was dropped in 100 ml of anhydrous Et₂O at – 15 °C. After 2 h at 0 °C the solvent was decanted off and the semisolid residue was triturated several times with fresh diethyl ether to obtain an off-white solid (60-93% yields).

3.2.3.1. *N*-ethyl-3-(nitrooxy)propan-1-ammonium nitrate (**4**).

¹H-NMR (DMSO-*d*₆): δ , 8.45 (s, 2H, exch. sign.); 4.60 (t, $J = 4.5$ Hz, 2H, CH₂ONO₂); 2.99 (m, 2H, CH₂CH₂CH₂); 1.17 (t, $J = 5.5$ Hz, 3H, CH₃). ¹³C-NMR (DMSO-*d*₆): δ , 70.8; 43.2; 42.2; 23.2; 11.0.

3.2.3.2. 4-[(nitrooxy)methyl]piperidinium nitrate (**12**).

¹H-NMR (DMSO-*d*₆): δ , 8.68 (br s, 1H, NH⁺); 8.34 (br s, 1H, NH⁺); 4.45 (d, $J = 6.4$ Hz, 2H, CH₂ONO₂); 3.34 – 3.30 (m, 2H, CH₂-piperidine); 2.98 – 2.86 (m, 2H, CH₂-piperidine); 2.10 – 2.04 (m, 1H, CH-piperidine); 1.87 – 1.82 (m, 2H, CH₂-piperidine); 1.51 – 1.38 (m, 2H, CH₂-piperidine).

¹³C-NMR (DMSO-*d*₆): δ , 76.6; 42.8; 31.4; 25.1. Spectral data were consistent with the reported ones[22].

3.2.3.3. 4-[2-(nitrooxy)ethyl]piperidinium nitrate (**13**).

¹H-NMR (CD₃OD): δ , 4.45 (t, $J = 6.3$ Hz, 2H, CH₂ONO₂); 3.30 – 3.25 (m, 2H, CH₂-piperidine); 2.91 – 2.83 (m, 2H, CH₂-piperidine); 1.88 – 1.83 (m, 2H, CH₂-piperidine); 1.64 – 1.58 (m, 3H, CH₂, CH); 1.37 – 1.30 (m, 2H, CH₂-piperidine). ¹³C-NMR (CD₃OD): δ , 71.5; 55.2; 53.3; 44.5; 33.0; 31.3; 29.0. Spectral data were consistent with the reported ones[24].

3.2.3.4. *1-[2-(nitrooxy)ethyl]piperazin-4-ium nitrate (14)*.

¹H-NMR (DMSO-*d*₆): δ, 9.09 (br s, 2H, 2NH⁺); 4.87 (t, *J* = 4.6 Hz, 2H, CH₂ONO₂); 3.62 (m, 2H, CH₂-lateral chain); 3.41 – 3.32 (m, 8H, 4CH₂-piperazine). ¹³C-NMR (DMSO-*d*₆): δ, 67.3; 52.6; 48.6; 40.4. Spectral data were consistent with the reported ones [25].

3.2.3.5. *1-[3-(nitrooxy)propyl]piperazin-4-ium nitrate (15)*.

¹H-NMR (DMSO-*d*₆): δ, 9.10 (br s, 2H, 2NH⁺); 4.61 (t, *J* = 6.0 Hz, 2H, CH₂ONO₂); 4.0 – 3.28 (m, 10H, 5 CH₂); 2.14 – 2.05 (m, 2H, CCH₂C-lateral chain). ¹³C-NMR (DMSO-*d*₆): δ, 70.8; 52.7; 48.5; 40.6; 21.4. Spectral data were consistent with the reported ones [25].

3.2.4. *2-hex-5-enyl-1H-isoindole-1,3(2H)-dione (17)*.

5-hexen-1-ol **16** (10 g, 10 mmol) was converted to the corresponding methanesulfonate according to a reported procedure [26]. The crude product was then suspended in CH₃CN with potassium phthalimide (1.5 eq) and a catalytic amount of potassium iodide. The mixture was refluxed for 72 h, then cooled and filtered on a sintered-glass funnel. The filtrate was purified by flash chromatography eluting with PE/EtOAc 10% to afford the desired product as a white solid (20 g, 86%). Spectral data were consistent with the reported ones [27]. ¹H-NMR (CDCl₃): δ, 7.86 - 7.80 (m, 2H arom); 7.64 - 7.68 (m, 2H arom); 5.85 - 5.71 (m, 1H, CH=); 5.04 - 4.92 (m, 2H, CH₂=); 3.69 (t, 2H, *J* = 7.2 Hz, CH₂N); 2.13 - 2.05 (m, 2H, CH₂CH); 1.72 - 1.67 (m, 2H, CH₂); 1.50 - 1.40 (m, 2H, CH₂). ¹³C-NMR (CDCl₃): δ, 168.4; 138.2; 133.8; 132.1; 123.1; 114.9; 37.8; 33.2; 28.0; 26.1; 21.0. MS CI (isobutane) (m/z): 230 [MH⁺]. M.p. (n-hexane): 52.5 °C.

3.2.5. *2-(5,6-dihydroxyhexyl)-1H-isoindole-1,3(2H)-dione (18)*.

To a solution of 5 g of **17** (22 mmol) in 200 ml of acetone cooled in an ice bath, a solution of KMnO₄ in 200 ml of water was added dropwise. After the addition was over the mixture was

allowed to raise to room temperature and kept under stirring for further 3 h; the brownish solid was filtered off on a sintered glass funnel and the filtrate was concentrated under reduced pressure. The residue was taken up with 100 ml of water and extracted with EtOAc (6 x 50 ml). The organic extracts were washed with brine (1 x 100 ml), dried and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel eluting with CH₂Cl₂/CH₃OH 5% to afford 3.16 g (56%) of the desired product as white solid. Spectral data were consistent with the reported ones [28]. ¹H-NMR (CD₃OD): δ, 7.85 - 7.76 (m, 4H_{arom}); 3.70 - 3.30 (m, 5H, CH₂N, CHOH, CH₂OH); 1.74 - 1.27 (m, 6H, CH₂CH₂CH₂). ¹³C-NMR (CD₃OD): δ, 169.8; 135.3; 133.4; 124.0; 73.0; 67.3; 38.8; 33.9; 29.6; 24.0; 23.3. MS (CI) (isobutane) (m/z): 264 [MH⁺]. M.p. (iPr₂O / iPrOH): 65 °C.

3.2.6. 2-[4-(2,2-dimethyl-1,3-dioxolan-4-yl)butyl]-1H-isoindole-1,3(2H)-dione (**19**).

To a solution of **18** (3.16 g; 12 mmol) in 200 ml of acetone, pyridinium p-toluenesulfonate (0.3 g; 1 mmol) was added. The mixture was stirred at room temperature for 18 h, then evaporated under reduced pressure and purified by flash chromatography on silica gel to afford 3.03 g (80 %) of title product as a white solid. Spectral data were consistent with the reported ones [28]. ¹H-NMR (CDCl₃): δ, 7.86 - 7.80 (m, 2H_{arom}); 7.74 - 7.68 (m, 2H_{arom}); 4.11 - 4.00 (m, 2H, 2CHO); 3.70 (t, *J* = 7.2 Hz, 2H, CH₂N); 3.54 - 3.44 (m, 1H, CHO); 1.77 - 1.46 (m, 6H, 3CH₂); 1.40 (s, 3H, CH₃); 1.34 (s, 3H, CH₃). ¹³C-NMR (CDCl₃): δ, 168.4; 165.7; 133.9; 132.1; 123.2; 108.72; 75.8; 71.9; 69.4; 37.8; 33.1; 28.6; 26.9; 25.7; 23.1. MS CI (isobutane) (m/z): 304 [MH⁺]. M.p. (n-hexane): 121 °C.

3.2.7. 4-(2,2-dimethyl-1,3-dioxolan-4-yl)butan-1-amine (**20**).

To a stirred solution of **19** (3 g; 10 mmol) in 100 ml of distilled THF, hydrazine monohydrate (3.9 ml; 8 eq.) was added and the mixture was stirred under reflux for 24 h. After cooling the mixture was filtered on a sintered-glass funnel and the filtrate was evaporated under reduced pressure. The

residue was purified by flash chromatography on silica gel eluting with DCM/MeOH 5%,

DCM/MeOH 10% NH₃ soln 1% affording the desired product as a colourless oil (0.9g 52%).

Spectral data were consistent with the reported ones [29]. ¹H-NMR (CDCl₃): δ, 4.13 - 4.01 (m, 2H, CH₂O); 3.54 - 3.47 (m, 1H, CH); 2,7 (t, 2H, *J* = 6.9 Hz, CH₂N); 1.95 (s, 3H, CH₂, CH); 1.40 (s, 3H, CH₃); 1.35 (s, 3H, CH₃). ¹³C-NMR (CDCl₃): δ, 108.6; 77.2; 75.9; 69.4; 41.8; 33.3; 26.9; 25.7; 23.2. MS CI (isobutane) (m/z): 174 [MH⁺].

3.2.8. *N*-[4-(2,2-dimethyl-1,3-dioxolan-4-yl)butyl]-2-nitrobenzenesulfonamide (**21**).

To a solution of 0.9 g (5 mmol) of **20** and Et₃N (1.1 eq) in 20 ml of DCM cooled in an ice bath, 2-nitrobenzenesulfonylchloride (1 eq) was added portionwise. The mixture was stirred at RT for 30 min, taken up with DCM and washed with 20 ml of 1N HCl; the aqueous phase was extracted with DCM (2 x 10 ml) and the organic phase was then washed with water and brine, dried over Na₂SO₄, filtered and evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel, eluting with PE/EtOAc 30% to afford the desired product as a colourless oil (1.55 g, 83%). ¹H-NMR (CDCl₃): δ, 8.13 – 8.10 (m, 1H arom); 7.86 – 7.77 (m, 3H arom); 5.76 - 5.73 (m, 1H, CH); 4.14 - 3.98 (m, 2H, CH₂O); 3.38 – 3.44 (m, 1H, NH); 3.13 (qt, 2H, *J* = 6.3 Hz, CH₂N); 1.60 - 1.36 (m, 6H, CH₂CH₂CH₂); 1.32 (s, 3H, CH₃); 1.26 (s, 3H, CH₃). ¹³C-NMR (CDCl₃): δ, 148.2; 135.6; 134.2; 133.6; 133.2; 131.1; 125.5; 108.8; 76.0; 69.5; 43.9; 33.2; 29.8; 27.2; 26.0; 25.5; 23.0; 14.5. MS CI (isobutane) (m/z): 359 [MH⁺].

3.2.9. *N*-[4-(2,2-dimethyl-1,3-dioxolan-4-yl)butyl]-*N*-ethyl-2-nitrobenzenesulfonamide (**22**).

To a stirred solution of **21** (1.55 g; 4.3 mmol) in 50 ml of DMF, K₂CO₃ (10 eq) and EtI (1.5 eq) were added. The solution was stirred at 60 °C for 10 hrs, taken up with 100 ml of Et₂O and washed with water (3 x 50 ml) and brine; the organic phase was dried over Na₂SO₄, filtered and dried under reduced pressure. The residue was purified by flash chromatography on silica gel eluting with PE/EtOAc 30%, to afford the desired product as a pale yellow oil (1.63 g 98%). ¹H-NMR (CDCl₃):

δ , 8.02 – 7.99 (m, 1H arom); 7.70 - 7.67 (m, 2H arom); 7.65 – 7.60 (m, 1H, arom); 4.07 – 3.99 (m, 2H, CH₂O); 3.50 – 3.46 (m, 1H, CH); 3.40 – 3.28 (m, 4H, NCH₂CH₃, NCH₂); 1.66 - 1.29 (m, 12H, 3 CH₂, CH₃CCH₃); 1.12 (t, $J = 7.2$ Hz, 3H, CH₃CH₂). ¹³C-NMR (CDCl₃): δ , 133.8, 133.4, 130.6, 127.0, 75.8, 69.3, 46.7, 42.0, 36.5, 33.0, 31.4, 28.3, 26.9, 25.7, 22.8, 13.7. MS CI (isobutane) (m/z): 387 [MH⁺].

3.2.10. *N*-[4-(2,2-dimethyl-1,3-dioxolan-4-yl)butyl]-*N*-ethylamine (**23**).

A solution of 1.08 ml (2.5 eq) of thiophenol in 15 ml of CH₃CN kept under N₂ was cooled in an ice bath. To this solution 0.60 g of KOH (2.5 eq) dissolved in 2ml of water were added dropwise. After 10 min 1.63 g of **22** (0.004 mol) dissolved in 10 ml of CH₃CN were added dropwise over a period of 20 min. The mixture was stirred at 50 °C for 4 h, then it was filtered and the filtrate was evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel, eluting with DCM, DCM/MeOH 5%, DCM/MeOH 10% NH₃ 1% to afford the title product as a colourless oil (0.65 g 77%). ¹H-NMR (CDCl₃): δ , 4.10 - 4.01 (m, 2H, CH₂O); 3.50 (t, 1H, $J = 7.09$ Hz, CH); 2.70 - 2.60 (m, 4H, CH₂NCH₂); 1.66 - 1.43 (m, 6H, CH₂CH₂CH₂); 1.41 (s, 3H, CH₃); 1.37 (s, 3H, CH₃); 1.12 (t, 3H, $J = 7.2$ Hz, CH₃CH₂). ¹³C-NMR (CDCl₃): δ , 108.7; 69.5; 49.5; 44.1; 33.5; 29.9; 27.0; 25.7; 23.6; 15.1. MS CI (isobutane) (m/z): 202 [MH⁺].

3.2.11. 6-(ethylamino)hexane-1,2-diol (**25**).

of Compound **23** (0.64 g; 3.2 mmol) was dissolved in 20 ml of 80% TFA in water. The mixture was refluxed for 24 hrs, then it was evaporated under reduced pressure. The residue was taken up with 20 ml of water and activated carbon was added; the suspension was heated to boiling and filtered through a bed of celite. The filtrate was evaporated under reduced pressure and the residue was eluted through an ion-exchange column over Amberlite IRA-400 resin to afford 0.42 g of the title product as a colourless oil (82% free base). ¹H-NMR (CD₃OD): δ , 3.61 - 3.59 (m, 1H, CHOH); 3.47 - 3.45 (m, 2H, CH₂OH); 3.10 - 2.97 (m, 4H, CH₂NHCH₂); 1.73 - 1.40 (m, 6H, CH₂CH₂CH₂); 1.31

(t, 3H, $J = 7.4$ Hz, CH₃). ¹³C-NMR (CD₃OD): δ , 72.8; 67.2; 48.4; 44.0; 33.7; 27.3; 23.6; 11.6. MS CI (isobutane) (m/z): 162 [MH⁺].

3.2.12. *N*-ethyl-5,6-bis(nitrooxy)hexan-1-ammonium nitrate (**26**).

To 20 ml of fuming nitric acid cooled at -15 °C, **25** (1.5 g; 9.3 mmol) was added dropwise over a period of 10 min. The mixture was then allowed to warm to room temperature and was kept under stirring for 40 h. After that period the solvent was distilled under reduced pressure and the residue was dropped and triturated in 150 ml of dry diethyl ether at -15 °C. The white solid thus obtained was collected through filtration and washed thoroughly with dry diethyl ether to afford 1.97 g of the title compounds (67% as nitric acid salt). ¹H-NMR (DMSO-*d*₆): δ , 8.21 (br s, 2H NH₂⁺); 5.46 - 5.39 (m, 1H, CHONO₂); 4.97 - 4.92 (dd, $J_1 = 2.4$ Hz, $J_2 = 12.9$ Hz, 1H, CH'H''ONO₂); 4.74 - 4.68 (dd, $J_1 = 6.2$ Hz, $J_2 = 12.8$ Hz, 1H, CH'H''ONO₂); 2.99 - 2.85 (m, 4H, CH₂NH₂⁺CH₂); 1.78 - 1.70 (m, 2H, CH₂); 1.63 - 1.37 (m, 6H, 2CH₂); 1.16 (t, 3H, $J = 7.2$ Hz, CH₃). ¹³C-NMR (DMSO-*d*₆): δ , 80.0; 71.8; 45.8; 41.8; 27.6; 25.1; 21.3; 10.9. M.p.: $64 - 65$ °C. Anal. Calc. for: C₈H₁₇N₃O₆·HNO₃·0.5H₂O C% 29.72, H% 5.92, N% 17.33; Found 29.61, H% 5.65, N% 17.53.

3.2.13. General procedure for the synthesis of the derivatives **28-35**.

The appropriate nitric ester and **27** (1 eq.) were suspended in a mixture of CH₃CN/DMF 1/1. To the suspension, cooled at 0 °C, 3.5 eq. of Et₃N were added dropwise. The mixture was kept under stirring at room temperature for 6 h. After that period the solvent was distilled under reduced pressure and the residue was taken up with water and extracted with EtOAc. The organic phase was washed with brine, dried over Na₂SO₄, filtered and evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel eluting with mixture of DCM/MeOH to afford the desired products as yellow solids in 35 – 56% yields.

3.2.13.1. 7-chloro-4-[[4-hydroxy-3-({[3-

(nitrooxy)propyl]ammonio)methyl]phenyl]amino}quinolinium dihydrochloride (28).

The free base was taken up with MeOH and HCl saturated Et₂O was added at 0 °C; the solution was kept under stirring at room temperature for two hours, then the solvent was evaporated under reduced pressure and the solid was freeze dried to give the product as the hydrochloride. ¹H-NMR (DMSO-*d*₆): δ, 11.20 (br s, 1H, exch. signal); 10.79 (br s, 1H, exch. signal); 9.50 (br s, 2H, exch. signal) 8.95 (d, *J* = 9.0 Hz, 1H arom.); 8.46 (d, *J* = 7.0 Hz, 1H arom.); 8.19 (d, *J* = 1.9 Hz, 1H arom.); 7.82 (dd, *J*₁ = 9.0 Hz, *J*₂ = 1.9 Hz, 1H arom.); 7.60 (d, *J* = 2.3 Hz, 1H arom.); 7.34 (d, *J*₁ = 8.6 Hz, *J*₂ = 2.3 Hz, 1H arom.); 7.19 (d, *J* = 8.6 Hz, 1H arom.); 6.86 (dd, *J* = 7.0 Hz, 1H arom.); 4.66 (t, *J* = 6.0 Hz, 2H, CH₂ONO₂); 4.13 (s, 2H, CH₂Ph); 3.06 (m, 2H, CH₂N); 2.17 (m, 2H, CCH₂C). ¹³C-NMR (DMSO-*d*₆): δ, 155.4; 155.3; 154.9; 142.9; 138.8; 138.2; 128.7; 127.8; 127.2; 125.9; 119.2; 119.0; 116.4; 115.5; 100.2; 70.7; 44.6; 43.2; 22.8. M. p.: 177 - 179 °C (dec.). Anal. Calc. for: C₁₉H₁₉ClN₄O₄·2HCl·1.7H₂O C% 45.07, H% 4.86, N% 11.06; Found 45.16, H% 4.49, N% 10.69.

3.2.13.2. 7-chloro-4-[[3-({ethyl[3-(nitrooxy)propyl]ammonio)methyl)-4-

hydroxyphenyl]amino}quinolinium dihydrochloride (29).

The free base was taken up with MeOH and HCl saturated Et₂O was added at 0 °C; the solution was kept under stirring at room temperature for two hours, then the solvent was evaporated under reduced pressure and the solid was freeze dried to give the product as the hydrochloride. ¹H-NMR (free base) (CDCl₃): δ, 8.42 (d, *J* = 5.6 Hz, 1H arom.); 7.97 – 7.93 (m, 2H arom.); 7.41 (dd, *J*₁ = 9.0 Hz, *J*₂ = 2.5 Hz, 1H arom.); 7.14 (dd, *J*₁ = 8.2 Hz, *J*₂ = 2.5 Hz, 1H arom.); 6.96 (d, *J* = 2.5 Hz, 1H arom.); 6.87 (d, *J* = 8.2 Hz, 1H arom.); 6.63 (d, *J* = 5.6 Hz, 1H arom.); 4.49 (t, *J* = 6.2 Hz, 2H, CH₂ONO₂); 3.77 (s, 2H, CH₂Ph); 2.67 (m, 4H, CH₂NCH₂); 1.99 (m, 2H, CCH₂C); 1.13 (t, *J* = 7.2 Hz, 3H, CH₃). ¹³C-NMR (free base) (CDCl₃): δ, 156.1; 150.7; 149.7; 148.3; 135.5; 129.9; 127.7; 125.8; 125.7; 125.2; 125.1; 117.1; 101.1; 70.8; 57.2; 49.1; 47.1; 24.0; 10.9. MS CI (free base)

(isobutane) (m/z): 431 / 433 [MH⁺]. M. p.: 182 - 183 °C (dec.). Anal. Calc. for:

C₂₁H₂₃ClN₄O₄·2HCl·1.5H₂O C% 47.51, H% 5.31, N% 10.55; Found 47.62, H% 5.02, N% 10.27.

3.2.13.3. *7-chloro-4-{{3-({ethyl[6-(nitrooxy)hexyl]ammonio)methyl}-4-hydroxyphenyl}amino}quinolinium dihydrochloride (30).*

The free base was taken up with MeOH and HCl saturated Et₂O was added at 0 °C; the solution was kept under stirring at room temperature for two hours, then the solvent was evaporated under reduced pressure and the solid was freeze dried to give the product as the hydrochloride. ¹H-NMR (CD₃OD): δ, 8.65 (d, *J* = 9.1 Hz, 1H arom.); 8.39 (d, *J* = 7.1 Hz, 1H arom.); 7.76 (dd, *J*₁ = 9.1 Hz, *J*₂ = 1.9 Hz, 1H arom.); 7.61 (d, *J* = 2.5 Hz, 1H arom.); 7.45 (dd, *J*₁ = 8.7 Hz, *J*₂ = 2.5 Hz, 1H arom.); 7.15 (d, *J* = 8.7 Hz, 1H arom.); 6.86 (d, *J* = 7.1 Hz, 1H arom.); 4.49 (t, *J* = 6.5 Hz, 2H, CH₂ONO₂); 4.23 (s, 2H, CH₂Ph); 3.30 – 3.20 (m, 4H, CH₂NCH₂); 1.83 (m, 2H, CH₂); 1.74 (m, 2H, CH₂) 1.42 (m, 7H, 2CH₂, CH₃). ¹³C-NMR (CD₃OD): δ, 157.8; 157.5; 144.2; 141.4; 140.5; 131.4; 130.5; 129.9; 129.1; 126.5; 120.4; 119.3; 118.0; 117.2; 101.7; 74.5; 53.7; 52.6; 49.8; 27.6; 27.3; 26.3; 24.6; 9.2. M. p.: 191 - 193 °C (dec.). Anal. Calc. for: C₂₄H₂₉ClN₄O₄·2HCl·H₂O C% 51.31, H% 5.77, N% 9.90; Found 51.12, H% 5.90, N% 9.94.

3.2.13.4. *6-[[5-[(7-chloroquinolin-4-yl)amino]-2-hydroxybenzyl](ethylamino)hexan-1,2-diyl dinitrate (31).*

¹H-NMR (CD₃OD): δ, 8.32 - 8.24 (m, 2H arom.); 7.85 (s, 1H arom.); 7.49 - 7.46 (m, 1H arom.); 7.16 (d, *J* = 4.5 Hz, 1H arom.); 7.08 (s, 1H arom.); 6.88 - 6.84 (m, 1H arom.); 6.66 - 6.64 (m, 1H arom.); 5.43 - 5.39 (m, 1H, CHONO₂); 4.96 - 4.88 (m, 1H, CH₂ONO₂); 4.63 - 4.56 (m, 1H, CH₂ONO₂); 3.84 (s, 2H, PhCH₂N); 2.73 - 2.61 (m, 4H, CH₂NCH₂); 1.82 - 1.49 (m, 6H, CH₂CH₂CH₂); 1.19 - 1.15 (m, 3H, CH₃). ¹³C-NMR (DMSO-*d*₆): δ, 154.8; 151.8; 149.44; 149.38; 133.7; 130.4; 127.5; 125.4; 124.6; 124.5; 124.2; 124.0; 117.6; 116.0; 100.4; 80.2; 71.8; 55.0; 51.9;

46.4; 28.0; 25.5; 22.1; 10.8. M. p.: 120 -122 °C (dec.). Anal. Calc. for C₂₄H₂₈ClN₅O₇ C% 53.09, H% 5.38, N% 12.90; Found C% 52.79, H% 5.12, N% 12.61.

3.2.13.5. *7-chloro-4-[[4-hydroxy-3-({4-[(nitrooxy)methyl]piperidinium-1-yl)methyl}phenyl)amino}quinolinium dihydrochloride (32).*

The free base was taken up with MeOH and HCl saturated Et₂O was added at 0 °C; the solution was kept under stirring at room temperature for two hours, then the solvent was evaporated under reduced pressure and the solid was freeze dried to give the product as the hydrochloride. ¹H-NMR (free base) (DMSO-*d*₆): δ, 8.90 (s, 1H arom.); 8.43 - 8.36 (m, 2H arom.); 7.85 (d, *J* = 1.8 Hz, 1H arom.); 7.52 (dd, *J*₁ = 7.2, Hz, *J*₂ = 1.8 Hz, 1H arom.); 7.10 – 7.08 (m, 2H arom.); 6.82 (d, *J* = 9.2 Hz, 1H arom.); 6.57 (d, *J* = 5.4 Hz, 1H arom.); 4.42 (d, *J* = 6.3 Hz, 2H, CH₂ONO₂); 3.64 (s, 2H, CH₂Ph); 2.91 – 2.51 (m, 2H, CH₂-piperidine); 2.11 – 2.04 (m, 2H, CH₂-piperidine); 1.79 – 1.70 (m, 3H, CH-piperidine, CH₂-piperidine) 1.32 – 1.28 (m, 2H, CH₂-piperidine). ¹³C-NMR (free base) (DMSO-*d*₆): δ, 154.0; 151.8; 149.4; 149.0; 133.7; 131.2; 129.4; 127.8; 127.5; 124.7; 124.1; 117.7; 116.8; 116.4; 100.8; 77.2; 65.3; 59.3; 54.0; 34.7; 28.2. M. p.: 170 - 172 °C (dec.). Anal. Calc. for: C₂₂H₂₅ClN₄O₄·2HCl·2H₂O C% 47.88, H% 5.30 N% 10.15; Found 47.60, H% 4.87, N% 10.07.

3.2.13.6. *7-chloro-4-[[4-hydroxy-3-({4-[2-(nitrooxy)ethyl]piperidinium-1-yl)methyl}phenyl)amino}quinolinium dihydrochloride (33).*

The free base was taken up with MeOH and HCl saturated Et₂O was added at 0 °C; the solution was kept under stirring at room temperature for two hours, then the solvent was evaporated under reduced pressure and the solid was freeze dried to give the product as the hydrochloride. ¹H-NMR (free base) (CD₃OD + CDCl₃): δ, 8.28 (d, *J* = 5.6 Hz, 1H arom.); 8.21 (d, *J* = 9.0 Hz, 1H arom.); 7.83 (d, *J* = 2.0 Hz, 1H arom.); 7.44 (dd, *J*₁ = 6.9, Hz, *J*₂ = 2.1 Hz, 1H arom.); 7.13 (dd, *J*₁ = 6.0, Hz, *J*₂ = 2.5 Hz, 1H arom.); 7.02 (d, *J* = 2.4 Hz, 1H arom.); 6.84 (d, *J* = 8.5 Hz, 1H arom.); 6.62 (d, *J* = 5.6 Hz, 1H arom.) 4.54 (t, *J* = 6.5 Hz, 2H, CH₂ONO₂); 3.73 (s, 2H, CH₂Ph); 3.07 – 3.03 (m, 2H,

CH₂-piperidine) 2.21 – 2.14 (m, 2H, CH₂-piperidine); 1.85 – 1.81 (m, 2H, CH₂-piperidine); 1.74 – 1.68 (m, 2H, CH₂-lateral chain); 1.63 – 1.55 (m, 1H, CH-piperidine); 1.46 – 1.33 (m, 2H, CH₂-piperidine). ¹³C-NMR (free base) (CD₃OD + CDCl₃): δ, 157.2; 156.3; 143.1; 141.1; 140.8; 139.7; 131.0; 129.3; 128.8; 125.8; 119.8; 117.5; 117.4; 116.5; 101.2; 71.1; 57.9; 55.3; 53.0; 49.7; 32.9; 31.2; 29.5. MS CI (free base) (isobutane) (m/z): 457 / 459 [MH⁺]. M. p.: 163 - 164 °C (dec.). Anal. Calc. for: C₂₃H₂₅ClN₄O₄·2HCl C% 52.14, H% 5.14 N% 10.57; Found 51.79, H% 5.34, N% 10.17.

3.2.13.7. *7-chloro-4-{{4-hydroxy-3-({4-[2-(nitrooxy)ethyl]piperazinediium-1-yl)methyl}phenyl)amino}quinolinium trihydrochloride (34).*

The free base was taken up with MeOH and HCl saturated Et₂O was added at 0 °C; the solution was kept under stirring at room temperature for two hours, then the solvent was evaporated under reduced pressure and the solid was freeze dried to give the product as the hydrochloride. ¹H-NMR (free base) (CD₃OD): δ, 8.30 - 8.23 (m, 2H arom.); 7.82 (d, *J* = 2.1 Hz, 1H arom.); 7.50 (dd, *J*₁ = 6.9 Hz, *J*₂ = 2.1 Hz, 1H arom.); 7.15 – 6.90 (m, 2H arom.); 6.84 (d, *J* = 8.5, Hz, 1H arom.); 6.62 (d, *J* = 5.6 Hz, 1H arom.); 4.63 (t, *J* = 6.5 Hz, 2H, CH₂ONO₂); 3.74 (s, 2H, CH₂Ph); 2.76 (t, *J* = 5.4 Hz, 2H, CH₂CH₂ONO₂) 2.57 (br s 8H, 4CH₂-piperazine). ¹³C-NMR (free base) (CD₃OD): δ, 156.7; 152.6; 152.2; 149.9; 136.7; 132.1; 127.6; 127.5; 126.9; 126.5; 124.6; 124.1; 119.0; 117.6; 101.8; 71.4; 60.9; 59.8; 55.9; 54.2; 53.8; 53.3. MS CI (free base) (isobutane) (m/z): 458 [MH⁺]. M. p.: 153 - 154 °C (dec.). Anal. Calc. for: C₂₂H₂₄ClN₅O₄·3HCl·2.5H₂O C% 43.15, H% 5.27 N% 11.44; Found 42.87, H% 4.93, N% 11.20.

3.2.13.8. *7-chloro-4-{{4-hydroxy-3-({4-[3-(nitrooxy)propyl]piperazinediium-1-yl)methyl}phenyl)amino}quinolinium trihydrochloride (35).*

The free base was taken up with MeOH and HCl saturated Et₂O was added at 0 °C; the solution was kept under stirring at room temperature for two hours, then the solvent was evaporated under reduced pressure and the solid was freeze dried to give the product as the hydrochloride. ¹H-NMR

(free base) (DMSO- d_6): δ , 8.91 (br s, exch. sign. 1H) 8.43 - 8.36 (m, 2H arom.); 7.85 (s, 1H arom.); 7.54 - 7.51 (m, 1H arom.); 7.11 - 7.09 (m, 2H arom.); 6.84 (d, $J = 8.9$, Hz, 1H arom.); 6.60 - 6.59 (m, 1H arom.); 4.55 (t, $J = 6.2$ Hz, 2H, CH₂ONO₂); 3.64 (s, 2H, CH₂Ph); 2.51 - 2.38 (m, 10H, 5CH₂) 1.85 - 1.81 (m 2H, CH₂). ¹³C-NMR (free base) (DMSO- d_6): δ , 155.7; 154.8; 153.3; 150.9; 135.4; 132.0; 129.0; 127.9; 127.3; 126.1; 125.8; 124.9; 119.2; 117.5; 102.0; 73.9; 59.8; 56.8; 55.2; 53.8; 48.3; 25.1; 14.9. M. p.: 167 - 168 °C (dec.). Anal. Calc. for: C₂₂H₂₄ClN₅O₄·3HCl·2.5H₂O C% 44.10, H% 5.47 N% 11.18; Found 43.81, H% 4.99, N% 10.88.

3.2.14. 4-[(7-Chloroquinolin-4-yl)amino]-2-[[ethyl-(2-hydroxyethyl)amino]methyl]phenol dihydrochloride (**36**)

To a stirred solution of 2-(ethylamino)ethanol (0.82 mL; 8.43 mmol) in CH₃CN (20 mL), kept under nitrogen, **27** (1 g; 2.81 mmol) was added. The yellow suspension was stirred for 18 h. The solvent was evaporated and the yellow residue was treated with water (15 mL) and stirred for 15 min. The solid was collected on a buchner funnel and washed with water (50 mL). After drying over P₂O₅ the product was purified by flash chromatography eluting with CH₂Cl₂/MeOH 20% to give **36** (0.72 g; 69%) as yellow solid. The product was converted into the corresponding hydrochloride by treatment with HCl saturated MeOH and recrystallised from dry MeOH/Et₂O to obtain an analytical sample. Mp: 228-229.5 °C (dec.) (230 °C dec. [30]). ¹H NMR (DMSO- d_6 +D₂O): δ , 8.33 (d, 1H, $J = 9.1$ Hz, AQ-H₅); 8.21 (d, 1H, $J = 7.1$ Hz, AQ-H₂); 7.82 (d, 1H, $J = 2$ Hz, AQ-H₈); 7.65 (dd, 1H, $J = 2, 9.1$ Hz, AQ-H₆); 7.38 (d, 1H, $J = 2.5$ Hz, AQ-H₃'); 7.34 (dd, 1H, $J = 2.5, 8.6$ Hz AQ-H₅'); 7.06 (d, 1H, $J = 8.6$ Hz, AQ-H₆'); 6.72 (d, 1H, $J = 7.1$ Hz, AQ-H₃); 4.32 (s, 2H, CH₂Ph); 3.79 (t, 2H, $J = 5.1$ Hz, CH₂O); 3.2 (m, 4H, 2(CH₂N)); 1.26 (t, 3H, $J = 7.2$ Hz, CH₃). ¹³C NMR (DMSO- d_6 +D₂O): δ , 154.0 (two overlapping peaks); 141.2; 138.1; 136.9; 128.1; 127.5; 126.9; 126.5; 123.3; 117.6; 116.2; 115.5; 113.9; 98.7; 53.6; 52.3; 50.6; 47.0; 6.9. MS (free base)

(EI) m/z : 371 (52), 340 (35), 283 (100). Anal. Calc. For $C_{20}H_{22}ClN_3O_2 \cdot 2 HCl \cdot 0.1 H_2O$ C% 53.78, H% 5.46, N% 9.40; found C% 53.58, H% 5.39, N% 9.33.

3.2.15. 4-[(7-Chloroquinolin-4-yl)amino]-2-[(ethyl-[2-(3-phenylfuroxan-4-yl)oxy]ethyl)amino)methyl]phenol dihydrochloride (**37**).

To a suspension of **36** (1.3 g; 2.92 mmol) in distilled THF/DMF 14/1 (27.5 mL), stirred under nitrogen at RT, 50% NaOH aqueous solution (14.6 mmol) was added dropwise followed by a solution of 3-phenyl-4-benzenesulfonyl furoxan (1.24 g; 4.1 mmol) in distilled THF. The reaction mixture was stirred for 5 h, then the mixture was concentrated under reduced pressure to leave a brown oil. This residue was taken up with water (20 mL) and extracted with EtOAc (4 x 30 mL). The organic phase was washed with brine (40 mL), dried and evaporated under reduced pressure to afford the crude product as brown solid. The solid was purified by flash chromatography eluting with $CH_2Cl_2/MeOH$ 3%. The fractions containing the desired product were collected in tubes filled with 0.2 mL of HCl saturated MeOH to readily convert the product into the corresponding dihydrochloride. After evaporation under reduced pressure **37** was obtained in 50% yield as bright-yellow solid. The product was recrystallised from MeOH/Et₂O. Mp: 229-231 °C (dec.) ¹H NMR (DMSO-d₆): δ, 11.24 (s, 1H, exch. signal); 11.15 (br s, 1H, exch. signal); 10.98 (br s, 1H, exch. signal); 8.94 (d, 1H, J = 8.8 Hz, AQ-H₅); 8.4 (d, 1H, J = 6.5 Hz, AQ-H₂); 8.18 (s, 1H, AQ-H₈); 8.02 (d, 2H, J = 6.8 Hz, FxPh-H_o); 7.81 (d, 1H, J = 8.8 Hz, AQ-H₆); 7.71 (s, 1H, AQ-H₃'); 7.52 (m, 3H, FxPh-H_m; FxPhH_p); 7.38 (d, 1H, J = 8.4 Hz, AQ-H₅'); 7.22 (d, 1H, J = 8.4 Hz, AQ-H₆'); 6.89 (d, 1H, J = 6.5 Hz, AQ-H₃); 4.97 (br m, 2H, CH₂O); 4.26 (s, 2H, PhCH₂N); 3.74 (br m, 2H, CH₂N); 3.29 (br m, 2H, CH₃CH₂N); 1.36 (br m, 3H, CH₃). ¹³C NMR (DMSO-d₆): δ, 161.6; 155.9; 154.6; 142.8; 138.7; 138.1; 130.5; 130.1; 128.7; 128.4; 127.8; 127.0; 126.3; 126.2; 121.5; 118.9; 117.0; 116.7; 115.5; 107.6; 100.4; 65.2; 50.2; 49.6; 47.9; 8.5. Anal. Calc. For $C_{28}H_{26}ClN_5O_4 \cdot 2 HCl$ C% 55.59, H% 4.66, N% 11.58; found C% 55.29, H% 4.64, N% 11.44.

3.2.16. 2-[[[3-Phenylsulfonylfuroxan-4-yl)oxy]ethyl]ethylamine hydrochloride (**39**)

To a stirred solution of 3,4-bis(phenylsulfonyl)furoxan (**38**) (1 g; 2.72 mmol) in dist. THF (14 mL) kept under nitrogen, 2-(ethylamino)ethanol (0.54 mL; 5.52 mmol) was added. The obtained mixture was cooled to 15 °C and 50% NaOH aqueous solution (0.64 g; 8.16 mmol) was added dropwise. The reaction mixture was stirred for further 40 min and the solvent evaporated under reduced pressure to give a yellow solid. The solid was taken up with water (30 mL) and extracted with CH₂Cl₂ (4 x 30 mL), dried (Na₂SO₄) and evaporated to give the crude product as an oil. The product was purified by FC eluting with CH₂Cl₂/MeOH 5% to obtain the title product (0.4 g; 48%) as colorless oil. The product was either used immediately in the next step or stored at -21 °C overnight. An analytical sample was obtained as the hydrochloride by treatment with HCl saturated MeOH, precipitation with dry Et₂O and recrystallisation from dry MeOH/Et₂O. Mp: 136.8-138. ¹H NMR (DMSO-*d*₆): δ, 9.49 (*s*, 2H, *exch.* signal); 8.08 (*d*, 2H, *J* = 7.5 Hz, Ph-H_o); 7.91 (*t*, 1H, *J* = 7.5 Hz, Ph-H_p); 7.76 (*t*, 2H, *J* = 7.7 Hz, Ph-H_m); 4.78 (*t*, 2H, *J* = 4.9 Hz, CH₂O); 3.35 (*m*, 2H, CH₂N); 3.08 (*q*, 2H, *J* = 7.2 Hz, NCH₂CH₃); 1.27 (*t*, 3H, *J* = 7.2, CH₃). ¹³C NMR (DMSO-*d*₆): δ, 158.5; 136.8; 136.1; 129.9; 128.5; 110.8; 67.0; 44.2; 42.3; 11.0. Anal. Calc. For C₁₂H₁₅N₃O₅S · HCl C% 41.21, H% 4.61, N% 12.01; found C% 41.51, H% 4.81, N% 11.88.

3.2.17. 4-[(7-Chloroquinolin-4-yl)amino]- 2-[[[2-(3-phenylsulfonylfuroxan-4-yl)oxy]ethyl]ethyl]amino]methyl]phenol dihydrochloride (**40**)

To a suspension of **27** (0.9 g; 2.53 mmol) in iPrOH/DMF 10/1 (16.5 mL), stirred under nitrogen at RT, triethylamine (10.1 mmol) was added. After 15 min of stirring a solution of **39** (0.7g; 3.54 mmol) in iPrOH/DMF 10/1 (10 mL) was added dropwise at RT. The reaction mixture was stirred for 24 h, the solvent evaporated under reduced pressure to leave an oily residue which was taken up with water (20 mL) and extracted with EtOAc (4 x 30 mL). The organic phase was washed with

brine (40 mL), dried and evaporated under reduced pressure to give the crude product as light-brown oil. The product was purified by flash chromatography eluting with CH₂Cl₂/MeOH 3%. The fractions containing the desired product were collected in tubes filled with 0.2 mL of HCl saturated MeOH to readily convert the product into the corresponding dihydrochloride. After evaporation under reduced pressure **40** was obtained in 40% yield as white solid. The product was recrystallised from MeOH. Mp: 214-215 °C (dec.) ¹H NMR (CD₃OD+D₂O): δ, 8.54 (d, 1H, *J* = 9.1 Hz, AQ-H₅); 8.3 (d, 1H, *J* = 7.1 Hz, AQ-H₂); 7.98 (d, 2H, *J* = 7.4 Hz, FxPh-H_o); 7.94 (d, 1H, *J* = 2 Hz, AQ-H₈); 7.81-7.74 (m, 2H, AQ-H₆, FxPh-H_p); 7.69-7.62 (m, 3H, AQ-H₃', FxPh-H_m); 7.48-7.45 (m, 1H, AQ-H₅'); 7.17 (d, 1H, *J* = 8.7 Hz, AQ-H₆'); 6.83 (d, 1H, *J* = 7.1 Hz, AQ-H₃); 4.95 (br m, 2H, CH₂O); 4.64 (s, 2H, PhCH₂N); 3.83 (br m, 2H, OCH₂CH₂N); 3.49 (q, 2H, *J* = 7.2 Hz, CH₃CH₂N); 1.38 (t, 3H, *J* = 7.2 Hz CH₃). ¹³C NMR (CD₃OD+D₂O): δ, 159.6; 157.7; 157.6; 143.9; 141.4; 140.3; 138.2; 137.3; 131.3; 131.0; 130.6; 129.9; 129.6; 129.2; 126.2; 120.4; 119.2; 118.2; 117.0; 111.9; 101.6; 67.1; 54.1; 52.0; 51.0; 9.6. Anal. Calc. For C₂₈H₂₆ClN₅O₆S · 2 HCl C% 50.27, H% 4.22, N% 10.47; found C% 50.29, H% 4.23, N% 10.27.

3.3. Dissociation constants determination

The ionisation constants of compounds were determined by potentiometric titration with the GLpK_a apparatus (Sirius Analytical Instruments Ltd, Forrest Row, East Sussex, UK). Apparent ionisation constants (p_sK_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 17-65 Wt% methanol for chloroquine derivatives and 40-65 Wt% methanol for amodiaquine derivatives) 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 10.5. The titrations were performed under argon at 25.0 ± 0.1 °C. The initial estimates of the apparent ionisation constants (p_sK_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 [31]. The molar % of species were calculated using the experimental pK_a values from the Henderson-Hasselbalch equation.

3.4.1 Parasite cultures

P. falciparum cultures were carried out according to Trager and Jensen's with slight modifications [32]. The CQ-sensitive, strain D10 and the CQ-resistant, strain W2 were maintained at 5 % hematocrit (human type A-positive red blood cells) in RPMI 1640 (EuroClone, Celbio) medium with the addition of 1% AlbuMax (Invitrogen, Milan, Italy), 0.01% hypoxanthine, 20 mM HEPES, and 2 mM glutamine. All the cultures were maintained at 37 °C in a standard gas mixture consisting of 1% O₂, 5% CO₂, 94% N₂.

3.4.2 Parasite growth and drug susceptibility assay

Compounds were dissolved in either water (chloroquine) or DMSO and then diluted with medium to achieve the required concentrations (final DMSO concentration <1 %, which is non-toxic to the parasite). Drugs were placed in 96 wells flat-bottom microplates (COSTAR) and serial dilutions made. Asynchronous cultures with parasitemia of 1-1.5 % and 1 % final haematocrit were aliquoted into the plates and incubated for 72 hours at 37 °C. Parasite growth was determined spectrophotometrically (OD₆₅₀) by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of Makler's method in control and drug-treated cultures [33,34]. Antimalarial activity is expressed as the 50 % inhibitory concentrations (IC₅₀); each IC₅₀ value is the mean and standard deviation of at least three separate experiments.

3.4.3 Vasodilator activity assay.

Thoracic aortas were isolated from male Wistar rats weighing 180-200 g. The endothelium was removed and the vessels were elically cut: three strips were obtained from each aorta. The tissues were mounted in organ baths containing 30 mL of Krebs-bicarbonate buffer of the following composition (mM): NaCl 111.2, KCl 5.0, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.0, NaHCO₃ 12, glucose 11.1 maintained at 37 °C and continuously gassed with 95% O₂-5% CO₂ (pH=7.4). The aortic strips were allowed to equilibrate for 90 min and then contracted with 1 μM (-) phenylephrine. When the response to the agonist reached a plateau, cumulative concentration–response curves were determined. Effect of 1 μM ODQ was evaluated in separate series of experiments in which they was added 5 min before the contraction. EC₅₀ values are the mean of at least 5 determinations. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab.

3.4.4. Mice, infection and treatment

Six to eight week old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All experimental protocols were reviewed and approved by LJBI Institutional Animal Care and Use Committee. *Plasmodium berghei* ANKA strain expressing the green fluorescent protein (PbA-GFP, a kind donation of MR4, Manassas, VA) was inoculated intraperitoneally (1x10⁶ parasitized red blood cells) and parasitemia, motor behavior, rectal temperature and weight were checked beginning on day 5 after infection. CM was defined as the presentation of one or more of the following clinical signs of neurological involvement: ataxia, limb paralysis, poor righting reflex, seizures, roll-over, coma. Additional clinical evaluation was performed using a set of six simple behavioral tests, as described [18]. Mice with late stage CM were treated with either amodiaquine (AQ) at 1mg per mouse, compound **40** or compound **31** at molar-equivalent doses (1.4mg and 1.15mg per mouse, respectively) daily for 5 days. Compound **40** was prepared in vehicle containaing 20% DMSO (Sigma, St Louis, MO), 20% polyethyleneglycol 400 (Sigma) and 60% saline, and compound **31** was prepared in saline. AQ was given in the control group in the same vehicle as the test compound. Each mouse received 100μL intraperitoneally.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version,

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Figure Captions

Scheme 1. Preparation of derivatives **3**, **4**, **7**, **12-15**. Reagents and conditions: (a) fuming HNO₃, -15 °C to RT, 24 h; (b) EtNH₂, EtOH, RT; 48 h; (c) 1N HCl; (d) ion-exchange chromatography, Amberlite IRA-400.

Scheme 2. Preparation of **26**. Reagents and conditions: (a) CH₃SO₂Cl, Et₃N, CH₂Cl₂, RT; (b) potassium phthalimide, KI cat., CH₃CN, reflux, 72 h. (c) KMnO₄, Acetone/H₂O, 0 °C to RT, 3 h; (d) Acetone, PPTS cat, RT, 18 h; (e) N₂H₄ · H₂O, dist. THF, reflux, 24 h; (f) 2-nitrobenzenesulfonyl chloride, Et₃N, CH₂Cl₂, 0 °C to RT, 30 min; (g) EtI, K₂CO₃, DMF, 60 °C, 10 h; (h) PhSH, KOH, CH₃CN, 0 °C to 50 °C, 4 h, N₂ atmosphere. (i) CF₃COOH/H₂O 8/2, reflux, 24 h; (j) ion-exchange chromatography, Amberlite IRA-400; (k) fuming HNO₃, -15 °C to RT, 40 h.

Scheme 3. Preparation of derivatives **28-35**. Reagents and conditions: (a) **3**, **4**, **7**, **26**, Et₃N, CH₃CN/DMF 1/1, RT, 6 h; (b) HCl satd Et₂O, MeOH; (c) **12-15**, Et₃N, CH₃CN/DMF 1/1, RT, 6 h.

Scheme 4. Preparation of derivatives **37**, **40**. Reagents and conditions: (a) 2-(ethylamino)ethanol, CH₃CN, RT, 20 h; (b) 3-phenyl-4-benzenesulfonyl furoxan, 50% aq NaOH, dist. THF/DMF, RT, N₂ atmosphere (c) **39**, Et₃N, iPrOH/DMF, RT, 24 h; (d) 2-(ethylamino)ethanol, 50% aq NaOH, dist. THF/DMF, RT, 20 h; N₂ atmosphere.

Figure 1. Efficacy of amodiaquine (AQ), compound **40** and compound **31** in rescuing mice with late stage CM. A and B: survival curves of of PbA-infected mice with late stage CM following treatment with: (A) AQ (solid circle) 1 mg per mouse (n = 12) or compound **40** (open square, dashed line) at an equivalent molar dose (1.4 mg per mouse, n = 12) and; (B) AQ (solid circle) 1 mg per mouse (n = 29) or compound **31** (open triangle, dashed line) also at an equivalent molar dose (1.15 mg per mouse, n = 19). C and D: parasitemia curves showing the profile of parasite clearance

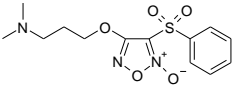
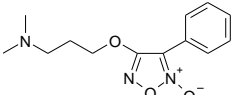
of the same treatment groups showed in A and B, respectively. Results in C and D are the mean \pm SEM.

Table 1. Dissociation constants of the final compounds

Compound	pK_{a1}^a	pK_{a2}^a	pK_{a3}^a
28	7.42	8.47	-
29	7.06	8.14	-
30	7.08	8.56	-
31	6.99	8.09	-
32	7.08	7.86	-
33	7.05	7.98	-
34	3.00	6.46	7.59
35	3.06	6.71	7.69
37	6.18	7.66	-
40	6.12	7.68	-

^a Determined by potentiometry; S.D. ≤ 0.07 .

Table 2. Antiplasmodial action and vasodilating ability of the synthesised compounds and reference derivatives

Compound	D10 (CQ-S) IC ₅₀ (μM) ^a	W2 (CQ-R) IC ₅₀ (μM) ^a	EC ₅₀ (μM) ^b	EC ₅₀ (μM) ^b + ODQ 1 μM
28	0.027 ± 0.012	0.062 ± 0.027	0.42 ± 0.09	11 ± 1
29	0.027 ± 0.016	0.031 ± 0.013	0.45 ± 0.12	3.3 ± 0.8
30	0.031 ± 0.012	0.028 ± 0.013	0.45 ± 0.05	> 30 ^c
31	0.038 ± 0.007	0.021 ± 0.004	0.22 ± 0.04	> 30 ^c
32	0.019 ± 0.007	0.028 ± 0.008	0.10 ± 0.04	> 30 ^c
33	0.019 ± 0.001	0.025 ± 0.012	0.10 ± 0.02	> 30 ^c
34	0.055 ± 0.007	0.138 ± 0.028	0.12 ± 0.02	6.5 ± 0.8
35	0.018 ± 0.003	0.034 ± 0.017	0.079 ± 0.012	3.6 ± 0.4
37	0.023 ± 0.005	0.024 ± 0.003	1.2 ± 0.2	> 10 ^c
40	0.046 ± 0.029	0.152 ± 0.075	0.017 ± 0.003	1.0 ± 0.1
3	> 5	> 5	-	-
7	> 5	> 5	-	-
12	> 5	> 5	-	-
13	> 5	> 5	-	-
14	> 5	> 5	-	-
15	> 5	> 5	-	-
26	> 5	> 5	-	-
	> 5	> 5	14.3 ± 3.8	> 10 ^c
A				
	> 5	4.94 ± 2.03	0.030 ± 0.004	-
B				
AQ	0.012 ± 0.001	0.016 ± 0.004	inactive	inactive
chloroquine	0.025 ± 0.007	0.632 ± 0.209	-	-

^a The IC₅₀ represents the μM equivalents of test compounds required to inhibit parasite growth by 50% (data are expressed as mean ± SD).

^b The EC₅₀ represents the μM equivalents of test compounds required to relax the precontracted rat aorta strips by 50% (data are expressed as means ± SE).

^c not able to reach 50% tissue relaxation at the maximum concentration tested.

- = not tested