# Synthesis, biological profiling and mechanistic studies of 4-aminoquinoline-based heterodimeric compounds with dual trypanocidal-antiplasmodial activity

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*ABSTRACT*

Dual submicromolar trypanocidal-antiplasmodial compounds have been identified by screening and chemical synthesis of 4-aminoquinoline-based heterodimeric compounds of three different structural classes. Inhibition of the enzyme trypanothione reductase seems to be involved in the potent trypanocidal activity of these heterodimers but likely it is not their main biological target within *Trypanosoma brucei*. Regarding their antiplasmodial activity, the heterodimers seem to share the mode of action of the antimalarial drug chloroquine, which involves inhibition of the haem detoxification process. Interestingly, all of these heterodimers display good brain permeabilities, thereby being potentially useful for late stage human African trypanosomiasis and cerebral malaria. Future optimization should mainly focus on decreasing cytotoxicity and acetylcholinesterase inhibitory activity.

## 1. Introduction

Human African trypanosomiasis (HAT or sleeping sickness), one of the 17 so-called neglected tropical diseases, and malaria have an enormous health and socioeconomic impact in the developing world.[1-3] Notwithstanding a wide-scale reduction in the number of infected people over recent years due to public health campaigns, HAT and malaria are still leading causes of morbidity and death and of loss of productivity especially in sub-Saharan Africa.[1,4,5] Indeed, some 48,000 people die annually from HAT,[6] whereas malaria annually kills more than 600,000 people.[3]

HAT and malaria are caused by protozoan parasites of the genus *Trypanosoma* and *Plasmodium*, which are transmitted to humans through the bite of blood-feeding infected tsetse flies and female *Anopheles* mosquitoes, respectively. The most common form of HAT in humans, accounting for nearly 95% of cases, is caused by *Trypanosoma brucei gambiense* and courses with a chronic infection that can last for years. A less common form of the disease with a more acute clinical presentation is caused by the subspecies *Trypanosoma brucei rhodesiense*. In the case of malaria, five species of *Plasmodium* may cause the disease, *Plasmodium falciparum* being the most common and deadly.

HAT begins with a hemolymphatic stage, where the parasite multiplies within the blood, lymph and subcutaneous tissue, which is characterized by the appearance of nonspecific symptoms such as fever and headache. Invasion of the central nervous system (CNS) by the parasite after crossing the blood–brain barrier (BBB) leads to the late-stage disease, the meningoencephalitic stage, giving rise to severe neurological symptoms such as psychiatric, motor and sleep disturbances, loss of consciousness, and, without treatment to coma and death. In malaria, the parasites initially infect the liver, and then are released into the bloodstream. The disease may progress to a severe form, where parasites can become sequestered within brain capillaries, particularly in children, evolving to cerebral malaria and eventually to coma and death.

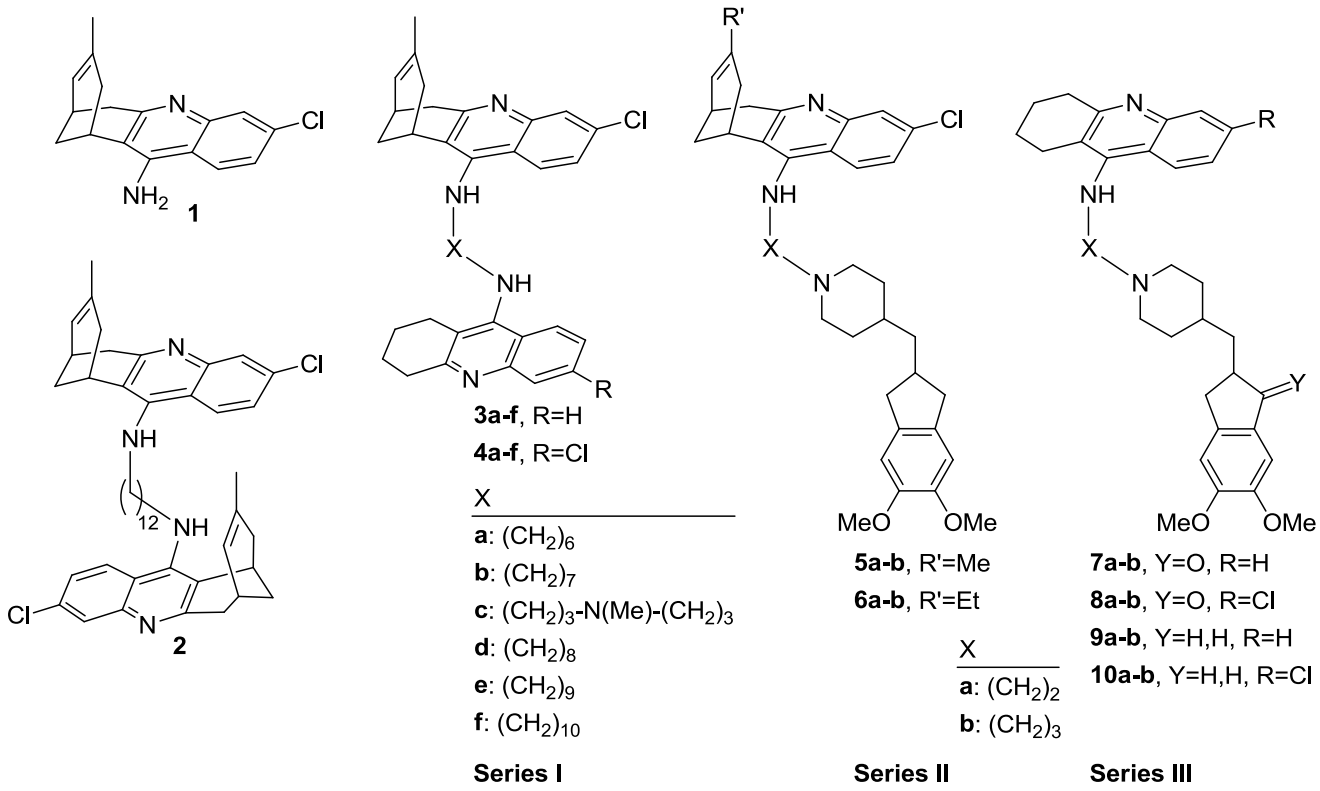



**Figure 1.** Structures of huprine Y, **1**, bis(4-aminoquinoline) homodimer **2** and bis- or mono-(4-aminoquinoline)-based heterodimers of series **I**-**III**

Vector control and public health interventions remain the main options for prevention, insofar as no licensed vaccine for either infection is yet available. Worryingly, currenty available chemotherapy against HAT and malaria suffer from serius limitations.[1,4,7-9] Five trypanocidal drugs (pentamidine, suramin, melarsoprol, nifurtimox and eflornithine) are used alone or in combinations specifically for one or another form or stage of HAT, but their relatively high cost, the requirement for long-lasting parenteral administration, often impracticable in the affected poor rural settings, the associated toxicity and emergence of parasite resistance, the two latter especially in the case of the arsenical melarsoprol,[10] challenge the widespread, safe and efficacious use of these drugs.[11,12] The emergence of resistance is the cause of the loss of effectivenes of chloroquine, after decades of being the mainstay for malaria treatment, and it starts to challenge also the effectiveness of the current first-line treatments based on artemisinin.[1]

In this scenario, it is of critical importance the enrichment of trypanocidal and antimalarial drug development pipelines with novel candidates that are devoid of the important flaws of existing drugs, i.e. new non toxic and inexpensive chemical entities that are effective against resistant parasite strains and are brain permeable, so that they may be useful for both disease stages in the case of HAT and for cerebral malaria, as well. Intensive research efforts involving phenotypic whole-cell screening of chemical libraries or newly synthesized compounds,[13-16] identification of novel key biological targets and subsequent target-based screening or rational design campaigns,[17-21] development of multitarget-directed ligands,[22-24] or drug repurposing programs[1] are being carried out in the pursuit of novel antiprotozoal compounds.

Because HAT and malaria afflict in a great part the same populations, development of compounds endowed with dual trypanocidal and antiplasmodial activity can be regarded a feasible economic therapeutic strategy.[25] A number of 4-amino-7-chloroquinolines and other aminoquinoline derivatives have been recently synthesized and found to be active against *T. brucei*, *P. falciparum* or both of them.[26-29] This prompted us to assess the antiprotozoal activity of huprines, a novel structural class of compounds featuring a 4-aminoquinoline moiety, which had been initially developed as brain permeable inhibitors of the enzyme acetylcholinesterase (AChE). Huprines turned out to be in general moderately potent and selective trypanocidal agents, a few of them being also active against the chloroquine-resistant K1 strain of *P. falciparum*.[30,31] The so-called huprine Y (**1**, Fig. 1) exhibited the most potent activity against *T. brucei* ($IC_{50}$ = 0.61 μM; $IC_{90}$ = 2.94 μM) and one of the best selectivity indices over rat myoblast L6 cells (SI = 13) but it was essentially devoid of antiplasmodial activity.[30]

Next, we turned our attention to the molecular dimerization of huprine Y because this approach had been successfully applied to other 4-aminoquinoline derivatives to overcome drug resistance.[32-36] One of the most interesting huprine dimers was the novel dodecamethylene-linked bis(4-aminoquinoline) compound **2** (Fig. 1), which tripled the potency and selectivity of the parent huprine Y against *T. brucei* but remained inactive against *P. falciparum*.[37]

The lack of potency of huprine Y and bis-huprines against *P. falciparum* was rather striking because: i) the huprine Y unit contains the 4-amino-7-chloroquinoline moiety of chloroquine, which is considered to be the pharmocophoric moiety responsible for the inhibition of heme dimerization by the antimalarial drug[28,38] and ii) in other bis(4-aminoquinoline) derivatives dimerization had been reported to increase antiplasmodial potency relative to the 4-aminoquinoline monomeric parent compounds due to the doubling of the number of protonatable

nitrogen atoms, which enabled a more efficient trapping of the dimeric compounds in the acidic digestive vacuole of *P. falciparum*, and hence, a more efficient inhibition of heme dimerization.[32,35] In this light, we hypothesized that neither huprine Y nor bis-huprines were able to hit the biological target of chloroquine and other bis(4-aminoquinoline) derivatives, despite their structural similarity.

To further deepening in the dimerization strategy and discovering new hits with dual trypanocidal-antiplasmodial activity, herein we report on: i) the screening against cultured bloodstream forms of *T. brucei* and *P. falciparum* and rat myoblast L6 cells of a small in-house library of brain permeable 4-aminoquinoline-based heterodimeric compounds, belonging to three distinct structural classes (series **I**-**III**, Fig. 1); ii) the synthesis of novel 4-aminoquinoline-based heterodimeric compounds of the most interesting series and the evaluation of their *T. brucei*, *P. falciparum*, rat myoblast L6 cells and human AChE inhibitory activities and brain permeability; and iii) the identification of the putative biological target in *T. brucei* and *P. falciparum* of selected hits of the different series and monomeric huprine Y by the *in vitro* evaluation of their inhibitory activity against *T. brucei* trypanothione reductase and β-haematin formation.

## 2. Results and discussion

### *2.1. Screening of trypanocidal, antiplasmodial and cytotoxic activity of 4-aminoquinoline-based heterodimeric compounds of series I-III*

The heterodimers of series **I**-**III** (Fig. 1) were recently developed by us as inhibitors of the enzyme AChE of potential interest for the treatment of Alzheimer's disease.[39-41] A high AChE inhibitory activity like that displayed by these heterodimers ($IC_{50}$ values in the low nanomolar range) is a drawback for antiprotozoal drug development. However, the presence of one or two 4-aminoquinoline moieties in the structures of these heterodimers and the fact that all of them had been found to be brain permeable encouraged us to screen them as potential hits of applicability for both stages of HAT and even for cerebral malaria, amenable to further optimization of the trypanocidal/anticholinesterase activity ratio.

Heterodimers of series **I** are a group of 14 racemic or enantiopure bis(4-aminoquinoline) derivatives that contain a unit of huprine Y and a unit of the structurally related tacrine (heterodimers **3a**-**f**) or 6-chlorotacrine (heterodimers **4a**-**f**), connected through linkers of different lenght and nature (hexa- to deca-methylene or 4-methyl-4-azaheptamethylene), which are di- or tri-protonated at physiological or acidic pH. The antiprotozoal activity of heterodimers of series **I** was assessed *in vitro* against cultured bloodstream forms of *T. brucei* and the chloroquine-resistant K1 strain of *P. falciparum* and their cytotoxicity determined against rat skeletal myoblast L6 cells. All of the heterodimers of series **I** are potent trypanocidal agents, exhibiting $IC_{50}$ and $IC_{90}$ values in the 0.15-0.56 μM and 0.29-0.79 μM range, respectively (Table 1). The main structural feature influencing distinctly the trypanocidal potency of heterodimers of series **I** is the length of the linker. Indeed, the trypanocidal potency seems to increase at increased tether length both in heterodimers containing an unsubstituted tacrine moiety (heterodimers **3**) and in those bearing a 6-chlorotacrine unit (heterodimers **4**). Thus, the trypanocidal activity peaks in the octa- and deca-methylene heterodimers **3d**, **4d**, **3f** and **4f** that are more than 3-fold more potent than their hexamethylene-linked counterparts. In contrast, the presence of a third protonatable nitrogen atom within the linker does not seem to have a significant influence on trypanocidal potency, heterodimers **3c** and **4c** being roughly equipotent to heptamethylene-linked heterodimers **3b** and **4b**, which have an equivalent tether length. Also, the presence or absence of a chlorine atom at the tacrine unit does not have much effect on trypanocidal activity, heterodimers **3a**-**f** being essentially equipotent to **4a**-**f**. Finally, there does not seem to be enantioselectivity in the trypanocidal activity of these heterodimers, as enantiopure heterodimers (7*R*,11*R*)-**3b** and (7*S*,11*S*)-**3b** and racemic **3b** display essentially the same potency. Of note, all of the heterodimers of series **I** are more potent than monomeric huprine Y (up to 4-fold when considering $IC_{50}$ values and up to 10-fold when considering $IC_{90}$ values). Overall, like in the homodimerization strategy leading to bis-huprines such as **2**, heterodimerization here results in increased trypanocidal potency.

Strikingly, unlike the parent huprine Y and homodimerization leading to bis-huprines, which were inactive against *P. falciparum*, heterodimerization resulted in potent antiplasmodial activities, most heterodimers of series **I** exhibiting $IC_{50}$ values in the submicromolar range (Table 1). Very interestingly, seven of these heterodimers were even 2-3-fold more potent than the reference compound chloroquine. Some clear structure-activity relationship trends were found for this activity in heterodimers of series **I**. Thus, the presence of an unsubstituted tacrine unit and increased tether lengths were the structural features leading to higher antiplasmodial potencies. The presence of a tertiary amino group within the linker only influenced antiplasmodial activity in the subseries bearing a chlorosubstituted tacrine unit. Like for trypanocidal activity, essentially no enantioselectivity was observed for antiplasmodial activity, enantiomeric (7*R*,11*R*)-**3b** and (7*S*,11*S*)-**3b** and racemic **3b** roughly displaying the same potencies.

With the exceptions of the nonamethylene-linked heterodimers **3e** and **4e**, which displayed a similar or a 5-fold lower cytotoxicity relative to the parent huprine Y, the rest of heterodimers of series **I** were more cytotoxic than huprine Y. Thus, heterodimers **3e** and **4e** showed the best selectivity indices against *T. brucei* (SI = 34 and 167, respectively), but only **3e** had a convenient selectivity index against *P. falciparum* (SI = 20), thereby emerging as the most interesting dual acting trypanocidal-antiplasmodial heterodimer of the series.

Heterodimers of series **II** are a group of 5 racemic or enantiopure compounds that contain a unit of huprine Y or its 9-ethyl analogue connected through an ethylene or trimethylene linker to a piperidine ring substituted at position 4 with a (5,6-dimethoxyindan-2-yl)methyl group, and are diprotonated at physiological or acidic pH. Again, all of the heterodimers of this series display potent trypanocidal agents, with $IC_{50}$ and $IC_{90}$ values in the 0.17-0.49 μM and 0.35-1.33 μM range, respectively (Table 1), they being up to 4-fold more potent than the parent huprine Y when considering $IC_{50}$ values and up to 8-fold when considering $IC_{90}$ values). The presence of a trimethylene linker seemed to have a positive effect on trypanocidal activity, whereas no clear trend was found regarding the presence of a methyl or an ethyl group at the huprine moiety. Also, there seems not to be any enantioselectivity in the trypanocidal action of these compounds, as enantiopure (7*S*,11*S*)-**6a** exhibits the same potency as the racemic compound.

**Table 1**
Trypanocidal, antiplasmodial and cytotoxic activity of 4-aminoquinoline–based heterodimeric compounds **3-10** and reference compounds **1** and chloroquine diphosphate[a]

| Compd | X | R | R' | Y | *T. brucei* $IC_{50}$ µM | *T. brucei* $IC_{90}$ µM | *P. falciparum* $IC_{50}$ µM | L6 cells $IC_{50}$ µM | $SI_{Tb}$[b] | $SI_{Pf}$[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| **3a** | $(CH_2)_6$ | H | | | 0.47 ± 0.05 | 0.66 ± 0.01 | 2.99 ± 0.85 | 1.17 ± 0.03 | 2.5 | 0.39 |
| **3b** | $(CH_2)_7$ | H | | | 0.24 ± 0.04 | 0.43 ± 0.02 | 0.47 ± 0.36 | <1.0 | <4.2 | <2.1 |
| (7*R*,11*R*)-**3b** | $(CH_2)_7$ | H | | | 0.21 ± 0.03 | 0.42 ± 0.01 | 0.35 ± 0.15 | 0.73 ± 0.28 | 3.5 | 2.1 |
| (7*S*,11*S*)-**3b** | $(CH_2)_7$ | H | | | 0.29 ± 0.04 | 0.49 ± 0.02 | 0.46 ± 0.19 | <1.0 | <3.4 | <2.2 |
| **3c** | $(CH_2)_3N(Me)(CH_2)_3$ | H | | | 0.32 ± 0.02 | 0.45 ± 0.01 | 0.43 ± 0.22 | <1.0 | <3.1 | <2.3 |
| **3d** | $(CH_2)_8$ | H | | | 0.15 ± 0.02 | 0.29 ± 0.01 | 0.39 ± 0.14 | 1.11 ± 0.15 | 7.4 | 2.8 |
| **3e** | $(CH_2)_9$ | H | | | 0.21 ± 0.03 | 0.45 ± 0.02 | 0.35 ± 0.06 | 7.15 ± 0.30 | 34 | 20 |
| **3f** | $(CH_2)_{10}$ | H | | | 0.14 ± 0.01 | 0.48 ± 0.02 | nd | 1.67 ± 0.04 | 12 | nd |
| **4a** | $(CH_2)_6$ | Cl | | | 0.56 ± 0.03 | 0.79 ± 0.03 | 4.40 ± 1.38 | 1.10 ± 0.01 | 2.0 | 0.25 |
| **4b** | $(CH_2)_7$ | Cl | | | 0.27 ± 0.06 | 0.43 ± 0.04 | >6.88 | <3.0 | <11 | <0.44 |
| **4c** | $(CH_2)_3N(Me)(CH_2)_3$ | Cl | | | 0.28 ± 0.04 | 0.40 ± 0.01 | 0.52 ± 0.13 | <1 | <3.6 | <1.9 |
| **4d** | $(CH_2)_8$ | Cl | | | 0.17 ± 0.01 | 0.46 ± 0.02 | >7.14 | 0.97 ± 0.13 | 5.7 | <0.14 |
| **4e** | $(CH_2)_9$ | Cl | | | 0.24 ± 0.04 | 0.52 ± 0.01 | >6.82 | 40.0 ± 5.1 | 167 | <5.9 |
| **4f** | $(CH_2)_{10}$ | Cl | | | 0.17 ± 0.01 | 0.49 ± 0.02 | 2.10 ± 0.72 | 2.63 ± 0.16 | 15 | 1.3 |
| **5a** | $(CH_2)_2$ | | Me | | 0.49 ± 0.12 | 1.18 ± 0.07 | >7.01 | 6.76 ± 0.28 | 14 | <1.0 |
| **5b** | $(CH_2)_3$ | | Me | | 0.17 ± 0.01 | 0.35 ± 0.03 | nd | 2.06 ± 0.17 | 12 | nd |
| **6a** | $(CH_2)_2$ | | Et | | 0.29 ± 0.03 | 0.70 ± 0.04 | >7.14 | 3.55 ± 0.17 | 12 | <0.50 |
| (7*S*,11*S*)-**6a** | $(CH_2)_2$ | | Et | | 0.29 ± 0.02 | 0.72 ± 0.03 | >7.05 | 6.29 ± 0.58 | 22 | <0.89 |
| (7*S*,11*S*)-**6b** | $(CH_2)_3$ | | Et | | 0.26 ± 0.03 | 1.33 ± 0.10 | 0.81 ± 0.20 | 2.15 ± 0.06 | 8.3 | 2.7 |
| **7a** | $(CH_2)_2$ | H | | O | 0.41 ± 0.03 | 0.88 ± 0.07 | >8.15 | <3.0 | <7.3 | <0.37 |
| **7b** | $(CH_2)_3$ | H | | O | 0.28 ± 0.02 | 0.62 ± 0.04 | nd | <1.0 | <3.6 | nd |
| **8a** | $(CH_2)_2$ | Cl | | O | 0.30 ± 0.03 | 0.68 ± 0.04 | 4.86 ± 3.30 | <3.0 | <10 | <0.62 |
| **8b** | $(CH_2)_3$ | Cl | | O | 0.12 ± 0.01 | 0.17 ± 0.03 | 0.36 ± 0.07 | 0.57 ± 0.19 | 4.7 | 1.6 |
| **9a** | $(CH_2)_2$ | H | | H,H | 0.38 ± 0.04 | 0.79 ± 0.01 | 2.60 ± 1.91 | <3.0 | <7.9 | <1.2 |
| **9b** | $(CH_2)_3$ | H | | H,H | 0.27 ± 0.03 | 0.66 ± 0.03 | 0.83 ± 0.44 | <3.0 | <11 | <3.6 |
| **10a** | $(CH_2)_2$ | Cl | | H,H | 0.97 ± 0.11 | 2.39 ± 0.08 | >7.72 | 2.42 ± 0.20 | 2.5 | <0.31 |
| **10b** | $(CH_2)_3$ | Cl | | H,H | 0.34 ± 0.07 | 0.66 ± 0.04 | 6.04 ± 5.94 | <3.0 | <8.8 | <0.5 |
| **1**[c] | | | | | 0.61 ± 0.03 | 2.94 ± 0.20 | >10 | 7.80 ± 0.47 | 13 | <0.78 |
| chloroquine | | | | | | | 0.93 ± 0.44 | | | |

[a] *In vitro* activity against bloodstream form of *T. brucei* (pH 7.4), *P. falciparum* (strain K1), and rat myoblast L6 cells expressed at the concentration that inhibited growth by 50% ($IC_{50}$) and 90% ($IC_{90}$, for trypanocidal activity). Data are the mean of triplicate experiments ± SEM.

[b] SI: Selectivity index is the ratio of cytotoxic to trypanocidal ($SI_{Tb}$) or antiplasmodial ($SI_{Pf}$) $IC_{50}$ values.

[c] Activity values of reference compound **1** taken from ref. 30.

In sharp contrast with series **I**, most heterodimers of series **II** were essentially inactive against *P. falciparum*, with the sole exception of **6b** ($IC_{50}$ = 0.81 µM, Table 1), which is roughly equipotent to the reference antimalarial drug chloroquine.

All of the heterodimers of series **II** turned out to be slightly more cytotoxic than the parent huprine Y. However, their superior trypanocidal potencies lead to similar or even better selectivity indices against *T. brucei* than that of huprine Y (in almost all cases over 10).

Heterodimers of series **III** form a group of 8 compounds that combine the 4-aminoquinoline unit of tacrine or 6-chlorotacrine with the 4-[(5,6-dimethoxyindan-2-yl)methyl]piperidine group that was also present in series **II** or with the indanone derivative thereof (Fig. 1). Like the other series, all of these heterodimers are submicromolar trypanocidal agents ($IC_{50}$ = 0.12-0.97 µM, $IC_{90}$ = 0.17-2.39 µM, Table 1), most potent than huprine Y. The presence of an indanone system and a trimethylene linker are the structural features that lead to higher trypanocidal potencies. As for the influence of the presence of an unsubstituted or a 6-chloro-substituted tacrine unit, the former seems to be optimal for the indane derivatives and the latter for the indanone analogues.

Most heterodimers of series **III** are submicromolar or low micromolar antiplasmodial compounds, one of them, **8b**, being 3-fold more potent than chloroquine. Like for the trypanocidal activity, in general the antiplasmodial potency of these heterodimers increased with the presence of an indanone system, a trimethylene linker and a tacrine or a 6-chlorotacrine unit for the indane or the indanone derivatives, respectively.

An important flaw of heterodimers of series **III** is their cytotoxicity, in general superior to that of huprine Y and the other series of heterodimers, which results in poor selectivity indices against both *T. brucei* and *P. falciparum*.

Overall, all of the 27 screened 4-aminoquinoline-based heterodimers turned out to be more potent trypanocidal agents than huprine Y and 10 of them (most belonging to series **I**) also exhibited more potent antiplasmodial activity than the antimalarial drug chloroquine, even though most heterodimers have inadequate selectivity indices for trypanocidal and antiplasmodial over cytotoxic activities. Likely the most interesting hit is heterodimer **3e** (series **I**), with a balanced dual trypanocidal ($IC_{50}$ = 0.21 μM) and antiplasmodial ($IC_{50}$ = 0.35 μM) activity and selectivity indices of 34 and 20 for these activities over L6 cell cytotoxicity.

### *2.2. Synthesis of novel 4-aminoquinoline-based heterodimeric compounds*

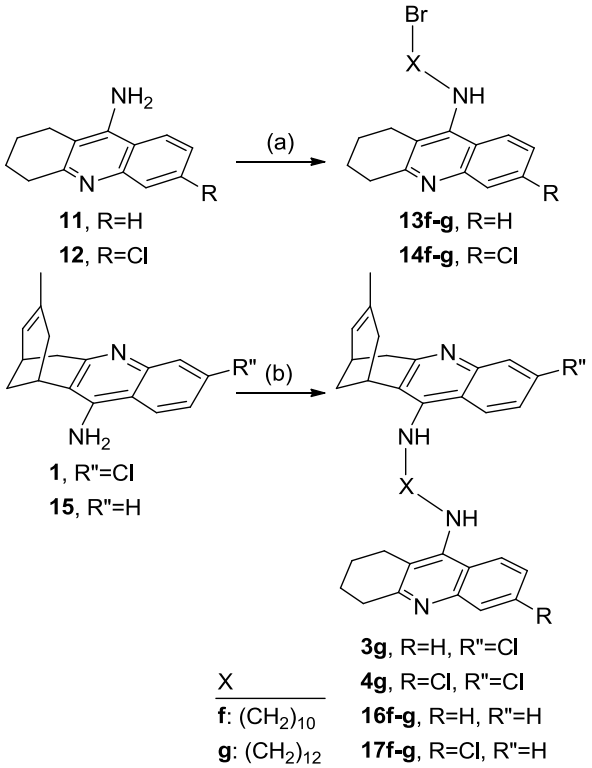
Taking into account: i) the most favourable dual trypanocidal/antiplasmodial profile of heterodimers of series **I**; ii) their easier and, hence, less expensive synthesis relative to heterodimers of series **II** and **III**; iii) and the trend towards increased trypanocidal and antiplasmodial potencies at increased tether lengths, we envisioned the synthesis and biological profiling of the upper dodecamethylene-linked homologues of series **I** **3g** and **4g** (Scheme 1). On the other hand, because removal of the chlorine atom at position 3 of the huprine moiety is known to decrease AChE inhibitory activity,[42] we also undertook the synthesis and evaluation of the deca- and dodeca-methylene-linked heterodimers **16f-g** and **17f-g** bearing a 3-unsubstituted huprine moiety (Scheme 1).

The synthesis of the novel heterodimers of series **I** involved an initial alkylation of tacrine, **11**, or 6-chlorotacrine, **12**, with 1,10-dibromodecane or 1,12-dibromododecane in the presence of KOH in DMSO, which afforded the known 10-bromodecyltacrines **13f**[39] and **14f**[39] and the novel 12-bromododecyltacrines **13g** and **14g** in moderate yields (30-39%) (Scheme 1). Subsequent alkylation of huprine Y, **1**, or its 3-unsubstituted derivative **15** with the ω-haloalkyltacrines **13f-g** or **14f-g** under similar conditions at rt for 4 days, afforded the novel heterodimers **3g**, **4g**, **16f-g** and **17f-g** in moderate to good yields (45-88%) after silica gel column chromatography purification of the reaction crudes.

The novel heterodimers were converted into the corresponding dihydrochlorides for their chemical characterization (melting point, IR, $^{1}H$ and $^{13}C$ NMR, HRMS and elemental analysis) and biological profiling.

### *2.3. Biological profiling of the novel 4-aminoquinoline-based heterodimeric compounds*

The putative dual trypanocidal/antiplasmodial and cytotoxic activities of the novel heterodimers **3g**, **4g**, **16f-g** and **17f-g** were evaluated *in vitro* against cultured bloodstream forms of *T. brucei*, the chloroquine-resistant K1 strain of *P. falciparum*, and rat skeletal myoblast L6 cells, respectively. Additionally, their inhibitory activity against human recombinant AChE was evaluated *in vitro* by the method of Ellman *et al.*[43] and their brain permeation assessed through the well-established parallel artificial membrane permeability assay (PAMPA-BBB).[44]




**Scheme 1.** Reagents and conditions: (a) **11** or **12**, KOH, DMSO, 2 h; then, 1,10-dibromodecane or 1,12-dibromododecane (1.2 eq), DMSO, rt, overnight; (b) **1** or **15**, KOH, DMSO, 2 h; then, **13f-g** or **14f-g** (1.2 eq), DMSO, rt, 4 days.

All of the novel heterodimers turned out to be more potent trypanocidal agents than the parent huprine (up to 3- and 10-fold when considering $IC_{50}$ or $IC_{90}$ values, respectively) (Table 2). Elongation of the tether chain from 10 methylenes in **3f** and **4f** to 12 methylenes in the novel heterodimers **3g** and **4g** resulted in a slightly decreased potency, so that the octa- and deca-methylene chains remained the most adequate linkers for trypanocidal activity of huprine Y-based heterodimers of series **I**, as found in the initial screening campaign. Substitution of the huprine Y unit of heterodimers of series **I** by a 3-unsubstituted huprine moiety led in general to slightly increased trypanocidal potencies, especially if $IC_{90}$ values are taken into account. Indeed, the novel heterodimer **16g** exhibited the lowest $IC_{90}$ value (0.28 μM) among all the heterodimers of series **I**.

Unlike most previously screened heterodimers of series **I**, the novel heterodimers **3g**, **4g**, **16f-g** and **17f-g** were found to be essentially inactive or only moderately potent antiplasmodial compounds, displaying one-digit micromolar activities in the best cases (Table 2). Thus, homologation of the tether chain to a dodecamethylene linker and removal of the chlorine atom at position 3 of the huprine unit are detrimental for antiplasmodial activity.

Similarly to most of the screened heterodimers of series **I**, the novel analogues had inadequate selectivity indices for both *T. brucei* and *P. falciparum* over rat L6 cells.

To complete the biological profiling of the novel heterodimers, their brain permeation and human AChE inhibitory activities were assessed.

**Table 2**
Trypanocidal, antiplasmodial, cytotoxic, and anticholinesterase activity and BBB permeabilities of the novel 4-aminoquinoline–based heterodimeric compounds **3g**, **4g**, **16f-g**, and **17f-g** and reference compounds **1** and chloroquine diphosphate[a]

| Compd | X | R | R" | *T. brucei* $IC_{50}$ µM | *T. brucei* $IC_{90}$ µM | *P. falciparum* $IC_{50}$ µM | L6 cells $IC_{50}$ µM | $SI_{Tb}$[b] | $SI_{Pf}$[b] | hAChE $IC_{50}$ (nM) | *Pe* ($10^{-6}$ cm $s^{-6}$)[c] (Prediction) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **3g** | $(CH_2)_{12}$ | H | Cl | 0.35 ± 0.01 | 0.52 ± 0.01 | >6.9 | 1.04 ± 0.02 | 3.0 | <0.15 | 1.92 ± 0.40 | 10.8 ± 1.0 (CNS+) |
| **4g** | $(CH_2)_{12}$ | Cl | Cl | 0.59 ± 0.05 | 0.77 ± 0.01 | >6.6 | 5.58 ± 0.53 | 9.5 | <0.85 | 3.46 ± 0.56 | 16.8 ± 0.3 (CNS+) |
| **16f** | $(CH_2)_{10}$ | H | H | 0.24 ± 0.01 | 0.31 ± 0.04 | 3.50 ± 2.29 | <1 | <4.2 | <0.29 | 6.46 ± 1.43 | 6.3 ± 1.1 (CNS+) |
| **16g** | $(CH_2)_{12}$ | H | H | 0.21 ± 0.00 | 0.28 ± 0.01 | >7.3 | <1 | <4.8 | <0.14 | 10.1 ± 1.19 | 7.3 ± 0.7 (CNS+) |
| **17f** | $(CH_2)_{10}$ | Cl | H | 0.34 ± 0.02 | 0.43 ± 0.01 | 4.36 ± 2.82 | 1.11 ± 0.04 | 3.3 | 0.25 | 1.48 ± 0.24 | 17.2 ± 1.6 (CNS+) |
| **17g** | $(CH_2)_{12}$ | Cl | H | 0.55 ± 0.01 | 0.75 ± 0.01 | >6.9 | 2.74 ± 0.29 | 5.0 | <0.40 | 3.66 ± 0.33 | 11.0 ± 1.0 (CNS+) |
| **1**[d] | | | | 0.61 ± 0.03 | 2.94 ± 0.20 | >10 | 7.80 ± 0.47 | 13 | <0.78 | 0.61 ± 0.03[e] | 23.8 ± 2.7 (CNS+)[f] |
| chloroquine | | | | | | 0.93 ± 0.44 | | | | | |

[a] *In vitro* activity against bloodstream form of *T. brucei* (pH 7.4), *P. falciparum* (strain K1), and rat myoblast L6 cells expressed at the concentration that inhibited growth by 50% ($IC_{50}$) and 90% ($IC_{90}$, for trypanocidal activity) and *in vitro* activity against human recombinant AChE expressed at the concentration that inhibited enzyme activity by 50% ($IC_{50}$). Data are the mean of triplicate experiments ± SEM.

[b] SI: Selectivity index is the ratio of cytotoxic to trypanocidal ($SI_{Tb}$) or antiplasmodial ($SI_{Pf}$) $IC_{50}$ values.

[c] Permeability values from the PAMPA-BBB assay. Values are expressed as the mean ± SD of three independent experiments.

[d] Antiprotozoal and cytotoxic activity values of reference compound **1** taken from ref. 30.

[e] Taken from ref. 40.

[f] Taken from ref. 45.

Brain permeation of the novel heterodimers was determined *in vitro* through the widely used PAMPA-BBB assay,[44] using a lipid extract of porcine brain as an artificial membrane model of BBB. The heterodimerization strategy, as it was also found for homodimerization,[37] results in dibasic bis(4-aminoquinoline) derivatives that will be mostly diprotonated under the assay conditions, which explains their lower permeabilities relative to the parent monobasic huprine Y (Table 2). However, all the heterodimers had permeabilities well above the threshold established for high BBB permeation ($P_e$ ($10^{-6}$ cm $s^{-1}$) > 5.1, CNS+, Table 2). As expected, elongation of the linker from 10 to 12 methylenes and the presence of a chlorine substituent at the huprine moiety lead to increased lipophilicity, and, hence, increased brain permeability.

As expected, replacement of the 3-chlorosubstituted huprine Y unit in these heterodimers by a 3-unsubstituted huprine moiety results in general in a reduced inhibitory activity against human recombinant AChE. However, the reduction of this activity was very modest, so that the novel heterodimers **16f-g** and **17f-g** remain very active anticholinesterasic compounds, as it is also the case for **3g** and **4g** (Table 2).

Thus, extension of series **I** with the novel heterodimers has resulted in the identification of two of the most potent trypanocidal compounds of the series, **16f** and **16g**, the latter displaying also significant antiplasmodial activity. Reduction of their cytotoxicity and AChE inhibitory activities will need to be addressed in future development of this structural class.

### *2.4. Determination of the trypanocidal and antiplasmodial mode of action of 4-aminoquinoline-based heterodimeric compounds of series I-III*

Identification of the biological target responsible or, at least, in part involved in the trypanocidal and antiplasmodial activity of the heterodimers of series **I-III** might enable further optimization of the initial hits through target-based screening or by structure-based rational design of novel analogues, when 3D structures of the biological target are available.

#### *2.4.1. Trypanothione reductase inhibitory activity*

Trypanothione reductase (TryR) is a NADPH-dependent disulfide oxidoreductase enzyme that is involved in the protection of trypanosomatid parasites from oxidative damage. Because it is unique and essential to these parasites, TryR constitutes a valuable target for trypanocidal drug discovery programs.[46,47]

A number of quinoline (ref 20) or aminoacridine derivatives have been reported to be inhibitors of TryR.[20,46,48,49] In these compounds the presence of a protonatable nitrogen atom seems to be crucial for the TryR inhibitory activity because it mimics the positively charged substrate, trypanothione disulfide. Interestingly, it has been reported that dimerization of several classes of compounds with known TryR inhibitory activity leads to increased potency,[50-52] which might be related to the presence of two interacting binding sites in the large active site of TryR.[49]

These precedents prompted us to determine whether TryR was a biological target for aminoquinoline-based heterodimers of series **I-III**. The TryR inhibitory activity of selected compounds of these series, namely heterodimers **3d**, **5b** and **8b**, as well as the parent huprine Y, **1**, was determined through a described methodology based on the colorimetric reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) by dithiol trypanothione ($T[SH]_2$), the product of the reaction catalyzed by TryR.[53,54]

The four tested compounds exhibited submicromolar to low micromolar $IC_{50}$ values for TryR inhibition (Table 3). The most potent compound turned out to be **3d** (series **I**) which was 6-8-fold more potent than the parent huprine and the heterodimers of the other series. Thus, as previously reported for other structural classes, dimerization of a 4-aminoquinoline motif in **3d** relative to huprine Y resulted in increased TryR inhibitory activity.

**Table 3**
Inhibitory activity of selected 4-aminoquinoline-based heterodimeric compounds and reference compounds **1**, tetracycline hydrochloride and chloroquine diphosphate against *T. brucei* trypanothione reductase and β-haematin formation[a]

| Compd | *Tb* TryR[b] | $BHIA_{50}$[c] |
|---|---|---|
| | $IC_{50}$ μM | |
| **3d** | 0.87 ± 0.10 | 0.19 ± 0.02 |
| **5b** | 4.88 ± 0.44 | 0.42 ± 0.06 |
| **8b** | 7.39 ± 0.43 | 0.54 ± 0.08 |
| **1** | 5.00 ± 0.31 | >4 |
| tetracycline | | >4 |
| chloroquine | | 0.14 ± 0.05 |

[a] Values are expressed as the mean ± SD of three independent experiments.

[b] *In vitro* activity against recombinant *T. brucei* TryR expressed at the concentration that inhibited enzyme activity by 50% ($IC_{50}$).

[b] β-Haematin inhibitory activity in equivalents of drug relative to haemin causing 50% inhibition.

Of note, no correlation was found between the trypanocidal and TryR inhibitory activities of these compounds. Indeed, all the tested compounds displayed more potent trypanocidal than TryR inhibitory activity (6-62-fold). These results might suggest that either TryR is not the sole target of these compounds or that they might be selectively concentrated into the cell or metabolically activated, as suggested for other trypanocidal compounds.[55]

### *2.4.2. Inhibition of β-haematin formation*

The 4-amino-7-chloroquinoline unit present in chloroquine and other antimalarial compounds is the pharmacophoric moiety responsible for the inhibition of dimerization and crystallization of free haem, generated during the digestion of hemoglobin in the host erythrocytes, into nontoxic insoluble haemozoin, which results in increased levels of toxic haem and, hence, in parasite death.[28,56]

Huprine Y, **1**, and bis-huprine **2** contain the 4-amino-7-chloroquinoline moiety but they are inactive against *P. falciparum*.[30,37] Surprisingly, many heterodimers of series **I**-**III**, which in most cases also feature one or two 4-amino-7-chloroquinoline motifs, have been found to be quite potent antiplasmodial compounds (Tables 1 and 2). In the light of these results, we assessed whether the antiplasmodial activity of these heterodimers relied on the same mechanism of action of chloroquine, by determining the inhibition of the formation of β-haematin, which is identical to haemozoin. Thus, the inhibitiory activity against β-haematin formation of heterodimers **3d**, **5b** and **8b** was evaluated following a described procedure,[57,58] and expressed as the 50% inhibitory concentration for β-haematin inhibition in equivalents of the tested compound relative to haemin ($BHIA_{50}$). The parent huprine Y and tetracycline were also evaluated as negative controls and chloroquine was used as a positive control. All the tested heterodimers inhibited β-haematin formation comparably to that seen with chloroquine, whereas huprine Y and tetracycline showed no inhibition (Table 3). Particularly, heterodimer **3d** (series **I**) was equipotent to chloroquine as inhibitor of β-haematin formation (Table 3) and heterodimers **5b** and **8b** turned out to be slightly less potent (3- and 4-fold). Because the β-haematin formation inhibitory activities of these heterodimers correlate well with their antiplasmodial activities, it might be suggested that inhibition of β-haematin formation is likely an important mechanim by which they inhibit the growth of malaria parasites.

## 3. Conclusion

We have identified a number of dual submicromolar trypanocidal-antiplasmodial compounds by phenotypic screening against bloodstream forms of *T. brucei* and the multidrug-resistant strain K1 of *P. falciparum* of a small library of 27 brain permeable 4-aminoquinoline-based heterodimeric compounds belonging to three distinct structural classes. Indeed, all of the tested compounds displayed submicromolar $IC_{50}$ values, and the vast majority also submicromolar $IC_{90}$ values against *T. brucei*, they being more potent trypanocidal agents than the parent huprine Y. One third of them exhibited also submicromolar $IC_{50}$ values against *P. falciparum* and increased potency relative to the reference antimalarial compound chloroquine. In a further extension of the most promising structural class (series **I**) to expand the structure-activity relationships studies and to decrease the AChE inhibitory activity, which was present in all the screened compounds, 6 novel heterodimers were synthesized and biologically evaluated. All of the novel compounds kept high trypanocidal potency and good BBB permeability and a few of them also moderately potent antiplasmodial activity, but also AChE inhibitory potency and not too adequate selectivity indices.

Mechanistic studies have shown that the high trypanocidal activity of these heterodimers can be only partly ascribed to inhibition of the enzyme TryR, whereas their antiplasmodial activity seems to arise from an inhibition of the haem detoxification process, like in the antimalarial drug chloroquine.

Heterodimer **3e** (series **I**) emerges as an interesting hit featuring balanced dual trypanocidal ($IC_{50}$ = 0.21 μM) and antiplasmodial ($IC_{50}$ = 0.35 μM) activity and selectivity indices of 34 and 20 for these activities over rat L6 cell cytotoxicity. Further optimization should address an improvement of the selectivity indices and a reduction of AChE inhibitory activities of these heterodimers.

## 4. Experimental

### *4.1. Chemistry*

Melting points were determined in open capillary tubes with a MFB 595010M Gallenkamp melting point apparatus. 400 MHz $^{1}H$ / 100.6 MHz $^{13}C$ NMR spectra were recorded on a Varian Mercury 400 spectrometer at the Centres Científics i Tecnològics of the University of Barcelona (CCiTUB). The chemical shifts are reported in ppm ($\delta$ scale) and coupling constants are reported in Hertz (Hz). The *syn* (*anti*) notation of the protons at position 13 of the huprine moiety of the heterodimers means that the corresponding proton at position 13 is on the same (different) side of the quinoline moiety with respect to the cyclohexene ring. IR spectra were run on a Perkin-Elmer Spectrum RX I spectrophotometer, using KBr pellets. Absorption values are expressed as wave-numbers ($cm^{-1}$); only significant absorption bands are given. Column chromatography was performed on silica gel 60 AC.C (40–60 μM, SDS, ref 2000027). Thin-layer chromatography was performed with aluminum-backed sheets with silica gel 60 $F_{254}$ (Merck, ref 1.05554), and spots were visualized with UV light and 1% aqueous solution of $KMnO_4$. Elemental analyses and high resolution mass spectra were carried

out at the Mycroanalysis Service of the IIQAB (CSIC, Barcelona, Spain) with a Carlo Erba 1106 analyzer, and at the CCiTUB with a LC/MSD TOF Agilent Technologies spectrometer, respectively. The analytical samples of all of the heterodimers that were subjected to pharmacological evaluation were dried at 65 ºC / 2 Torr at least for 2 days (standard conditions) and possess a purity ≥95% as evidenced by their elemental analyses.

#### *4.1.1. 9-(12-Bromododecyl)amino-1,2,3,4-tetrahydroacridine (13g)*

A suspension of tacrine, **11** (3.00 g, 15.2 mmol, 1 eq), and finely powdered KOH (85% purity reagent, 1.60 g, 24.3 mmol, 1.6 eq) in anhydrous DMSO (37 mL) was stirred, heating every 10 min approximately with a heat gun for 1 h and at rt for an additional hour. This mixture was added dropwise during 1.5 h to a solution of 1,12-dibromododecane (5.98 g, 18.2 mmol, 1.2 eq) in anhydrous DMSO (20 mL), containing 4 Å molecular sieves. The reaction mixture was stirred at rt overnight, diluted with 10N NaOH (150 mL) and $H_2O$ (200 mL) and extracted with EtOAc (3×200 mL). The combined organic extracts were washed with $H_2O$ (2×200 mL), dried over anhydrous $Na_2SO_4$, and evaporated at reduced pressure to give a yellow oil (8.02 g), which was purified by column chromatography (40–60 µm silica gel, $CH_2Cl_2$/50% aq. $NH_4OH$ 100:0.2), to afford bromoalkyl derivative **13g** (2.65 g, 40% yield); $R_f$ 0.27 ($CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 9:1:0.05).

A solution of **13g** (106 mg, 0.24 mmol) in MeOH (10 mL) was filtered through a 0.2 µm NYL filter and treated with 45% aq. HBr (0.50 mL, 4.14 mmol, 17 eq). The resulting solution was evaporated at reduced pressure and the solid was taken in MeOH (0.1 mL) and precipitated upon addition of AcOEt (1.2 mL). The precipitated solid was separated and washed with pentane (3×2 mL) to give, after drying under standard conditions, **13g**·HBr (49 mg) as a yellowish solid: mp 131–133 ºC; IR (KBr) *ν* 3500–2500 (max at 3255, 3044, 3004, 2915, 2846 and 2793, $N^+$–H, N–H, and C–H st), 1629, 1588, 1573 and 1517 (Ar–C–C, Ar–C–N st) $cm^{-1}$; $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 1.29–1.47 (complex signal, 16H, 3'-$H_2$, 4'-$H_2$, 5'-$H_2$, 6'-$H_2$, 7'-$H_2$, 8'-$H_2$, 9'-$H_2$, 10'-$H_2$), 1.79–1.87 (complex signal, 4H, 2'-$H_2$, 11'-$H_2$), 1.94–2.01 (complex signal, 4H, 2-$H_2$, 3-$H_2$), 2.71 (m, 2H, 1-$H_2$), 3.02 (m, 2H, 4-$H_2$), 3.43 (t, *J* = 6.6 Hz, 2H, 12'-$H_2$), 3.96 (t, *J* = 7.2 Hz, 2H, 1'-$H_2$), 4.85 (s, NH, $NH^+$), 7.59 (ddd, *J* = 8.6 Hz, *J'* = 7.0 Hz, *J''* = 1.6 Hz, 1H, 7-H), 7.75 (dd, *J* = 8.4 Hz, *J'* = 0.8 Hz, 1H, 5-H), 7.86 (ddd, *J* = 8.3 Hz, *J'* = 6.9 Hz, *J''* = 1.3 Hz, 1H, 6-H), 8.40 (d, *J* = 8.4 Hz, 1H, 8-H); $^{13}$C NMR (100.6 MHz, $CD_3OD$) $\delta$ 21.8 ($CH_2$, C3), 23.0 ($CH_2$, C2), 24.9 ($CH_2$, C1), 27.7 ($CH_2$ C3'), 29.1 ($CH_2$, C10'), 29.3 ($CH_2$, C4), 29.8 ($CH_2$), 30.2 ($CH_2$), 30.51 (3 $CH_2$) and 30.54 ($CH_2$), (C4', C5', C6', C7', C8', C9'), 31.5 ($CH_2$) and 34.0 ($CH_2$) (C2', C11'), 34.5 ($CH_2$, C8'), 49.2 (2 $CH_2$, C1', C12'), 112.8 (C) and 117.0 (C) (C8a, C9a) 120.1 (CH, C5), 126.3 (CH, C7), 126.5 (CH, C8), 134.1 (C, C6), 139.7 (C, C10a), 151.6 (C, C4a), 158.0 (C, C9); HRMS (ESI), calcd for [$C_{25}H_{37}BrN_2$ + $H^+$] 445.2213, found 445.2212.

#### *4.1.2. 9-(12-Bromododecyl)amino-6-chloro-1,2,3,4-tetrahydroacridine (14g)*

It was prepared as described for **13g**. Starting from 6-chlorotacrine, **12** (2.00 g, 8.60 mmol, 1 eq), and a solution of 1,12-dibromododecane (3.38 g, 10.3 mmol, 1.2 eq) in anhydrous DMSO (20 mL), a brown oil (4.70 g) was obtained and subjected to column chromatography purification (40–60 µm silica gel, $CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ mixtures, gradient elution). On elution with $CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 100:0:0.2, bromoalkyl derivative **14g** (546 mg) was isolated. On elution with $CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 100:0:0.2 to 90:10:0.2, a mixture of **14g** and starting **12** (2.60 g) was obtained. Column chromatography purification of this mixture (40–60 µm silica gel, $CH_2Cl_2$/50% aq. $NH_4OH$ 100:0.2) afforded more **14g** (691 mg, 30% total yield); $R_f$ 0.46 ($CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 9:1:0.05).

A solution of **14g** (100 mg, 0.21 mmol) in MeOH (18 mL) was filtered through a 0.2 µm NYL filter and treated with 45% aq. HBr (0.5 mL, 4.14 mmol, 20 eq). The resulting solution was evaporated at reduced pressure and the solid was washed with pentane (3×2 mL) to give, after drying under standard conditions, **14g**·HBr (87 mg) as a beige solid: mp 122–124 ºC; IR (KBr) *ν* 3500–2500 (max at 3241, 3134, 3044, 2921, 2850 and 2791 $N^+$–H, N–H, and C–H st), 1630, 1620, 1588, 1572, 1546 and 1521, (Ar–C–C, Ar–C–N st) $cm^{-1}$; $^1$H NMR (400 MHz, CD3OD) $\delta$ 1.29–1.43 (complex signal, 16H, 3'-$H_2$, 4'-$H_2$, 5'-$H_2$, 6'-$H_2$, 7'-$H_2$, 8'-$H_2$, 9'-$H_2$, 10'-$H_2$), 1.78–1.87 (complex signal, 4H, 2'-$H_2$, 11'-$H_2$), 1.96–1.97 (complex signal, 4H, 2-$H_2$, 3-$H_2$), 2.68 (m, 2H, 1-$H_2$), 3.00 (m, 2H, 4-$H_2$), 3.43 (t, *J* = 6.6 Hz, 2H, 12'-$H_2$), 3.94 (t, *J* = 7.6 Hz, 2H, 1'-$H_2$), 4.85 (s, NH, $NH^+$), 7.56 (dd, *J* = 9.2 Hz, *J'* = 2.0 Hz, 1H, 7-H), 7.77 (d, *J* = 2 Hz, 1H, 5-H), 8.39 (d, *J* = 9.2 Hz, 1H, 8-H); $^{13}$C NMR (100.6 MHz, $CD_3OD$) $\delta$ 21.8 ($CH_2$, C3), 22.9 ($CH_2$, C2), 24.7 ($CH_2$, C1), 27.7 ($CH_2$, C3'), 29.1 ($CH_2$, C10'), 29.3 ($CH_2$, C4), 29.8 ($CH_2$), 30.2 ($CH_2$) and 30.5 (4 $CH_2$) (C4', C5', C6', C7', C8', C9'), 31.3 ($CH_2$) and 34.0 ($CH_2$) (C2', C11'), 34.4 ($CH_2$, C8'), 49.2 (2 $CH_2$, C1', C12'), 113.3 (C) and 115.4 (C) (C8a, C9a), 119.1 (CH, C5), 126.8 (CH, C7), 128.8 (CH, C8), 140.1 (C, C6), 140.5 (C, C10a), 152.1 (C, C4a), 157.9 (C, C9); HRMS (ESI), calcd for [$C_{25}H_{36}BrClN_2$ + $H^+$] 479.1823, found 479.1830.

#### *4.1.3. 3-Chloro-6,7,10,11-tetrahydro-9-methyl-12-{{12-[(1,2,3,4-tetrahydroacridin-9-yl)amino]dodecyl}amino}-7,11-methanocycloocta[b]quinoline (3g)*

A mixture of finely powdered KOH (85% purity reagent, 209 mg, 3.16 mmol, 3 eq), huprine **1** (300 mg, 1.05 mmol, 1 eq), 4 Å molecular sieves (approximately 690 mg) in dry DMSO (4 mL) was thoroughly stirred for 1 h heating with a heatgun every 10 min and for one additional hour at rt. The resulting mixture was added dropwise during 1 h to a mixture of bromoalkyltacrine **13g** (562 mg, 1.26 mmol, 1.2 eq) in dry DMSO (6 mL). The reaction mixture was vigorously stirred at rt for 4 days, diluted with 10 N NaOH (150 mL), $H_2O$ (200 mL), and extracted with AcOEt (3×200 mL). The combined organic extracts were washed with 1N NaOH (3×200 mL), dried with anhydrous $Na_2SO_4$ and evaporated at reduced pressure to give a brown oily residue (820 mg), which was subjected to column chromatography (40–60 mesh silica gel, $CH_2Cl_2$/50% aqueous $NH_4OH$ 100:0.2), to afford heterodimer **3g** (507 mg, 71% yield) as a yellow oil; $R_f$ 0.48 ($CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 9:1:0.05).

A solution of **3g** (507 mg, 0.78 mmol) in $CH_2Cl_2$ (26 mL) was filtered through a 0.2 µm NYL filter, and treated with an excess of a methanolic solution of HCl (1.45 N, 4.9 mL, 7.10 mmol) and the resulting solution was concentrated in vacuo to dryness. The solid was taken in MeOH (1.5 mL) and precipitated upon addition of AcOEt (12 mL). The precipitated solid was separated, washed with pentane (3×2), and dried under standard conditions, to give **3g**·2HCl (362 mg) as a yellow solid: mp: 165-168 ºC; IR (KBr) *ν* 3500–2500 (max at 3380, 3234, 3108, 3048, 3007, 2923, 2850, 2788 and 2651, $N^+$–H, N–H, and C–H st), 1632, 1583 and 1521 (Ar–C–C, Ar–C–N st) $cm^{-1}$; $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 1.27–1.46 (complex signal, 16H, 3'-$H_2$, 4'-$H_2$, 5'-$H_2$, 6'-$H_2$, 7'-$H_2$, 8'-$H_2$, 9'-$H_2$, 10'-$H_2$), 1.58 (s, 3H, 9-$CH_3$), 1.79–1.95 (complex signal, 10H, 10-$H_{endo}$, 13-$H_{syn}$, 2'-$H_2$, 11'-$H_2$, 2''-$H_2$, 3''-$H_2$), 2.08 (d, *J* = 12.4 Hz, 1H, 13-$H_{anti}$), 2.55 (br dd, *J* = 17.2 Hz, *J'* = 3.6 Hz, 1H, 10-$H_{exo}$), 2.70 (m, 2H, 1''-$H_2$), 2.77 (br m, 1H, 7-

H), 2.88 (d, *J* = 17.6 Hz, 1H, 6-$H_{endo}$), 3.02 (m, 2H, 4"-$H_2$), 3.20 (dd, *J* = 18.0 Hz, *J'* = 5.2 Hz, 1H, 6-$H_{exo}$), 3.46 (br m, 1H, 11-H), 3.96 (m, 4H, 1'-$H_2$, 12'-$H_2$), 4.85 (s, NH, $NH^+$), 5.58 (br d, *J* = 5.2 Hz, 1H, 8-H), 7.54 (d, *J* = 9.2 Hz, 1H, 2-H), 7.56 (t, *J* = 7.6 Hz, 1 H, 7"-H), 7.80 (s, 1H, 4-H), superimposed in part 7.77 (d, *J* = 7.2 Hz, 1H, 5"-H), superimposed in part 7.83 (dd, *J* = 14.8 Hz, *J'* = 8.0 Hz, 1H, 6"-H), 8.38 (dd, *J* = 8.8 Hz, *J'* = 2.4 Hz, 2 H, 1-H, 8''-H); $^{13}$C NMR (100.6 MHz, $CD_3OD$) $\delta$ 21.8 ($CH_2$, C3"), 23.0 ($CH_2$, C2"), 23.5 ($CH_3$, 9-$CH_3$), 24.9 ($CH_2$, C1"), 27.3 (CH, C11), 27.68 (CH), 27.77 ($CH_2$) and 27.81 (CH) (C7, C3', C10'), 29.3 (2 $CH_2$) (C13, C4"), 30.2 ($CH_2$), 30.3 ($CH_2$) and 30.6 (4 $CH_2$) (C4', C5', C6', C7', C8', C9'), 31.2 ($CH_2$) and 31.5 ($CH_2$) (C2', C11'), 36.0 ($CH_2$) and 36.1 ($CH_2$) (C6, C10), 49.1 ($CH_2$) and 49.6 ($CH_2$) (C1', C12'), 112.8 (C), 115.6 (C), 117.0 (C) and 117.6 (C) (C11a, C12a, C8a", C9a"), 119.1 (CH) and 120.1 (CH) (C4, C5"), 125.1 (CH, C8), 126.3 (CH, C7"), 126.5 (CH, C8"), 126.6 (CH, C2), 129.4 (CH, C1), 134.1 (CH, C6"), 134.5 (C, C9), 139.7 (C), 140.2 (C) and 141.0 (C) (C3, C4a, C10a"), 151.2 (C) and 151.6 (C) (C5a, C4a"), 156.8 (C), 158.0 (C) (C12, C9"); HRMS (ESI), calcd for [$C_{42}H_{54}{}^{35}ClN_4$ + $H^+$] 649.4032, found 649.4019; Anal. calcd. for $C_{42}H_{53}ClN_4 \cdot 2HCl \cdot 1.5H_2O$: C, 67.32; H, 7.80; N, 7.48; Cl, 14.19. Found: C, 67.33; H, 7.88; N, 7.29; Cl, 14.47.

#### *4.1.4. 3-Chloro-6,7,10,11-tetrahydro-9-methyl-12-{{12-[(6-chloro-1,2,3,4-tetrahydroacridin-9-yl)amino]dodecyl}amino}-7,11-methanocycloocta[b]quinoline (4g)*

It was prepared as described for **3g**. Starting from huprine **1** (290 mg, 1.02 mmol, 1 eq), and a solution of of bromoalkyltacrine **14g** (587 mg, 1.22 mmol, 1.2 eq) in anhydrous DMSO (6 mL), a brown oily residue (1.20 g) was obtained and subjected to column chromatography purification (40–60 μm silica gel, $CH_2Cl_2$/50% aq. $NH_4OH$ 100:0.2), to afford the heterodimer **4g** (311 mg, 45% yield) as a yellow oil; $R_f$ 0.67 ($CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 9:1:0.05).

A solution of **4g** (122 mg, 0.18 mmol) in $CH_2Cl_2$ (6 mL) was filtered through a 0.2 μm NYL filter, and treated with an excess of a methanolic solution of HCl (1.45 N, 1.1 mL, 1.59 mmol) and the resulting solution was concentrated in vacuo to dryness. The solid was taken in MeOH (0.5 mL) and precipitated upon addition of AcOEt (3 mL). The precipitated solid was separated, washed with pentane (3×2), and dried under standard conditions, to give **4g**·2HCl (49 mg) as a yellow solid: mp 178–181 ºC; IR (KBr) *ν* 3500–2500 (max at 3224, 3034, 2924, 2854 and 2786, $N^+$–H, N–H, and C–H st), 1632, 1583, 1572 and 1519 (Ar–C–C, Ar–C–N st) $cm^{-1}$; $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 1.27–1.44 (complex signal, 16H, 3'-$H_2$, 4'-$H_2$, 5'-$H_2$, 6'-$H_2$, 7'-$H_2$, 8'-$H_2$, 9'-$H_2$, 10'-$H_2$), 1.58 (s, 3H, 9-$CH_3$), 1.79–1.95 (complex signal, 10H, 10-$H_{endo}$, 13-$H_{syn}$, 2'-$H_2$, 11'-$H_2$, 2"-$H_2$, 3"-$H_2$), 2.08 (br dm, *J* = 12.4 Hz, 1H, 13-$H_{anti}$), 2.55 (br dd, *J* = 17.2 Hz, *J'* = 4.4, 1H, 10-$H_{exo}$), 2.67 (br m, 2H, 1"-$H_2$), 2.77 (br m, 1H, 7-H), 2.87 (d, *J* = 18.0 Hz, 1H, 6-$H_{endo}$), 3.00 (br m, 2H, 4"-$H_2$), 3.20 (dd, *J* = 18.0 Hz, *J'* = 5.6 Hz, 1H, 6-$H_{exo}$), 3.45 (br m, 1H, 11-H), 3.96 (m, 4H, 1'-$H_2$,12'-$H_2$), 4.85 (s, NH, $NH^+$), 5.58 (br d, *J* = 4.4 Hz, 1H, 8-H), 7.55 (d, *J* = 9.6 Hz, 1 H, 2-H, 7"-H), 7.79 (d, *J* = 3.6 Hz, 1H, 4-H, 5"-H), 8.38 (dd, *J* = 9.0 Hz, *J'* = 4.2 Hz, 2 H, 1-H, 8"-H); $^{13}$C NMR (100.6 MHz, $CD_3OD$) $\delta$ 21.8 ($CH_2$, C3"), 22.8 ($CH_2$, C2"), 23.5 ($CH_3$, 9-$CH_3$), 24.5 ($CH_2$, C1"), 24.7 (CH), 27.3 (CH, C11), 27.7 (CH), 27.79 ($CH_2$) and 27.81 (CH) (C7, C3', C10'), 28.4 ($CH_2$), 29.3 ($CH_2$) (2 $CH_2$, C13, C4"), 30.2 (2 $CH_2$), 30.58 (2 $CH_2$) and 30.60 (2 $CH_2$) (C4', C5', C6', C7', C8', C9'), 31.2 ($CH_2$) and 31.3 ($CH_2$) (C2', C11'), 36.0 ($CH_2$, C10), 36.1 ($CH_2$, C6), 49.1 ($CH_2$) and 49.6 ($CH_2$) (C1', C12'), 113.3 (C), 115.4 (C), 115.6 (C) and 117.6 (C) (C11a, C12a, C8a" and C9a"), 119.2 (CH) (C4, C5"), 125.1 (CH, C8), 126.6 (CH) and 126.7 (CH) (C2, C7"), 128.7 (CH) and 129.4 (CH) (C1, C8"), 134.5 (CH, C9), 140.1 (C) and 140.2 (C) (C3, C6") 140.5 (C) and 141.0 (C) (C4a, C10a"), 151.2 (C) and 152.1 (C) (C5a, C4a"), 156.9 (C), 157.8 (C) (C12, C9"); HRMS (ESI), calcd for [$C_{42}H_{52}{}^{35}Cl_2N_4$ + $H^+$] 638.3641, found 683.3622; Anal. calcd. for $C_{42}H_{52}Cl_2N_4 \cdot 2HCl \cdot 1.5H_2O$: C, 64.37; H, 7.33; N, 7.15; Cl, 18.09. Found: C, 64.04; H, 7.08; N, 6.81; Cl, 17.71.

#### *4.1.5. 6,7,10,11-Tetrahydro-9-methyl-12-{{10-[(1,2,3,4-tetrahydroacridin-9-yl)amino]decyl}amino}-7,11-methanocycloocta[b]quinoline (16f)*

It was prepared as described for **3g**. Starting from huprine **15** (250 mg, 1.00 mmol, 1 eq), and a solution of of bromoalkyltacrine **13f** (551 mg, 1.32 mmol, 1.3 eq) in anhydrous DMSO (6 mL), a brown oily residue (1.04 g) was obtained and subjected to column chromatography purification (40–60 μm silica gel, $CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ mixtures, gradient elution). On elution with $CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 100:0:0.2 to 95:5:0.2, the heterodimer **16f** (518 mg, 88% yield) was isolated as a yellow oil; $R_f$ 0.44 ($CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 9:1:0.05).

A solution of **16f** (518 mg, 0.88 mmol) in $CH_2Cl_2$ (25 mL) was filtered through a 0.2 μm NYL filter, and treated with an excess of a methanolic solution of HCl (1.70 N, 4.65 mL, 7.90 mmol) and the resulting solution was concentrated in vacuo to dryness. The solid was taken in MeOH (1.5 mL) and precipitated upon addition of AcOEt (11 mL). The precipitated solid was separated, washed with pentane (3×2), and dried under standard conditions, to give **16f**·2HCl (481 mg) as a yellow solid: mp 168–170 ºC; IR (KBr) *ν* 3500–2500 (max at 3380, 3237, 3120, 3055, 3013, 2925, 2852, 2805, 2722 and 2663, $N^+$–H, N–H, and C–H st), 1633, 1585 and 1522 (Ar–C–C, Ar–C–N st) $cm^{-1}$; $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 1.27–1.44 (complex signal, 12H, 3'-$H_2$, 4'-$H_2$, 5'-$H_2$, 6'-$H_2$, 7'-$H_2$, 8'-$H_2$), 1.57 (s, 3H, 9-$CH_3$), 1.79–1.96 (complex signal, 10H, 10-$H_{endo}$, 13-$H_{syn}$, 2'-$H_2$, 9'-$H_2$, 2"-$H_2$, 3"-$H_2$), 2.08 (dm, *J* = 12.4 Hz, 1H, 13-$H_{anti}$), 2.55 (br dd, *J* = 17.1 Hz, *J'* = 3.5 Hz, 1H, 10-$H_{exo}$), 2.70 (m, 1H, 1"-$H_2$), 2.77 (br m, 1H, 7-H), 2.90 (d, *J* = 18.0 Hz, 1H, 6-$H_{endo}$), 3.02 (m, 2H, 4"-$H_2$), 3.21 (dd, *J* = 18.0 Hz, *J'* = 5.6 Hz, 1H, 6-$H_{exo}$), 3.47 (br m, 1H, 11-H), 3.97 (complex signal, 4H, 1'-$H_2$, 10'-$H_2$), 4.85 (s, NH, $NH^+$), 5.58 (br d, *J* = 4.4 Hz, 1H, 8-H), 7.57 (t, *J* = 6 Hz, 2H, 4-H, 5"-H), 7.78 (t, *J'* = 7.0 Hz, 2H) and 7.84 (t, *J'* = 7.0 Hz, 2H) (2-H, 3-H, 6"-H, 7"-H), 8.39 (dd , *J* = 8.6 Hz, *J'* = 3.8 Hz, 2 H, 1-H, 8"-H); $^{13}$C NMR (100.6 MHz, $CD_3OD$) $\delta$ 21.9 ($CH_2$, C3"), 23.0 ($CH_2$, C2"), 23.5 ($CH_3$, 9-$CH_3$), 24.9 ($CH_2$, C1"), 27.3 (CH, C11), 27.7 (CH), 27.8 ($CH_2$) and 27.9 (CH) (C7, C3', C8'), 29.3 ($CH_2$, C13), 29.4 ($CH_2$, C4"), 30.26 ($CH_2$), 30.29 ($CH_2$), 30.48 ($CH_2$) and 30.53 ($CH_2$) (C4', C5', C6', C7'), 31.4 ($CH_2$) and 31.5 ($CH_2$) (C2', C9'), 36.0 ($CH_2$) and 36.3 ($CH_2$) (C6, C10), 49.1 ($CH_2$) and 49.6 ($CH_2$) (C1', C10'), 112.8 (C), 117.0 (C), 117.15 (C) and 117.21 (C) (C11a, C12a, C8a", C9a"), 120.1 (CH) and 125.2 (CH) (C4, C5"), 126.1 (CH) and 126.3 (CH) (C8, C7"), 126.5 (CH, C8"), 127.3 (CH), 134.1 (CH), 134.2 (CH), 134.5 (C), 139.8 (C) and 140.2 (C) (C1, C2, C3, C4a, C9, C6", C10a"), 150.7 (C) and 151.6 (C) (C5a, C4a"), 157.0 (C) and 158.0 (C) (C12, C9"); HRMS (ESI), calcd for [$C_{40}H_{50}N_4$ + $H^+$] 587.4108, found 587.4099; Anal. calcd. for $C_{40}H_{50}N_4 \cdot 2HCl \cdot 2H_2O$: C, 69.05; H, 8.11; N, 8.05; Cl, 10.19. Found: C, 68.68; H, 8.08; N, 7.76; Cl, 10.18.

#### *4.1.6. 6,7,10,11-Tetrahydro-9-methyl-12-{{12-[(1,2,3,4-tetrahydroacridin-9-yl)amino]dodecyl}amino}-7,11-methanocycloocta[b]quinoline (16g)*

It was prepared as described for **3g**. Starting from huprine **15** (300 mg, 1.20 mmol, 1 eq), and a solution of of bromoalkyltacrine **13g** (642 mg, 1.44 mmol, 1.2 eq) in anhydrous DMSO (6.5 mL), a brown oily residue (805 mg) was obtained and subjected to column chromatography purification (40–60 μm silica gel, $CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ mixtures, gradient elution. On elution with $CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 99:1:0.2, the heterodimer **16g** (609 mg, 83% yield) was isolated as a yellow oil; $R_f$ 0.53 ($CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 9:1:0.05).

A solution of **16g** (609 mg, 0.99 mmol) in $CH_2Cl_2$ (33 mL) was filtered through a 0.2 μm NYL filter, and treated with an excess of a methanolic solution of HCl (1.45 N, 6.2 mL, 8.99 mmol) and the resulting solution was concentrated in vacuo to dryness. The solid was taken in MeOH (2 mL) and precipitated upon addition of AcOEt (14 mL). The precipitated solid was separated, washed with pentane (3×2), and dried under standard conditions, to give **16g**·2HCl (612 mg) as a yellow solid: mp 159–162 ºC; IR (KBr) $\nu$ 3500–2500 (max at 3386, 3236, 3114, 3054, 3007, 2923, 2851, 2800, 2722 and 2663, $N^+$–H, N–H, and C–H st), 1633, 1585, 1568 and 1520 (Ar–C–C, Ar–C–N st) $cm^{-1}$; $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 1.28–1.46 (complex signal, 16H, 3'-$H_2$, 4'-$H_2$, 5'-$H_2$, 6'-$H_2$, 7'-$H_2$, 8'-$H_2$, 9'-$H_2$, 10'-$H_2$), 1.58 (s, 3H, 9-$CH_3$), 1.79-1.97 (complex signal, 10H, 10-$H_{endo}$, 13-$H_{syn}$, 2'-$H_2$, 11'-$H_2$, 2"-$H_2$ and 3"-$H_2$), 2.09 (dm, $J$ = 11.0 Hz, 1H, 13-$H_{anti}$), 2.56 (br dd, $J$ = 17.6 Hz, $J'$ = 4.4 Hz, 1H, 10-$H_{exo}$), 2.71 (m, 1H, 1"-$H_2$), 2.77 (br m, 1H, 7-H), 2.90 (d, $J$ = 18.0 Hz, 1H, 6-$H_{endo}$), 3.02 (m, 2H, 4"-$H_2$), 3.21 (dd, $J$ = 17.8 Hz, $J'$ = 5.4 Hz, 1H, 6-$H_{exo}$), 3.48 (br m, 1H, 11-H), 3.95 (t, $J$ = 7.8 Hz, 2H) and 4.00 (td, $J$ = 10.4 Hz, $J'$ = 7.2 Hz, $J''$ = 3.4 Hz, 2H) (1'-$H_2$, 12'-$H_2$), 4.85 (s, NH, $NH^+$), 5.58 (br d, $J$ = 4.4 Hz, 1H, 8-H), 7.57 (tdd, $J$ = 7.6 Hz, $J'$ = 2.8 Hz, $J''$ = 1.3 Hz, 2 H, 4-H, 5"-H), 7.78 (t, $J$ = 7.6 Hz, 2H) and 7.84 (t, $J$ = 7.6 Hz, 2H) (2-H, 3-H, 6"-H and 7"-H), 8.40 (dd , $J$ = 8.6 Hz, $J'$ = 4.2 Hz, 2 H, 1-H, 8"-H); $^{13}$C NMR (100.6 MHz, $CD_3OD$) $\delta$ 21.9 ($CH_2$, C3"), 23.0 ($CH_2$, C2"), 23.5 ($CH_3$, 9-$CH_3$), 24.9 ($CH_2$, C1"), 27.3 (CH, C11), 27.7 (CH), 27.8 ($CH_2$), 27.9 (CH) (C7, C3', C10'), 29.3 ($CH_2$, C13), 29.4 ($CH_2$, C4"), 30.3 ($CH_2$), 30.59 (3 $CH_2$) and 30.62 (2 $CH_2$) (C4', C5', C6', C7', C8', C9'), 31.4 ($CH_2$) and 31.5 ($CH_2$) (C2', C11'), 36.0 ($CH_2$) and 36.3 ($CH_2$) (C6, C10), 49.1 ($CH_2$) and 49.6 ($CH_2$) (C1', C12'), 112.8 (C), 117.0 (C), 117.15 (C) and 117.21 (C) (C11a, C12a, C8a" and C9a"), 120.1 (CH) and 125.2 (CH) (C4 and C5"), 126.1 (CH) and 126.3 (CH) (C8, C7"), 126.5 (CH, C8"), 127.3 (CH), 134.1 (CH), 134.2 (CH), 134.5 (C), 139.8 (C) and 140.3 (C) (C1, C2, C3, C4a, C9, C6", C10a"), 150.7 (C) and 151.6 (C) (C5a, C4a"), 157.0 (C) and 158.0 (C) (C12, C9"); HRMS (ESI), calcd for [$C_{42}H_{54}N_4$ + H $^+$] 615.4421, found 615.4423; Anal. calcd. for $C_{42}H_{54}N_4$·2HCl·2$H_2O$: C, 69.69; H, 8.35; N, 7.74; Cl, 9.80. Found: C, 69.75; H, 8.40; N, 7.49; Cl, 9.62.

### *4.1.7. 6,7,10,11-Tetrahydro-9-methyl-12-{{10-[(6-chloro-1,2,3,4-tetrahydroacridin-9-yl)amino]decyl}amino}-7,11-methanocycloocta[b]quinoline (17f)*

It was prepared as described for **3g**. Starting from huprine **15** (274 mg, 1.10 mmol, 1 eq), and a solution of of bromoalkyltacrine **14f** (596 mg, 1.32 mmol, 1.3 eq) in anhydrous DMSO (6 mL), a brown oily residue (810 mg) was obtained and subjected to column chromatography purification (40–60 μm silica gel, EtOAc/hexane/$Et_3N$ mixtures, gradient elution). On elution with EtOAc/hexane/$Et_3N$ 50:50:0.2 to 100:0:0.2, the heterodimer **17f** (477 mg, 70% yield) was isolated as a yellow oil; $R_f$ 0.51 ($CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 9:1:0.05).

A solution of **17f** (450 mg, 0.72 mmol) in $CH_2Cl_2$ (48 mL) was filtered through a 0.2 μm NYL filter, and treated with an excess of a methanolic solution of HCl (0.75 N, 9.0 mL, 6.75 mmol) and the resulting solution was concentrated in vacuo to dryness. The solid was taken in MeOH (1.5 mL) and precipitated upon addition of AcOEt (10 mL). The precipitated solid was separated, washed with pentane (3×2), and dried under standard conditions, to give **17f**·2HCl (351 mg) as a yellow solid: mp 176–178 ºC; IR (KBr) $\nu$ 3500–2500 (max at 3351, 3232, 3114, 3048, 3001, 2924, 2852, 2270, 2792, and 2651, $N^+$–H, N–H, and C–H st), 1632, 1585, 1571 and 1519 (Ar–C–C, Ar–C–N st) $cm^{-1}$; $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 1.32–1.44 (complex signal, 12H, 3'-$H_2$, 4'-$H_2$, 5'-$H_2$, 6'-$H_2$, 7'-$H_2$, 8'-$H_2$), 1.57 (s, 3H, 9-$CH_3$), 1.80–1.96 (complex signal, 10H, 10-$H_{endo}$, 13-$H_{syn}$, 2'-$H_2$, 9'-$H_2$, 2"-$H_2$, 3"-$H_2$), 2.08 (dm, $J$ = 12.4 Hz, 1H, 13-$H_{anti}$), 2.56 (br dd, $J$ = 16.4 Hz, 1H, 10-$H_{exo}$), 2.68 (m, 2H, 1"-$H_2$), 2.77 (br m, 1H, 7-H), 2.91 (d, $J$ = 18.0 Hz, 1H, 6-$H_{endo}$), 3.01 (m, 2H, 4"-$H_2$), 3.21 (dd, $J$ = 17.8 Hz, $J'$ = 5.0 Hz, 1H, 6-$H_{exo}$), 3.48 (br m, 1H, 11-H), 3.93 (t, $J$ = 6.8 Hz, 2H) and 3.99 (t, $J$ = 6.8 Hz, 2H) (1'-$H_2$, 10'-$H_2$), 4.85 (s, NH, $NH^+$), 5.58 (br d, $J$ = 4.4 Hz, 1H, 8-H), 7.53–7.58 (complex signal, 2 H, 4-H, 7"-H), 7.80 (s, 1H, 5"-H), superimposed in part 7.79 (t, $J$ = 8.0 Hz) and 7.83 (t, $J$ = 8.0 Hz) (2-H, 3-H), 8.38 (dd, $J$ = 8.0 Hz, $J'$ = 5.2 Hz, 2 H, 1-H, 8"-H); $^{13}$C NMR (100.6 MHz, $CD_3OD$) $\delta$ 21.8 ($CH_2$, C3"), 22.9 ($CH_2$, C2"), 23.5 ($CH_3$, 9-$CH_3$), 24.8 ($CH_2$, C1"), 27.3 (CH, C11), 27.7 ($CH_2$), 27.8 ($CH_2$) and 27.9 (CH) (C7, C3', C8'), 29.3 ($CH_2$), 29.4 ($CH_2$) (C13, C4"), 30.3 (2 $CH_2$), 30.46 ($CH_2$) and 30.51 ($CH_2$) (C4', C5', C6', C7'), 31.35 ($CH_2$) and 31.41 ($CH_2$) (C2', C9'), 36.0 ($CH_2$, C10), 36.3 ($CH_2$, C6), 49.2 ($CH_2$) and 49.6 ($CH_2$) (C1', C10'), 113.3 (C), 115.4 (C), 117.1 (C) and 117.2 (C) (C11a, C12a, C8a", C9a"), 119.1 (CH) and 120.1 (C), (C4, C5"), 125.2 (CH, C8), 126.1 (CH) and 126.7 (CH) (C2, C7"), 127.3 (CH) and 128.8 (CH) (C1, C8"), 134.2 (CH), 134.5 (C), 140.0 (C), 140.2 (C) and 140.5 (C) (C3, C4a, C9, C6", C10a"), 150.7 (C) and 152.1 (C) (C5a, C4a"), 157.0 (C) and 157.8 (C) (C12, C9"); HRMS (ESI), calcd for [$C_{40}H_{49}{}^{35}ClN_4$ + H $^+$] 621.3719, found 621.3717; Anal. calcd. for $C_{40}H_{49}ClN_4$·2HCl·2$H_2O$: C, 65.79; H, 7.59; N, 7.67; Cl, 14.56. Found: C, 66.10; H, 7.35; N, 7.56; Cl, 14.59.

### *4.1.8. 6,7,10,11-Tetrahydro-9-methyl-12-{{12-[(6-chloro-1,2,3,4-tetrahydroacridin-9-yl)amino]dodecyl}amino}-7,11-methanocycloocta[b]quinoline (17g)*

It was prepared as described for **3g**. Starting from huprine **15** (278 mg, 1.11 mmol, 1 eq), and a solution of of bromoalkyltacrine **14g** (639 mg, 1.33 mmol, 1.2 eq) in anhydrous DMSO (6 mL), a brown oily residue (1.30 g) was obtained and subjected to column chromatography purification (40–60 μm silica gel, $CH_2Cl_2$/50% aq. $NH_4OH$ 100:0.2), to afford the heterodimer **17g** (449 mg, 62% yield) as a yellow oil; $R_f$ 0.58 ($CH_2Cl_2$/MeOH/50% aq. $NH_4OH$ 9:1:0.05).

A solution of **17g** (143 mg, 0.22 mmol) in $CH_2Cl_2$ (7 mL) was filtered through a 0.2 μm NYL filter, and treated with an excess of a methanolic solution of HCl (1.45 N, 1.35 mL, 2.00 mmol) and the resulting solution was concentrated in vacuo to dryness. The resulting solid was washed with pentane (3×2), and dried under standard conditions, to give **17g**·2HCl (121 mg) as a yellow solid: mp 182–185 ºC; IR (KBr) $\nu$ 3500–2500 (max at 3366, 3240, 3050, 2922, 2852 and 2796, $N^+$–H, N–H, and C–H st), 1631, 1584, 1572 and 1519 (Ar–C–C, Ar–C–N st) $cm^{-1}$; $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 1.29–1.45 (complex signal, 16H, 3'-$H_2$, 4'-$H_2$, 5'-$H_2$, 6'-$H_2$, 7'-$H_2$, 8'-$H_2$, 9'-$H_2$, 10'-$H_2$), 1.58 (s, 3H, 9-$CH_3$), 1.82–1.97 (complex signal, 10H, 10-$H_{endo}$, 13-$H_{syn}$, 2'-$H_2$, 11'-$H_2$, 2"-$H_2$, 3"-$H_2$), 2.09 (dm, $J$ = 13.2 Hz, 1H, 13-$H_{anti}$), 2.56 (br dd, $J$ = 15.6 Hz, 1H, 10-$H_{exo}$), 2.68 (m, 2H, 1"-$H_2$), 2.78

(br m, 1H, 7-H), 2.89 (d, $J$ = 17.6 Hz, 1H, 6-$H_{endo}$), 3.00 (m, 2H, 4"-$H_2$), 3.21 (dd, $J$ = 17.8 Hz, $J'$ = 5.0 Hz, 1H, 6-$H_{exo}$), 3.47 (br m, 1H, 11-H), 3.95 (t, $J$ = 6.2 Hz, 2H), 4.00 (br t, 2H) (1'-$H_2$, 12'-$H_2$), 4.85 (s, NH, $NH^+$), 5.59 (br d, $J$ = 3.6 Hz, 1H, 8-H), 7.57 (complex signal, 2H, 4-H, 5"-H), 7.79 (s, 1H, 5"-H), superimposed in part 7.77 (t, $J$ = 7.6 Hz) and 7.84 (t, $J$ = 7.6 Hz) (2-H, 3-H), 8.40 (t, $J$ = 7.6 Hz, 2H, 1-H, 8"-H); $^{13}$C NMR (100.6 MHz, $CD_3OD$) $\delta$ 21.8 ($CH_2$, C3"), 22.9 ($CH_2$, C2"), 23.5 ($CH_3$, 9-$CH_3$), 24.5 ($CH_2$, C1"), 24.8 ($CH_2$), 27.3 (CH, C11), 27.7 ($CH_2$), 27.88 ($CH_2$) and 27.93 (CH) (C7, C3', C10'), 29.35 ($CH_2$) and 29.44 ($CH_2$) (C13, C4"), 30.3 (2 $CH_2$) and 30.7 (4 $CH_2$) (C4', C5', C6', C7', C8', C9'), 31.4 ($CH_2$) and 31.5 ($CH_2$) (C2', C10'), 36.0 ($CH_2$, C10), 36.3 ($CH_2$, C6) 49.3 ($CH_2$) and 49.7 ($CH_2$) (C1', C12'), 113.3 (C), 115.5 (C), 117.17 (C) and 117.25 (C) (C11a, C12a, C8a" and C9a"), 119.2 (CH) and 120.1 (C) (C4, C5"), 125.2 (CH, C8), 126.2 (CH) and 126.8 (CH) (C2, C7"), 127.3 (CH) and 128.8 (CH) (C1, C8"), 134.2 (CH), 134.6 (C), 140.1 (C), 140.3 (C) and 140.6 (C) (C3, C4a, C9 and C6", C10a"), 150.7 (C) and 152.1 (C) (C5a, C4a"), 157.1 (C) and 157.9 (C) (C12, C9"); HRMS (ESI), calcd for [$C_{42}H_{53}{}^{35}ClN_4$ + $H^+$] 649.4032, found 649.4022; Anal. calcd. for $C_{42}H_{53}ClN_4 \cdot 2HCl \cdot 2H_2O$: C, 66.52; H, 7.84; N, 7.39; Cl, 14.03. Found: C, 66.95; H, 7.99; N, 6.97; Cl, 13.83.

**Biological profiling**

#### *4.1.9. T. brucei culturing and evaluation of trypanocidal activity*

Bloodstream form *T. brucei* (strain 221) were cultured at 37 ºC in modified Iscove's medium.[59] Trypanocidal activity was assessed by growing parasites in the presence of various concentrations of the 4-aminoquinoline-based heterodimeric compounds and determining the levels which inhibited growth by 50% ($IC_{50}$) and 90% ($IC_{90}$). *T. brucei* in the logarithmic phase of growth were diluted back to 0.25 × 105 $mL^{-1}$ and aliquoted into 96-well plates. Heterodimeric compounds were then added at a range of concentrations and the plates incubated at 37 °C. Each drug concentration was tested in triplicate. Resazurin was added after 48 h and the plates incubated for a further 16 h and the plates then read in a Spectramax plate reader. Results were analysed using GraphPad Prism.

#### *4.1.10. P. falciparum culturing and evaluation of antiplasmodial activity*

Malaria parasites were maintained in human $A^+$ erythrocytes suspended in RPMI 1640 medium supplemented with $A^+$ serum and D-glucose according to previously published methods.[60,61] Cultures containing predominantly early ring stages were used for testing. Compounds were dissolved in DMSO and further diluted with RPMI 1640 medium (the final DMSO concentration did not exceed 0.5% which did not affect parasite growth). Two-fold serial dilutions were made in 96-well microtitre plates in duplicate and infected erythrocytes were added to give a final volume of 100 µL with haematocrit 2.5% and 1% parasitaemia. Chloroquine diphosphate was used as a positive control and uninfected and infected erythrocytes without compounds were included in each test. Plates were placed into a modular incubator gassed with nitrogen 93%, oxygen 3%, carbon dioxide 4% and incubated at 37 ºC for 48 h. Parasite growth was assessed by measuring lactate dehydrogenase activity.[62] The reagent used contained the following in each mL: acetylpyridine adenine dinucleotide (APAD), 0.74 mg; lithium lactate, 19.2 mg; diaphorase, 0.1 mg; triton X-100, 2 µL; and nitroblue tetrazolium, 1 mg. Fifty µL of this reagent was added to each well and mixed, and plates were incubated for 10–15 min at 37 ºC. Absorbances were read at 550 nm using a Dynatech Laboratories MRX microplate reader and % inhibition of growth was calculated by comparison with control values. $IC_{50}$ values were determined using linear regression analysis (Microsoft Excel).

#### *4.1.11. Cytotoxic activity against rat skeletal myoblast L6 cells*

Cytotoxicity against mammalian cells was assessed using microtitre plates following a described procedure.[63] Briefly, L6 cells (a rat skeletal muscle line) were seeded at 1 × $10^4$ $mL^{-1}$ in 200 µL of growth medium containing different compound concentrations. The plates were incubated for 6 days at 37 °C and 20 µL resazurin was then added to each well. After a further 8 h incubation, the fluorescence was determined using a Spectramax plate reader.

#### *4.1.12. Acetylcholinesterase inhibitory activity*

The inhibitory activities of the novel 4-aminoquinoline-based heterodimeric compounds **3g**, **4g**, **16f-g**, and **17f-g** against human recombinant AChE (Sigma-Aldrich) were evaluated spectrophotometrically by the method of Ellman et al.[43] The reactions took place in a final volume of 300 µL of 0.1 M phosphate-buffered solution pH 8.0, containing hAChE (0.02 u/mL) and 333 µM 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB; Sigma-Aldrich) solution used to produce the yellow anion of 5-thio-2-nitrobenzoic acid. Inhibition curves were performed in duplicates using at least 10 increasing concentrations of inhibitors and preincubated for 20 min at 37 ºC before adding the substrate. One duplicate sample without inhibitor was always present to yield 100% of AChE activity. Then, substrate acetylthiocholine iodide (450 µM; Sigma-Aldrich) was added and the reaction was developed for 5 min at 37 ºC. The colour production was measured at 414 nm using a labsystems Multiskan spectrophotometer.

Data from concentration–inhibition experiments of the compounds were calculated by non-linear regression analysis, using the GraphPad Prism program package (GraphPad Software; San Diego, USA), which gave estimates of the $IC_{50}$ (concentration of drug producing 50% of enzyme activity inhibition). Results are expressed as mean ± S.E.M. of at least 4 experiments performed in duplicate.

#### *4.1.13. Determination of brain permeability: PAMPA-BBB assay*

The *in vitro* permeability ($P_e$) of the novel 4-aminoquinoline-based heterodimeric compounds **3g**, **4g**, **16f-g**, and **17f-g** and fourteen known drugs through lipid extract of porcine brain membrane was determined by using a parallel artificial membrane permeation assay,[44] using a mixture PBS:EtOH 70:30. Assay validation was made by comparison of the experimental $P_e$ values of the known drugs with their reported values, which showed a good correlation: $P_e$ (exp) = 1.4974 $P_e$ (lit) – 0.8434 ($R^2$ = 0.9428). From this equation and the limits established by Di *et al.* for BBB permeation,[44] three ranges of permeability were established: compounds of high BBB permeation (CNS+): $P_e$ ($10^{-6}$ cm $s^{-1}$) > 5.10; compounds of low BBB permeation (CNS–): $P_e$ ($10^{-6}$ cm $s^{-1}$) < 2.15; and compounds of uncertain BBB permeation (CNS+/–): 5.10 > $P_e$ ($10^{-6}$ cm $s^{-1}$) > 2.15.

**Table 4**
Reported and experimental permeability values ($P_e$ $10^{-6}$ cm $s^{-1}$) of 14 commercial drugs used for the PAMPA-BBB assay validation

| Compound | Literature value[a] | Experimental value[b] |
|---|---|---|
| Cimetidine | 0.0 | 0.70 ± 0.03 |
| Lomefloxacin | 1.1 | 0.75 ± 0.02 |

| | | |
|---|---|---|
| Norfloxacin | 0.1 | 0.90 ± 0.02 |
| Ofloxacin | 0.8 | 0.97 ± 0.01 |
| Hydrocortisone | 1.9 | 1.40 ± 0.05 |
| Piroxicam | 2.5 | 1.71 ± 0.02 |
| Clonidine | 5.3 | 6.50 ± 0.05 |
| Corticosterone | 5.1 | 6.70 ± 0.10 |
| Imipramine | 13 | 12.3 ± 0.10 |
| Promazine | 8.8 | 13.8 ± 0.30 |
| Progesterone | 9.3 | 16.8 ± 0.30 |
| Desipramine | 12 | 17.8 ± 0.10 |
| Testosterone | 17 | 24.0 ± 0.14 |
| Verapamil | 16 | 25.3 ± 0.78 |

[a] Taken from ref. 44.

[b] Values are expressed as the mean ± SD of three independent experiments.

#### *4.1.14. Trypanothione reductase inhibitory activity*

The TryR inhibitory activity of selected compounds was determined through a described methodology based on the colorimetric reduction of DTNB by dithiol trypanothione ($T[SH]_2$).[53,54] The assay mixture consisted of: 40 mM HEPES pH 7.4, 1 mM EDTA, 6 μM trypanothione disulfide ($T[S]_2$), 50 μM DTNB, 2 mU $mL^{-1}$ TryR and 150 μM NADPH. $IC_{50}$ values were determined using 11 serial dilutions. Starting with 12 μL of a 10 mM DMSO solution of each compound, 6 μL was removed and added to another Eppendorf tube containing 6 μL of DMSO. The tube was sealed, mixed and then briefly centrifuged to ensure that the sample was a the bottom of the tube. This procedure was repeated 11 times to produce 12 serial 50% dilutions. Assays were performed in triplicate, by adding 158.2 mL of reagents (TryR + trypanothione + DTNB in assay buffer) to 1.8 μL of the inhibitor solution, and finally 20 μL of NADPH solution to start the assay. Absorbance at 412 nm was monitored at 25 ºC for 15 min using a SpectraMax 340PC (Molecular Devices) plate reader. Data were inspected for linearity and $IC_{50}$ values determined by nonlinear regression to the following four-paramenter equation: $y = (\text{range}/(1+x/IC_{50})^{\text{Slope factor}}) + \text{background}$. The choice of disulphide concentration represents $[S] \approx K_m$.

#### *4.1.15. β-Haematin inhibitory activity*

The quantitative β-haematin inhibitory activity (BHIA) assay is based on the differential solubility of haemin and β-haematin in DMSO (haemin is soluble while β-haematin is insoluble).[57,58] The method determines a 50% inhibitory concentration for β-haematin inhibition in equivalents of the compound under test with respect to haemin ($BHIA_{50}$). Drug samples were dissolved in DMSO (25%) to give concentrations of 32 mM. Fifty μL aliquots of DMSO (25%) were placed in wells of 96-well microplates and serial dilutions of drugs were made to give concentrations of 0.5–32 mM. Fifty μL haemin chloride (8 mM) in DMSO was then added to each well. Drug free controls were prepared by adding haemin chloride solution (50 μL) to 50 μL DMSO (25%) in place of drug solution; chloroquine diphosphate and tetracycline hydrochloride were used as positive and negative controls respectively. β-haematin formation was initiated by the addition of 100 μL of 8 M acetate buffer (pH 5.0) and the plates were then incubated at 37 ºC for 18 h.

Following incubation, microplates were inspected visually. In wells where β-haematin formation was strongly inhibited clumps of black precipitate consisting of drug-haem complex were observed with a clear supernatant whereas less or no inhibition was indicated by wells with a precipitate evenly distributed throughout. The minimum inhibitory concentration was taken to be the lowest concentration of drug that was see to produce clumps of precipitate. The quantitative estimation of β-haematin formation using methodology similar to that reported by Basilico *et al*.,[57] was carried out as follows. Samples were transferred to Eppendorf tubes, centrifuged and the supernatants discarded. DMSO (200 μL) was used to wash out any remaining precipitate from the microplate wells and then thoroughly mixed with the precipitate in the Eppendorf tubes. After centrifuging again, the supernatants were discarded. Insoluble β-haematin was then dissolved by adding 200 μL 0.1M NaOH and mixing thoroughly. Aliquots of 75 μL were transferred to a fresh microplate and optical densities read at 400 nm using a Dynatech Laboratories MRX microplate reader and percent inhibition of β-haematin compared to drug-free controls calculated using linear regression analysis. A minimum of three separate determinations was carried out for each compound except for inactive compounds in which two determinations were carried out.

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