Overcoming Chloroquine Resistance in Malaria: Design, Synthesis, and Structure-Activity Relationships of Novel Hybrid Compounds

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Resistance to antimalarial therapies, including artemisinin, has emerged as a significant challenge. Reversal of acquired resistance can be achieved using agents that resensitize resistant parasites to a previously efficacious therapy. Building on our initial work describing novel chemoreversal agents (CRAs) that resensitize resistant parasites to chloroquine (CQ), we herein report new hybrid single agents as an innovative strategy in the battle against resistant malaria. Synthetically linking a CRA scaffold to chloroquine produces hybrid compounds with restored potency toward a range of resistant malaria parasites. A preferred compound, compound 35, showed broad activity and good potency against seven strains resistant to chloroquine and artemisinin. Assessment of aqueous solubility, membrane permeability, and *in vitro* toxicity in a hepatocyte line and a cardiomyocyte line indicates that compound 35 has a good therapeutic window and favorable drug-like properties. This study provides initial support for CQ-CRA hybrid compounds as a potential treatment for resistant malaria.

Malaria is a global infectious disease caused by a parasitic protozoan of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*) that exhibits a complex life cycle involving an insect vector (mosquito) and a vertebrate host (human) (1). The World Health Organization (WHO) estimates that there were 214 million cases and 438,000 deaths in 2015 (2). Early diagnosis and treatment of malaria reduce disease burden, prevent death, and contribute to reducing malaria transmission. Historically, chloroquine (CQ) (compound 1; Fig. 1) was widely used to treat malaria due to its good efficacy, low toxicity, and affordability. However, *P. falciparum*, the predominant species infecting humans, has developed mechanisms to effectively neutralize the effects of compound 1 and as a result became resistant to the effects of compound 1. The current best available treatment, particularly for *P. falciparum* malaria, is an artemisinin-based combination therapy (ACT). Unfortunately, ACT has already shown prolonged parasite clearance times in Thailand and Cambodia (3–5). The constant threat of developing resistance against antimalarial drugs has led to the search for innovative therapies, such as new compounds with novel mechanisms of action, or new combination therapies to prevent drug resistance (6, 7).

In our previous work, we have described the reversal of chloroquine resistance with novel chemoreversal agents (CRAs) (A. Boudhar, X. W. Ng, C. Y. Loh, W. N. Chia, Z. M. Tan, F. Nosten, B. W. Dymock, and K. S. W. Tan, submitted for publication). We demonstrated the use of a fluorescent probe in screens for new chemosensitizing compounds (8; Boudhar et al., submitted), the design of analogues of hit compounds, as well as their activity and improved potential as CRAs (Boudhar et al., submitted).

Herein we report our results developing single-agent hybrid compounds based on these CRAs. The concept of hybrid compounds, combining more than one biological activity in a single compound, is a relatively unexplored concept in antimalarial-resistance-related drug discovery (9). Hybrid compounds have several advantages, including a lower risk of drug-drug interactions and simpler pharmacokinetics and dosing regimens compared to

combinations of single-mechanism drugs. Our aim was to achieve the combined bioactive effects of each of the pharmacophores via linking, fusing, or merging. One example of a combination strategy to create single-molecule drugs is to combine artemisinin and quinine (compound 2) (10–12); another strategy is to combine antimalarial agents with other scaffolds such as compound 3 (Fig. 2) (13, 14). There are also a few reported examples of CRAs combined to compound 1, aiming to have antimalarial activity of CQ and at the same time blocking the *P. falciparum* chloroquine resistance transporter (PfCRT) with the CRA (15–17).

With CRAs 4 to 6 (Fig. 3) at hand, we investigated their utility in hybrid compounds with compound 1 as the antimalarial component, seeking novel structures retaining good physicochemical properties with improved potency against resistant strains. Thus, a series of hybrid compounds were designed, linking the scaffold from compound 1 via its terminal secondary or tertiary amine with CRAs 4 to 6 (Fig. 1 and Fig. 3). The 4-aminoquinoline scaffold has been shown to be essential for the binding of CQ to free heme (18), and both the terminal tertiary amino group and the basicity of the quinolyl nitrogen are key elements (19, 20). Ideally, the length of the aliphatic side chain should be 2 to 4 carbons;

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FIG 1 Structure-activity relationship (SAR) summary of chloroquine (compound 1) antimalarial activity and application to the design of chloroquine-CRA hybrids.

however, the methyl group has little influence on the activity and was thus not retained.

MATERIALS AND METHODS

All reagents purchased from commercial sources were of the highest purity available and were used without further purification. Commercially available analytical reagent (AR) grade solvents or anhydrous solvents packed in resealable bottles were used as received. All reaction temperatures stated in the procedures are external bath temperatures. Nonaqueous reactions were performed under a positive pressure of nitrogen in oven-dried glassware. Yields refer to chromatographically and spectroscopically homogeneous materials, unless otherwise stated. Reaction progress was monitored by analytical thin-layer chromatography (TLC) with 0.25-mm Merck precoated silica gel plates (60F-254) using UV light (254 nm) as the visualizing agent and ceric ammonium molybdate or potassium permanganate solutions as the developing stain. Flash chromatography was performed on silica gel 60 (0.040 to 0.063 mm) purchased from SiliCycle or Merck. The structures of synthesized compounds were verified by ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, and mass spectrometry (MS). ^1H (400 MHz) and ^{13}C (101 MHz) NMR spectra were measured in CDCl_3 on a Bruker Avance III 400 (UltraShield Plus) spec-

trometer. Chemical shifts are reported in parts per million (ppm) using the residual CDCl_3 peak at 7.26 (^1H) or 77.16 (^{13}C) as the internal standard. ^1H NMR coupling constants (J) are reported in hertz (Hz), and multiplicities are presented as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broad). Mass spectra were obtained on a Bruker amaZon X ion trap instrument for the nominal mass or on a Bruker micrOTOFQII spectrometer for the high-resolution mass analysis. The purity of the compounds was assessed by high-pressure liquid chromatography (HPLC) detecting at 254 nm using an Agilent 1200 series HPLC system with a Zorbax SB-C₁₈ column (5 μm ; 4.6 by 250 mm) using a gradient elution starting from a 5:95 solution of acetonitrile-water with 1% trifluoroacetic acid (TFA) to 100% acetonitrile and 1% TFA with a flow rate of 0.5 ml per min over 15 min. HPLC purity is greater than 95% unless stated. All compounds synthesized were stored in a -20°C freezer.

Chemistry. Synthesis of precursor compounds 13 to 17 is described in the supplemental material.

(i) **N¹-(7-chloroquinolin-4-yl)-N⁴-(quinuclidin-3-yl)butane-1,4-diamine (compound 24).** To a stirred solution of quinuclidin-3-one hydrochloride (compound 23) (154 mg, 0.95 mmol, 1.0 equivalent [equiv.]) in dry methanol (1.40 ml), under inert conditions, was added a 1 M solution of ZnCl_2 in diethyl ether (Et_2O) (0.19 ml, 0.19 mmol, 0.2 equiv.). After the solution was stirred at room temperature (RT) for 30 min, N¹-(7-chloroquinolin-4-yl)butane-1,4-diamine (compound 19c) (476 mg, 1.91 mmol, 2.0 equiv.) was added. After the solution was stirred for another hour at room temperature, solid sodium cyanoborohydride (120 mg, 1.91 mmol, 2.0 equiv.) was added in portions. The reaction mixture was then stirred for 4 h at room temperature and quenched by the addition of water (about 5 ml). The quenched reaction mixture was partitioned between 5 M aqueous NaOH (NaOH_{aq}) and dichloromethane (DCM). The aqueous layer was extracted with DCM (three times), and the combined organic layers were dried with Na_2SO_4 , filtered, and concentrated. The crude residue was purified by flash chromatography (eluent DCM/methanol [MeOH] ratio of 95/5 to 8/2), yielding 9.9 mg (3%) of N¹-(7-chloroquinolin-4-yl)-N⁴-(quinuclidin-3-yl)butane-1,4-diamine (compound 24) as a yellow oil. In addition, 263 mg (55%) of the starting material N¹-(7-chloroquinolin-4-yl)butane-1,4-diamine (compound 19c) was isolated in a second fraction.

R_f (DCM/MeOH ratio of 95/5) = 0.20; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.50 (1 H, d, J = 5.3 Hz, H-3), 7.94 (1 H, d, J = 2.0 Hz, H-8), 7.74 (1 H, d, J = 9.0 Hz, H-5), 7.33 (1 H, dd, J = 9.0 Hz, J = 2.0 Hz, H-7), 6.40 (1 H, d, J = 5.3 Hz, H-2), 5.42 (1 H, bs, NH), 3.34 (2 H, m, H-10), 3.20 (2 H, m, H-13), 3.30 (4 H, m, H-16), 2.62 (3 H, m, H-14 and H-15), 1.82 (12 H, m, H-12, H-11, H-17, H-18 and NH); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 151.7 (C-3), 150.1 (C-1), 148.8 (C-4), 135.2 (C-6), 128.5 (C-5), 125.5 (C-8), 121.5 (C-7), 117.3 (C-9), 99.1 (C-2), 60.5 (C-15), 53.5 (C-14), 52.8 (C-10), 52.2 (C-13), 47.0 (C-16), 43.3 (C-16), 28.0 (C-11), 26.7

FIG 2 Examples of hybrid compounds linking two pharmacophores: linking two antimalarial agents artemisinin (gray line) and quinine (gray dashed line) to give compound 2, and combining the heme-binding 4-amino-quinoline scaffold (gray dashed line) with a dihydropteroate synthase-inhibiting sulfonamide group (gray line) to give compound 3.

FIG 3 Reported CRA compounds from our earlier studies: compound 4 derived from L,703,606, compound 5 derived from loperamide, and compound 6 derived from octoclotheptin. Ph, phenyl.

(C-12), 25.1 (C-18), 23.9 (C-17), 18.9 (C-17); MS (ion trap-time of flight [IT-TOF]) $m/z = 359.2$ $[M+H]^+$.

(ii) **4-((2-((7-chloroquinolin-4-yl)amino)ethyl)(ethyl) amino)-2,2-diphenylbutanenitrile (compound 26)**. To a solution of N^1 -(7-chloroquinolin-4-yl)- N^2 -ethylethane-1,2-diamine (compound 21a) (426 mg, 1.71 mmol, 1.0 equiv.) and 4-bromo-2,2-diphenylbutanenitrile compound 11 (512 mg, 1.71 mmol, 1.0 equiv.) in dry acetonitrile (MeCN) (5.70 ml), under inert conditions, was added diisopropylethylamine (DIPEA) (0.91 ml, 5.13 mmol, 3.0 equiv.). After the solution was stirred at reflux for 5 days, the solvent was evaporated under reduced pressure. The crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 95/5 to 9/1), yielding 321 mg (40%) of 4-((2-((7-chloroquinolin-4-yl)amino)ethyl)(ethyl)amino)-2,2-diphenylbutanenitrile (compound 26) as a yellow oil.

R_f (DCM/MeOH ratio of 9/1) = 0.53; 1H NMR (400 MHz, $CDCl_3$) δ (ppm) = 8.54 (1 H, d, $J = 5.3$ Hz, H-3), 7.98 (1 H, d, $J = 2.0$ Hz, H-8), 7.72 (1 H, d, $J = 9.0$ Hz, H-5), 7.39 (1 H, dd, $J = 9.0$ Hz, $J = 2.0$ Hz, H-7), 7.27 (10 H, m, H-Ph), 6.31 (1 H, d, $J = 5.3$ Hz, H-2), 5.89 (1 H, bs, NH), 3.14 (2 H, q, $J = 5.6$ Hz, H-12), 2.79 (2 H, t, $J = 5.7$ Hz, H-10), 2.64 (4 H, m, H-14 and H-11), 2.55 (2 H, m, H-15), 1.06 (3 H, t, $J = 7.0$ Hz, H-13); ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) = 151.2 (C-3), 150.0 (C-1), 148.8 (C-4), 139.8 (C-18), 135.1 (C-6), 129.1 (C-19), 128.9 (C-5), 128.2 (C-21), 126.7 (C-20), 125.5 (C-8), 122.4 (C-9), 121.4 (C-7), 117.6 (C-17), 99.61 (C-2), 51.7 (C-11), 50.0 (C-16), 49.4 (C-14), 47.5 (C-12), 40.1 (C-10), 37.3 (C-15), 12.0 (C-13); MS (electrospray ionization [ESI]) $m/z = 469.1$ $[M+H]^+$; high-resolution mass spectrometry (HRMS) (ESI) $m/z = 469.2168$ $[M+H]^+$, calculated: 469.2154, differential (Diff.): 3.1 ppm.

(iii) **N^1 -(3-(2-chloro-10H-phenothiazin-10-yl)propyl)- N^3 -(2-((7-chloroquinolin-4-yl)amino)ethyl)- N^3 -ethylpropane-1,3-diamine (compound 42)**. 2-Chlorophenothiazine (compound 39) (1.01 g, 4.30 mmol, 1.0 equiv.) was dissolved under nitrogen in 14.3 ml of dry tetrahydrofuran (THF) with the aid of heat. This solution was added to a suspension of NaH (60% [by weight] in oil, 172 mg, 4.30 mmol, 1.0 equiv.) under nitrogen in 7.41 ml of dry dimethyl sulfoxide (DMSO) and 1.48 ml of dry THF. The mixture was stirred at 0°C for 30 min and then added to a solution of 1-chloro-3-iodopropane (compound 33) (0.48 ml, 4.52 mmol, 1.05 equiv.) under nitrogen in 1.48 ml of DMSO. The reaction mixture was stirred at room temperature for 5 h. The reaction mixture was then poured into ice water and extracted with DCM (three times). The combined extracts were washed with water, dried over Na_2SO_4 , filtered, and concentrated under reduced pressure to a pink oil. The crude was purified by flash chromatography (eluent hexane/DCM ratio of 97/3 to 8/2), yielding 615 mg (58%) of 2-chloro-10-(3-chloropropyl)-10H-phenothiazine (compound 40) as a colorless oil. In addition, 119 mg of the starting material compound 39 was isolated in a second fraction (based on recovered starting material [brsm] yield 66%).

R_f (hexane/DCM = 9/1) = 0.33; 1H NMR (400 MHz, $CDCl_3$) δ (ppm) = 7.17 (2 H, m, H-2 and H-3), 7.05 (1 H, d, $J = 9.0$ Hz, H-5), 6.92 (4 H, m, H-4, H-8, H-9 and H-11), 4.06 (2 H, t, $J = 6.9$ Hz, H-13), 3.67 (2 H, t, $J = 6.4$ Hz, H-15), 2.24 (2 H, m, H-14). The analytical data matched the published data (21).

Compound 41 was obtained using the same conditions as for compound 14, employing N^1 -(7-chloroquinolin-4-yl)- N^2 -ethylethane-1,2-diamine (compound 21a) (209 mg, 0.84 mmol, 1.0 equiv.), (9H-fluoren-9-yl)methyl (3-oxopropyl)carbamate (compound 13) (297 mg, 1.01 mmol, 1.2 equiv.), dry DCM (21 ml) and sodium triacetoxyborohydride [$NaBH(OAc)_3$] (356 mg, 1.68 mmol, 2.0 equiv.). The reaction mixture was stirred for 5 h, and the crude compound was purified by flash chromatography (eluent DCM/MeOH ratio of 95/5 to 9/1), yielding 363 mg (82%) of desired 9-fluorenylmethoxy carbonyl (Fmoc)-protected amine as a white foam.

R_f (DCM/MeOH ratio of 9/1) = 0.35; 1H NMR (400 MHz, $CDCl_3$) δ (ppm) = 8.49 (1 H, d, $J = 5.3$ Hz, H-3), 7.95 (1 H, s, H-8), 7.72 (3 H, m, H-5 and H-fluorene), 7.54 (2 H, m, H-fluorene), 7.32 (5 H, m, H-fluorene and H-Ar), 6.34 (1 H, d, $J = 5.3$ Hz, H-2), 5.98 (1 H, bs, NH), 5.20 (1 H, bs, NH), 4.39 (2 H, d, $J = 6.7$ Hz, H-18), 4.16 (1 H, t, $J = 6.7$ Hz, H-19), 3.32 (4 H, m, H-10 and H-16), 2.79 (2 H, m, H-12), 2.56 (4 H, m, H-11 and H-14), 1.69 (2 H, m, H-15), 1.04 (3 H, t, $J = 6.9$ Hz, H-13); MS (ESI) $m/z = 529.2$ $[M+H]^+$; HRMS (ESI) $m/z = 529.2364$ $[M+H]^+$, calculated: 529.2365, Diff.: 0.2 ppm.

To a stirred solution of the Fmoc-protected amine (880 mg, 1.66 mmol, 1.0 equiv.) in dry DCM (33 ml), under nitrogen and at room temperature, was then added piperidine (0.82 ml, 8.30 mmol, 5.0 equiv.). The reaction mixture was stirred for 36 h, and the solvent was removed. The crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 9/1 to 8/2 with 0.1% triethylamine [TEA]), yielding 437 mg (86%) of the desired free amine compound 41 as a colorless oil.

R_f (DCM/MeOH ratio of 9/1) = 0.35; 1H NMR (400 MHz, $CDCl_3$) δ (ppm) = 8.50 (1 H, d, $J = 5.3$ Hz, H-3), 7.93 (1 H, d, $J = 2.1$ Hz, H-8), 7.70 (3 H, d, $J = 8.8$ Hz, H-5), 7.33 (1 H, dd, $J = 8.8$ Hz, $J = 2.2$ Hz, H-7), 6.35 (1 H, d, $J = 5.3$ Hz, H-2), 6.14 (1 H, bs, NH), 3.22 (2 H, m, H-10), 2.78 (4 H, m, H-12 and H-16), 2.59 (4 H, m, H-11 and H-14), 2.26 (2 H, bs, NH_2), 1.63 (2 H, m, H-15), 1.06 (3 H, t, $J = 7.2$ Hz, H-13); ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) = 152.2 (C-3), 150.1 (C-1), 149.2 (C-4), 134.9 (C-6), 128.8 (C-5), 125.4 (C-8), 121.4 (C-7), 117.6 (C-9), 99.4 (C-2), 51.5 (C-11), 51.1 (C-14), 47.2 (C-12), 40.7 (C-10), 40.1 (C-16), 31.0 (C-15), 12.0 (C-13); MS (ESI) $m/z = 307.2$ $[M+H]^+$; HRMS (ESI) $m/z = 307.1688$ $[M+H]^+$, calculated: 307.1684, Diff.: 1.4 ppm.

A mixture of free amine compound 41 (64 mg, 0.21 mmol, 1.0 equiv.), 2-chloro-10-(3-chloropropyl)-10H-phenothiazine (compound 40) (65 mg, 0.21 mmol, 1.0 equiv.) and K_2CO_3 (29 mg, 0.21 mmol, 1.0 equiv.) in 4.2 ml MeOH, under nitrogen, was heated and stirred at reflux for 4 days. After cooling, the solvent was removed. The crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 9/1 to 8/2 with 0.1% TEA), yielding 53 mg of the desired compound with major impurities. This was further purified by HPLC, yielding 14 mg (12%) of N^1 -(3-(2-chloro-10H-phenothiazin-10-yl)propyl)- N^3 -(2-((7-chloroquinolin-4-yl)amino)ethyl)- N^3 -ethylpropane-1,3-diamine (compound 42) as a yellowish oil.

R_f (DCM/MeOH ratio of 8/2) = 0.14; 1H NMR (400 MHz, $CDCl_3$) δ (ppm) = 8.53 (1 H, d, $J = 5.1$ Hz, H-3), 7.95 (1 H, d, $J = 2.1$ Hz, H-8), 7.68 (3 H, d, $J = 8.8$ Hz, H-5), 7.33 (1 H, dd, $J = 8.8$ Hz, $J = 2.1$ Hz, H-7), 7.11 (2 H, m, H-21 and H-22), 7.00 (1 H, d, $J = 7.9$ Hz, H-24), 6.87 (4 H, m, H-23, H-27, H-28 and H-30), 6.36 (1 H, d, $J = 5.1$ Hz, H-2), 6.11 (1 H, bs,

NH), 3.82 (2 H, t, J = 6.8 Hz, H-19), 3.24 (2 H, m, H-10), 2.77 (2 H, t, J = 5.2 Hz, H-14), 2.55 (8 H, m, H-11, H-12, H-16 and H-17), 1.85 (2 H, m, H-18), 1.62 (2 H, m, H-15), 1.06 (3 H, t, J = 6.8 Hz, H-13); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 152.3 (C-3), 150.0 (C-1), 149.3 (C-4), 146.7 (C-31), 144.7 (C-20), 134.9 (C-6), 144.7 (C-29), 128.9 (C-5), 128.1 (C-22), 127.7 (C-24), 127.6 (C-27), 125.4 (C-8), 125.2 (C-25), 123.9 (C-26), 123.1 (C-23), 122.5 (C-28), 121.5 (C-7), 117.6 (C-9), 116.0 (C-21), 115.9 (C-30), 99.5 (C-2), 51.5 (C-11), 51.3 (C-14), 48.6 (C-12), 47.5 (C-19), 47.3 (C-10), 45.5 (C-16), 40.1 (C-17), 27.7 (C-15), 27.1 (C-18), 12.1 (C-13); MS (ESI) m/z = 580.1 $[\text{M}+\text{H}]^+$; HRMS (ESI) m/z = 580.2059 $[\text{M}+\text{H}]^+$, calculated: 580.2063, Diff.: 0.8 ppm.

Method A for the synthesis of hybrid compounds. (i) ***tert*-Butyl 3-((2-((7-chloroquinolin-4-yl)amino)ethyl)amino)piperidine-1-carboxylate (compound 27a).** Under inert conditions, *tert*-butyl piperidin-4-ylcarbamate (199 mg, 0.99 mmol, 1.0 equiv.) and 2-((7-chloroquinolin-4-yl)amino)ethyl methanesulfonate (compound 22a) (298 mg, 0.99 mmol, 1.0 equiv.) were suspended in 4.97 ml THF in a microwave tube, and then TEA (0.28 ml, 1.98 mmol, 2.0 equiv.) was added. The tube was sealed, and the reaction mixture was heated in a microwave reactor at 120°C for 4 h. The reaction mixture was poured into saturated K_2CO_3 (aqueous) and extracted with DCM (three times). The united organic phase was concentrated under reduced pressure, and the crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 99/1 to 9/1), yielding 136 mg (34%) of *tert*-butyl 3-((2-((7-chloroquinolin-4-yl)amino)ethyl)amino)piperidine-1-carboxylate (compound 27a) as a yellow oil.

R_f (DCM/MeOH ratio of 9/1) = 0.48; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.49 (1 H, d, J = 5.2 Hz, H-3), 7.93 (1 H, d, J = 1.8 Hz, H-8), 7.72 (1 H, d, J = 8.8 Hz, H-5), 7.34 (1 H, dd, J = 8.8 Hz, J = 1.8 Hz, H-7), 6.36 (1 H, d, J = 5.2 Hz, H-2), 6.01 (1 H, bs, NH), 3.90 (1 H, m, H-13b), 3.66 (1 H, m, H-13b), 3.32 (2 H, m, H-10), 3.07 (3 H, m, H-11 and NH), 2.89 (1 H, m, NH), 2.64 (1 H, m, H-12), 2.15 (2 H, m, H-14), 1.92 (1 H, m, H-15a), 1.69 (1 H, m, H-15b), 1.46 (11 H, bs, H-19 and H-16); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 155.1 (C-17), 151.8 (C-3), 150.2 (C-1), 148.8 (C-4), 135.1 (C-6), 128.5 (C-5), 125.5 (C-8), 121.6 (C-7), 117.4 (C-9), 99.3 (C-2), 79.8 (C-17), 53.4 (C-12), 48.9 (C-13), 45.0 (C-10), 44.9 (C-14), 44.2 (C-11), 31.6 (C-15), 28.4 (C-19), 23.5 (C-16); MS (ESI) m/z = 405.1 $[\text{M}+\text{H}]^+$; HRMS (ESI) m/z = 405.2067 $[\text{M}+\text{H}]^+$, calculated: 405.2052, Diff.: 3.7 ppm.

(ii) ***tert*-Butyl 3-((3-((7-chloroquinolin-4-yl)amino)propyl)amino)piperidine-1-carboxylate (compound 27b).** Compound 27b was obtained by method A, using the same conditions as for compound 27a: employing *tert*-butyl piperidin-4-ylcarbamate (compound 7 in Boudhar et al., submitted) (199 mg, 0.99 mmol, 1.05 equiv.), 3-((7-chloroquinolin-4-yl)amino)propyl methanesulfonate (compound 22b) (298 mg, 0.94 mmol, 1.0 equiv.) and TEA (0.28 ml, 1.98 mmol, 2.1 equiv.) in 4.95 ml THF. The reaction mixture was heated in a microwave reactor at 120°C for 5 h, and the crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 95/5 to 8/2), yielding a 136 mg fraction of the desired compound 27b together with some unreacted starting material compound 7 (yield compound 27b: 28%, based on 86% [by weight] calculated with the ^1H NMR). This was further purified by HPLC, yielding 40 mg of *tert*-butyl 3-((3-((7-chloroquinolin-4-yl)amino)propyl)amino)piperidine-1-carboxylate (compound 27b) as a yellowish oil.

R_f (DCM/MeOH ratio of 9/1) = 0.48; ^1H NMR (400 MHz, $\text{MeOH}-D_4$) δ (ppm) = 8.43 (2 H, m, H-3 and H-8), 7.90 (1 H, m, H-5), 7.69 (1 H, m, H-7), 6.93 (1 H, m, H-2), 4.09 (1 H, m, H-14a), 3.73 (3 H, m, H-14b and H-15), 3.58 (1 H, m, NH), 3.42–2.95 (5 H, m, H-10, H-12 and NH), 2.22 (4 H, m, H-13, H-11 and H-16a), 1.69 (3 H, m, H-16b and H-17), 1.46 (9 H, bs, H-20); ^{13}C NMR (400 MHz, $\text{MeOH}-D_4$) δ (ppm) = 163.0 (C-18), 157.8 (C-1), 144.0 (C-3), 141.1 (C-4), 140.1 (C-6), 128.8 (C-5), 126.1 (C-8), 120.3 (C-7), 117.1 (C-9), 99.8 (C-2), 81.9 (C-19), 53.1 (C-13), 44.9 (C-14), 44.7 (C-10), 44.1 (C-15), 43.9 (C-12), 41.7 (C-17), 28.5 (C-20), 26.0 (C-11), 21.5 (C-16); MS (ESI) m/z = 419.2 $[\text{M}+\text{H}]^+$; HRMS (ESI) m/z = 419.2218 $[\text{M}+\text{H}]^+$, calculated: 419.2208, Diff.: 2.4 ppm.

(iii) ***tert*-Butyl 3-((3-((2-((7-chloroquinolin-4-yl)amino)ethyl)amino)propyl) (2-iodobenzyl)amino)piperidine-1-carboxylate (compound 28).** Compound 28 was obtained by method A, using the same conditions as for compound 27a: employing crude *tert*-butyl 3-((3-aminopropyl)(2-iodobenzyl)amino)piperidine-1-carboxylate (compound 15) (79 mg, 0.11 mmol, 1.0 equiv.), 3-((7-chloroquinolin-4-yl)amino)ethyl methanesulfonate (compound 22a) (33 mg, 0.11 mmol, 1.0 equiv.) and TEA (30 μl , 0.22 mmol, 2.0 equiv.) in 2.20 ml THF. The reaction mixture was heated in a microwave reactor at 120°C for 5 h, and the crude residue was purified by HPLC, yielding 15 mg (21% over two steps) of *tert*-butyl 3-((3-((2-((7-chloroquinolin-4-yl)amino)ethyl)amino)propyl) (2-iodobenzyl)amino)piperidine-1-carboxylate (compound 28) as a yellowish oil.

^1H NMR (400 MHz, $\text{MeOH}-D_4$) δ (ppm) = 8.49 (1 H, d, J = 6.9 Hz, H-3), 8.43 (1 H, d, J = 9.0 Hz, H-8), 7.93 (2 H, m, H-26 and H-5), 7.71 (1 H, m, H-7), 7.61 (1 H, m, H-28), 7.46 (1 H, m, H-27), 7.13 (1 H, m, H-29), 6.99 (1 H, d, J = 6.9 Hz, H-12), 4.31 (3 H, m, H-16 and H-17a), 3.99 (3 H, m, H-23 and H-17b), 3.44 (2 H, m, H-10), 3.14 (4 H, m, H-11 and H-12), 2.24 (1 H, m, H-15), 2.11 (2 H, m, H-19), 1.83 (2 H, m, H-18), 1.42 (11 H, bs, H-22 and H-13); MS (ESI) m/z = 678.1 $[\text{M}+\text{H}]^+$; HRMS (ESI) m/z = 678.2093 $[\text{M}+\text{H}]^+$, calculated: 678.2066, Diff.: 3.9 ppm.

(iv) **N^1 -(2-((7-chloroquinolin-4-yl)amino)ethyl)- N^3 -(2,2-diphenylethyl)- N^3 -(2-iodobenzyl)propane-1,3-diamine (compound 29).** Compound 29 was obtained by method A, using the same conditions as for compound 27a: employing N^1 -(2,2-diphenylethyl)- N^1 -(2-iodobenzyl)propane-1,3-diamine (compound 17) (48 mg, 0.11 mmol, 1.0 equiv.), 3-((7-chloroquinolin-4-yl)amino)ethyl methanesulfonate (compound 22a) (50 mg, 0.16 mmol, 1.5 equiv.) and TEA (30 μl , 0.22 mmol, 2.0 equiv.) in 2.1 ml THF. The reaction mixture was heated in a microwave reactor at 120°C for 10 h, and the crude residue was purified by HPLC, yielding 24 mg (34%) of N^1 -(2-((7-chloroquinolin-4-yl)amino)ethyl)- N^3 -(2,2-diphenylethyl)- N^3 -(2-iodobenzyl)propane-1,3-diamine (compound 29) as a yellowish oil.

^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.53 (1 H, d, J = 5.4 Hz, H-3), 7.94 (1 H, d, J = 2.1 Hz, H-8), 7.71 (1 H, dd, J = 7.9 Hz, J = 1.0 Hz, H-26), 7.58 (1 H, d, J = 8.9 Hz, H-5), 7.34 (1 H, dd, J = 8.9 Hz, J = 2.1 Hz, H-7), 7.24 (4 H, m, H-Ar), 7.17 (7 H, m, H-Ar), 6.98 (2 H, m, H-Ar), 6.34 (1 H, d, J = 5.4 Hz, H-2), 5.79 (1 H, bs, NH), 4.16 (1 H, m, H-16), 3.64 (2 H, s, H-23), 3.15 (4 H, m, H-15 and H-10), 2.83 (2 H, t, J = 5.7 Hz, H-11), 2.62 (2 H, t, J = 6.5 Hz, H-14), 2.50 (2 H, t, J = 6.5 Hz, H-12), 1.63 (3 H, m, H-13 and NH); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 151.2 (C-3), 150.0 (C-1), 149.3 (C-4), 143.6 (C-17), 141.5 (C-24), 139.3 (C-26), 134.9 (C-6), 130.5 (C-28), 128.9 (C-5), 128.7 (C-27), 128.5 (C-19), 128.4 (C-18), 128.0 (C-8), 126.5 (C-20), 125.4 (C-29), 121.4 (C-7), 117.5 (C-9), 100.3 (C-25), 99.3 (C-2), 63.7 (C-14), 60.3 (C-15), 52.8 (C-23), 49.8 (C-16), 47.9 (C-10), 47.5 (C-11), 41.9 (C-15), 27.4 (C-13); MS (ESI) m/z = 675.3 $[\text{M}+\text{H}]^+$; HRMS (ESI) m/z = 675.1760 $[\text{M}+\text{H}]^+$, calculated: 675.1746, Diff.: 2.2 ppm.

(v) **N^1 -(7-chloroquinolin-4-yl)- N^2 -(2,2-diphenylethyl)ethane-1,2-diamine (compound 30).** Compound 30 was obtained by method A, using the same conditions as for compound 27a: employing 2,2-diphenylethane-1-amine (compound 9 in Part I) (145 mg, 0.48 mmol, 1.0 equiv.), 3-((7-chloroquinolin-4-yl)amino)ethyl methanesulfonate (compound 22a) (95 mg, 0.48 mmol, 1.0 equiv.) and TEA (0.13 ml, 0.96 mmol, 2.0 equiv.) in 4.80 ml THF. The reaction mixture was heated in a microwave reactor at 120°C for 7 h, and the crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 95/5 to 8/2), yielding 80 mg (42%) of N^1 -(7-chloroquinolin-4-yl)- N^2 -(2,2-diphenylethyl)ethane-1,2-diamine (compound 30) as a yellowish oil.

R_f (DCM/MeOH ratio of 9/1) = 0.44; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.49 (1 H, d, J = 5.1 Hz, H-3), 7.93 (1 H, d, J = 1.7 Hz, H-8), 7.24 (10 H, m, H-Ar), 6.31 (1 H, d, J = 5.1 Hz, H-2), 5.76 (1 H, bs, NH), 4.17 (1 H, t, J = 8.1 Hz, H-16), 3.32 (2 H, d, J = 8.1 Hz, H-12), 3.26 (2 H, m, H-10), 3.05 (2 H, m, H-11), 1.58 (1 H, bs, NH); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 151.8 (C-3), 150.1 (C-1), 148.8 (C-4), 142.8 (C-14),

FIG 4 Additional preferred CRA components used for hybrid compound synthesis.

135.1 (C-6), 128.8 (C-16), 128.4 (C-5), 128.1 (C-15), 126.8 (C-17), 125.4 (C-8), 117.4 (C-9), 99.2 (C-2), 53.8 (C-12), 51.5 (C-13), 47.3 (C-10), 47.8 (C-11); MS (ESI) $m/z = 402.2$ $[M+H]^+$; HRMS (ESI) $m/z = 402.1746$ $[M+H]^+$, calculated: 402.1732, Diff.: 3.6 ppm.

(vi) **7-Chloro-N-(2-(4-(10,11-dihydrodibenzo[b,f]thiepin-10-yl)piperazin-1-yl)ethyl)quinolin-4-amine (compound 31)**. Compound 31 was obtained by method A, using the same conditions as for compound 27a: employing 1-(10,11-dihydrodibenzo[b,f]thiepin-10-yl)piperazine (56 mg, 0.19 mmol, 1.0 equiv.), 2-((7-chloroquinolin-4-yl)amino)ethyl methanesulfonate (compound 22a) (57 mg, 0.19 mmol, 1.0 equiv.) and TEA (53 μ l, 0.38 mmol, 2.0 equiv.) in 1.90 ml THF. The mixture was heated in a microwave reactor at 120°C for 7 h, and the crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 95/5 to 9/1). Thus, 21 mg of hybrid compound 31 as a fluffy yellow solid and 26 mg of a second impure fraction were obtained (compound 31/impurity ~ 4/1, overall yield ~44%).

R_f (DCM/MeOH ratio of 9/1) = 0.17; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.53 (1 H, d, $J = 5.3$ Hz, H-3), 7.96 (1 H, d, $J = 2.1$ Hz, H-8), 7.72 (1 H, d, $J = 8.5$ Hz, H-5), 7.62 (1 H, dd, $J = 7.9$ Hz, $J = 1.4$ Hz, H-23), 7.52 (1 H, dd, $J = 7.7$ Hz, $J = 1.2$ Hz, H-7), 7.40 (2 H, m, H-17 and H-18), 7.22 (3 H, m, H-25, H-24 and H-22), 7.10 (2 H, m, H-16 and H-19), 6.37 (1 H, d, $J = 5.3$ Hz, H-2), 5.98 (1 H, bs, NH), 4.05 (1 H, m, H-14), 3.91 (1 H, m, H-27a), 3.30 (2 H, m, H-10), 3.21 (1 H, m, H-27b), 2.73 (6 H, m, H-13 and H-11), 2.54 (4 H, m, H-12); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 152.2 (C-3), 150.0 (C-1), 149.2 (C-4), 142.3 (C-20), 140.3 (C-21), 137.1 (C-26), 135.4 (C-6), 137.1 (C-15), 132.6 (C-23), 131.6 (C-18), 131.4 (C-25), 129.7 (C-24), 128.9 (C-16), 128.6 (C-17), 127.2 (C-5), 126.9 (C-19), 126.5 (C-22), 125.5 (C-8), 121.3 (C-7), 117.5 (C-9), 99.4 (C-2), 65.9 (C-14), 55.7 (C-11), 53.4 (C-12), 50.0 (C-13), 39.1 (C-10), 33.6 (C-27); MS (ESI) $m/z = 501.1$ $[M+H]^+$; HRMS (ESI) $m/z = 501.1885$ $[M+H]^+$, calculated: 501.1874, Diff.: 2.1 ppm.

(vii) **7-Chloro-N-(2-(4-(8-chloro-10,11-dihydrodibenzo[b,f]thiepin-10-yl)piperazin-1-yl)ethyl)quinolin-4-amine (compound 32)**. Compound 32 was obtained by method A, using the same conditions as for compound 27a: employing 1-(8-chloro-10,11-dihydrodibenzo[b,f]thiepin-10-yl)piperazine (36 mg, 0.12 mmol, 1.0 equiv.), 2-((7-chloroquinolin-4-yl)amino)ethyl methanesulfonate (compound 22a) (36 mg, 0.12 mmol, 1.0 equiv.), and TEA (33 μ l, 0.24 mmol, 2.0 equiv.) in 2.40 ml THF; the mixture was heated in a microwave reactor at 120°C for 7 h, and the crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 95/5 to 9/1), yielding 37 mg (58%) of hybrid compound 32 as a colorless oil.

FIG 5 Synthesis of compounds 14 to 17. Reagents and conditions: (a) 13, $\text{NaHB}(\text{OAc})_3$, DCM, 0°C to RT, 5 h, 76%; (b) piperidine, DCM, RT, used crude (15); 85% (17); (c) 13, $\text{NaHB}(\text{OAc})_3$, DCM, 0°C to RT, 3 h, 98%.

R_f (DCM/MeOH ratio of 9/1) = 0.55; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.53 (1 H, d, $J = 5.4$ Hz, H-3), 7.97 (1 H, d, $J = 2.1$ Hz, H-8), 7.69 (2 H, m, H-22 and H-5), 7.51 (1 H, d, $J = 7.3$ Hz, H-23), 7.52 (1 H, dd, $J = 8.9$ Hz, $J = 2.1$ Hz, H-7), 7.35 (1 H, d, $J = 7.9$ Hz, H-18), 7.27 (2 H, m, H-25 and H-24), 7.09 (2 H, m, H-16 and H-19), 6.38 (1 H, d, $J = 5.3$ Hz, H-2), 5.99 (1 H, bs, NH), 3.92 (2 H, m, H-14 and H-27a), 3.32 (2 H, m, H-10), 3.18 (1 H, m, H-27b), 2.77 (6 H, m, H-13 and H-11), 2.77 (4 H, m, H-12); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 152.1 (C-3), 150.0 (C-1), 149.1 (C-4), 142.3 (C-20), 142.2 (C-21), 136.4 (C-26), 135.1 (C-6), 133.9 (C-15), 133.2 (C-17), 132.9 (C-23), 132.2 (C-18), 131.5 (C-25), 129.7 (C-24), 129.0 (C-16), 128.8 (C-5), 127.0 (C-19), 126.7 (C-22), 125.5 (C-8), 121.3 (C-7), 117.5 (C-9), 99.4 (C-2), 65.6 (C-14), 55.6 (C-11), 53.3 (C-12), 48.9 (C-13), 39.2 (C-10), 32.8 (C-27); MS (ESI) $m/z = 535.1$ $[M+H]^+$; HRMS (ESI) $m/z = 535.1484$ $[M+H]^+$, calculated: 535.1484, Diff.: 0.2 ppm.

Method B for the synthesis of hybrid compounds. (i) **N^1 -(3-chloropropyl)- N^2 -(7-chloroquinolin-4-yl)- N^1 -ethylethane-1,2-diamine (compound 34)**. To a solution of N^1 -(7-chloroquinolin-4-yl)- N^2 -ethylethane-1,2-diamine (compound 21a) (580 mg, 2.32 mmol, 1.0 equiv.) and K_2CO_3

FIG 6 Synthesis of intermediate compounds 19 to 22. NHet, ethylamine; OMs, mesyl. Reagents and conditions: (a) neat, reflux, 6 to 20 h, 74 to 100%; (b) MsCl , Et_3N , DCM, 0°C, 3 h, 87 to 100%.

FIG 7 Preparation of hybrid compounds 27 to 31. Reagents and conditions: (a) R^1NH_2 or R^1R^2NH , NEt_3 , THF, microwave, 28 to 58%.

(962 mg, 6.96 mmol, 3.0 equiv.) in dry MeCN (42 ml), under inert conditions, was added 1-chloro-3-iodopropane (compound 33) (0.50 ml, 4.64 mmol, 2.0 equiv.). After the solution was stirred at room temperature for 4 days, the reaction was quenched with water and extracted with DCM (three times). The combined organic phase was dried over Na_2SO_4 and filtered, and the solvent was removed under reduced pressure. The crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 97/3 to 9/1), yielding 383 mg (51%) of N^1 -(3-chloropropyl)- N^2 -(7-chloroquinolin-4-yl)- N^1 -ethylethane-1,2-diamine (compound 34) as an off-white solid.

R_f (DCM/MeOH ratio of 9/1) = 0.38; 1H NMR (400 MHz, $CDCl_3$) δ (ppm) = 8.53 (1 H, d, $J = 5.3$ Hz, H-3), 7.96 (1 H, d, $J = 2.1$ Hz, H-8), 7.65 (1 H, d, $J = 9.0$ Hz, H-5), 7.38 (1 H, dd, $J = 9.0$ Hz, $J = 2.1$ Hz, H-7), 6.38

(1 H, d, $J = 5.3$ Hz, H-2), 5.90 (1 H, bs, NH), 3.61 (2 H, t, $J = 6.2$ Hz, H-10), 3.29 (2 H, q, $J = 5.4$ Hz, H-12), 2.84 (2 H, t, $J = 5.7$ Hz, H-11), 2.70 (2 H, t, $J = 7.0$ Hz, H-12), 2.61 (2 H, q, $J = 7.0$ Hz, H-16), 1.95 (2 H, m, H-15), 1.08 (3 H, t, $J = 7.1$ Hz, H-13); ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) = 152.2 (C-3), 150.0 (C-1), 149.2 (C-4), 135.0 (C-6), 128.9 (C-5), 125.5 (C-8), 121.1 (C-9), 117.5 (C-7), 99.5 (C-2), 51.6 (C-11), 50.0 (C-14), 46.9 (C-12), 43.0 (C-10), 40.0 (C-16), 30.2 (C-15), 11.9 (C-13); MS (ESI) $m/z = 325.9$ $[M+H]^+$.

(ii) N^1 -benzyl- N^1 -((2R,3R)-2-benzylquinuclidin-3-yl)- N^3 -(2-((7-chloroquinolin-4-yl)amino)ethyl)- N^3 -ethylpropane-1,3-diamine (compound 35). By method B, under inert conditions, N^1 -(3-chloropropyl)- N^2 -(7-chloroquinolin-4-yl)- N^1 -ethylethane-1,2-diamine (compound 34) (45 mg, 0.14 mmol, 1.0 equiv.) and compound 4 (42 mg, 0.14

FIG 8 Synthesis of hybrid compound 24. Reagents and conditions: (a) ZnCl_2 , NaBH_3CN , MeOH, RT, 20 h, 3%.

mmol, 1.0 equiv.) were suspended in 1.40 ml MeCN in a microwave tube, and then K_2CO_3 (39 mg, 0.28 mmol, 2.0 equiv.) and NaI (4 mg, 0.03 mmol, 0.2 equiv.) were added. The tube was sealed, and the reaction mixture was heated in a microwave reactor at 100°C for 90 min. The solvent was removed under reduced pressure, and the crude residue was purified by flash chromatography (eluent DCM/MeOH ratio of 95/5 to 85/15 with 0.1% TEA), yielding 35 mg of a fraction with major TEA impurities. This was dissolved in DCM, stirred with Amberlyst 21 for 1 h, and filtered, and the solvent was removed under reduced pressure. Thus, 19 mg (22%) of hybrid compound 35 as a colorless oil (with minor impurities) was obtained.

R_f (DCM/MeOH ratio of 9/1) = 0.11; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.52 (1 H, d, J = 5.2 Hz, H-3), 7.94 (1 H, d, J = 2.1 Hz, H-8), 7.85 (1 H, d, J = 8.8 Hz, H-5), 7.37 (1 H, dd, J = 8.8 Hz, J = 2.1 Hz, H-7), 7.25 (8 H, m, H-Ph), 7.00 (2 H, m, H-Ph), 6.37 (1 H, d, J = 5.2 Hz, H-2), 5.91 (1 H, bs, NH), 3.86 (2 H, t, J = 5.4 Hz, H-11), 3.73 (1 H, d, J = 13.0 Hz, H-27a), 3.43 (4 H, m, H-17, H27b and H-16), 3.22 (2 H, m, H-10), 3.01 (3 H, m, H-22a and H-21a), 2.84 (4 H, m, H-18, H-22b and H-21b), 2.74 (2 H, t, J = 6.0 Hz, H-14), 2.62 (2 H, q, J = 7.1 Hz, H-12), 1.74 (3 H, m, H-15 and H-19), 1.56 (2 H, m, H-20a), 1.40 (2 H, m, H-20b), 1.07 (3 H, t, J = 7.1 Hz, H-13); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 151.9 (C-3), 149.8 (C-1), 149.1 (C-4), 140.0 (C-28), 139.2 (C-23), 134.9 (C-6), 128.7 (C-24), 128.6 (C-25), 128.3 (C-30), 127.8 (C-29), 126.9 (C-31), 126.2 (C-26), 125.4 (C-7), 125.4 (C-8), 121.6 (C-5), 117.5 (C-9), 98.9 (C-2), 63.9 (C-22), 60.2 (C-17), 54.8 (C-18), 53.4 (C-11), 52.1 (C-14), 51.9 (C-16), 48.9 (C-12), 47.9 (C-10), 41.9 (C-21), 40.6 (C-21), 33.3 (C-22), 28.3 (C-20), 24.6 (C-20), 24.5 (C-19), 19.2 (C-15), 11.7 (C-13); MS (ESI) m/z = 596.4 $[\text{M}+\text{H}]^+$; HRMS (ESI) m/z = 596.3527 $[\text{M}+\text{H}]^+$, calculated: 596.3515, Diff.: 2.1 ppm.

(iii) *tert*-Butyl 3-((3-((2-((7-chloroquinolin-4-yl)amino)ethyl)(ethyl)amino)propyl)amino)piperidine-1-carboxylate (compound 36). Compound 36 was obtained by method B, using the same conditions as for compound 35: employing N^1 -(3-chloropropyl)- N^2 -(7-chloroquinolin-4-yl)- N^1 -ethylethane-1,2-diamine (compound 34) (102 mg, 0.31 mmol, 1.0 equiv.), *tert*-butyl piperidin-4-ylcarbamate (63 mg, 0.31 mmol, 1.0 equiv.), K_2CO_3 (86 mg, 0.63 mmol, 2.0 equiv.), and NaI (9 mg, 0.06 mmol, 0.2 equiv.) in 3.13 ml MeCN. The mixture was heated in a microwave reactor at 100°C for 90 min. After flash chromatography (eluent DCM/MeOH ratio of 95/5 to 8/2 with 0.1% TEA) and treatment with Amberlyst 21, 81 mg (44%) of hybrid compound 36 as a fluffy yellowish solid with some minor impurities was thus obtained.

R_f (DCM/MeOH ratio of 9/1) = 0.36; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.52 (1 H, d, J = 5.3 Hz, H-3), 7.94 (1 H, d, J = 2.1 Hz, H-8), 7.70 (1 H, d, J = 8.8 Hz, H-5), 7.36 (1 H, dd, J = 8.8 Hz, J = 2.1 Hz, H-7), 6.36

(1 H, d, J = 5.3 Hz, H-2), 6.08 (1 H, bs, NH), 3.88 (3 H, m, H-18 and NH), 3.26 (2 H, m, H-10), 2.64 (11 H, m, H-21, H-17, H-11, H-12, H-14 and H-16), 1.65 (6 H, m, H-19, H-20 and H-15), 1.43 (9 H, s, H-24), 1.07 (3 H, t, J = 13.8 Hz, H-13); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 154.0 (C-22), 151.6 (C-3), 150.5 (C-1), 148.7 (C-4), 135.3 (C-6), 128.2 (C-7), 125.6 (C-8), 122.2 (C-5), 117.6 (C-9), 99.2 (C-2), 80.0 (C-23), 54.0 (C-17), 52.3 (C-11), 51.3 (C-18), 47.8 (C-21), 47.5 (C-14), 46.1 (C-10), 46.0 (C-16), 40.3 (C-12), 28.6 (C-15), 28.6 (C-24), 26.4 (C-19), 23.5 (C-20), 9.3 (C-13); MS (ESI) m/z = 490.2 $[\text{M}+\text{H}]^+$; HRMS (ESI) m/z = 490.2939 $[\text{M}+\text{H}]^+$, calculated: 490.2943, Diff.: 0.9 ppm.

(iv) N^1 -(2-((7-chloroquinolin-4-yl)amino)ethyl)- N^3 -(2,2-diphenylethyl)- N^1 -ethylpropane-1,3-diamine (compound 37). Under inert conditions, N^1 -(3-chloropropyl)- N^2 -(7-chloroquinolin-4-yl)- N^1 -ethylethane-1,2-diamine (compound 34) (58 mg, 0.18 mmol, 1.0 equiv.) and 2,2-diphenylethylamine (compound 9) (35 mg, 0.18 mmol, 1.0 equiv.) were suspended in 1.80 ml MeCN in a microwave tube, and then K_2CO_3 (50 mg, 0.36 mmol, 2.0 equiv.) was added. The tube was sealed, and the reaction mixture was heated in a microwave reactor at 100°C for 90 min. The solvent was removed under reduced pressure, and the crude residue was purified by flash chromatography (solid deposit, eluent DCM/MeOH ratio of 95/5 to 85/15), yielding 40 mg (46%) of N^1 -(2-((7-chloroquinolin-4-yl)amino)ethyl)- N^3 -(2,2-diphenylethyl)- N^1 -ethylpropane-1,3-diamine (compound 37) as a colorless oil. Two additional fractions were also obtained: 15 mg (26%) of chloride compound 34 as well as 24 mg of a mixture of compound 34 and 2,2-diphenylethylamine (compound 9).

R_f (DCM/MeOH ratio of 9/1) = 0.28; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.53 (1 H, d, J = 5.7 Hz, H-3), 7.95 (1 H, d, J = 2.1 Hz, H-8), 7.76 (1 H, d, J = 8.8 Hz, H-5), 7.32 (1 H, dd, J = 8.8 Hz, J = 2.1 Hz, H-7), 7.26 (4 H, m, H-Ph), 7.16 (6 H, m, H-Ph), 6.35 (1 H, d, J = 5.7 Hz, H-2), 6.25 (1 H, bs, NH), 4.10 (1 H, t, J = 7.7 Hz, H-18), 3.18 (4 H, m, H-14 and H-10), 2.74 (4 H, m, H-17 and H-12), 2.48 (4 H, m, H-16 and H-11), 2.03 (1 H, bs, NH), 1.66 (2 H, m, H-15), 0.96 (3 H, t, J = 7.1 Hz, H-13); MS (ESI) m/z = 487.2 $[\text{M}+\text{H}]^+$; HRMS (ESI) m/z = 487.2644 $[\text{M}+\text{H}]^+$, calculated: 487.2623, Diff.: 4.2 ppm.

(v) N^1 -(3-(4-(8-chloro-10,11-dihydrodibenzo[b,f]thiepin-10-yl)piperazin-1-yl)propyl)- N^2 -(7-chloroquinolin-4-yl)- N^1 -ethylethane-1,2-diamine (compound 38a). Compound 38a was obtained by method B, using the same conditions as for compound 35: employing N^1 -(3-chloropropyl)- N^2 -(7-chloroquinolin-4-yl)- N^1 -ethylethane-1,2-diamine (compound 34) (32 mg, 0.10 mmol, 1.0 equiv.), 1-(10,11-dihydrodibenzo[b,f]thiepin-10-yl)piperazine (30 mg, 0.10 mmol, 1.0 equiv.), K_2CO_3 (27 mg, 0.20 mmol, 2.0 equiv.), and NaI (3 mg, 0.02 mmol, 0.2 equiv.) in 1.00 ml MeCN. The mixture was heated in a microwave reactor at 100°C for 3 h. After flash chromatography (eluent DCM/MeOH

FIG 9 Synthesis of hybrid compound 26. Reagents and conditions: (a) MeCN, DIPEA, reflux, 5 d, 40%.

FIG 10 Synthesis of chloride compound 34 and preparation of hybrid compounds 35 to 38. Reagents and conditions: (a) MeCN, RT, 4 d, 51%; (b) $R^1R^2\text{NH}$, K_2CO_3 , NaI, MeCN, μW (100°C, 90 min), 22 to 75%.

ratio of 95/5 to 8/2 with 0.1% TEA), 36 mg (62%) of hybrid compound 38a as a fluffy yellowish solid with some TEA impurities was obtained.

R_f (DCM/MeOH ratio of 8/2) = 0.69; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.52 (1 H, d, $J = 5.4$ Hz, H-3), 7.94 (1 H, d, $J = 2.1$ Hz, H-8), 7.67 (1 H, d, $J = 8.8$ Hz, H-5), 7.58 (1 H, dd, $J = 7.6$ Hz, $J = 0.9$ Hz, H-28), 7.58 (1 H, dd, $J = 7.6$ Hz, $J = 1.1$ Hz, H-23), 7.37 (2 H, m, H-7 and H-30), 7.22 (2 H, m, H-29 and H-27), 7.09 (3 H, m, H-21, H-22 and H-24), 6.37 (1 H, d, $J = 5.4$ Hz, H-2), 6.06 (1 H, bs, NH), 3.94 (1 H, m, H-19), 3.84 (1 H, m, H-32a), 3.23 (2 H, m, H-10), 3.13 (1 H, m, H-32b), 2.81 (6 H, m, H-12, H-17 and H-18), 2.61 (4 H, m, H-17 and H-18), 2.53 (2 H, m, H-11), 2.34 (4 H, m, H-14 and H-16), 1.67 (2 H, m, H-15), 1.06 (1 H, t, $J = 7.1$ Hz, H-13); ^{13}C NMR (400 MHz, CDCl_3) δ (ppm) = 152.2 (C-3), 150.0 (C-1), 149.2 (C-4), 142.6 (C-25), 140.3 (C-26), 137.0 (C-31), 135.2 (C-6), 134.9 (C-20), 132.6 (C-28), 131.5 (C-23), 131.3 (C-30), 129.6 (C-29), 128.8 (C-21), 128.6 (C-22), 127.1 (C-5), 126.8 (C-24), 126.3 (C-27), 125.4 (C-8), 121.3 (C-7), 117.5 (C-9), 99.4 (C-2), 65.8 (C-19), 56.7 (C-10), 54.0 (C-11), 51.4 (C-14), 51.0 (C-16), 48.5 (C-17), 47.1 (C-12), 46.1 (C-18), 39.1 (C-10), 33.3 (C-32), 24.7 (C-15), 10.0 (C-13); MS (ESI) m/z = 586.2 $[\text{M}+\text{H}]^+$; HRMS (ESI) m/z = 586.2777 $[\text{M}+\text{H}]^+$, calculated: 586.2766, Diff.: 1.9 ppm.

(vi) N^1 -(7-chloroquinolin-4-yl)- N^2 -(3-(4-(10,11-dihydrodibenzo[b,f]thiepin-10-yl)piperazin-1-yl)propyl)- N^2 -ethylethane-1,2-diamine (compound 38b). Compound 38b was obtained by method B, using the same conditions as for compound 35: employing N^1 -(3-chloropropyl)- N^2 -(7-chloroquinolin-4-yl)- N^1 -ethylethane-1,2-diamine (compound 34) (36 mg, 0.11 mmol, 1.0 equiv.), 1-(8-chloro-10,11-dihydrodibenzo[b,f]thiepin-10-yl)piperazine (34 mg, 0.11 mmol, 1.0 equiv.), K_2CO_3 (30 mg, 0.22 mmol, 2.0 equiv.), and NaI (3 mg, 0.02 mmol, 0.2 equiv.) in 1.10 ml MeCN. The mixture was heated in a microwave reactor at 100°C for 90 min. After flash chromatography (eluent DCM/MeOH ratio of 95/5 to 8/2 with 0.1% TEA) and treatment with Amberlyst 21, 52 mg (75%) of hybrid compound 38b as a yellowish oil was obtained.

R_f (DCM/MeOH ratio of 8/2 with 0.1% TEA) = 0.57; ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 8.54 (1 H, d, $J = 5.2$ Hz, H-3), 7.96 (1 H, d, $J = 2.1$ Hz, H-8), 7.64 (2 H, m, H-5 and H-28), 7.49 (1 H, m, H-23), 7.37 (1 H, dd, $J = 8.8$ Hz, $J = 2.1$ Hz, H-7), 7.33 (1 H, d, $J = 8.3$ Hz, H-30), 7.22 (2 H, m, H-29 and H-27), 7.10 (1 H, m, H-21), 7.03 (1 H, m, H-24), 6.38 (1 H, d, $J = 5.2$ Hz, H-2), 6.03 (1 H, bs, NH), 3.87 (2 H, m, H-19 and H-32a), 3.27 (2 H, m, H-10), 3.12 (1 H, m, H-32b), 2.88 (2 H, t, $J = 5.5$ Hz, H-11), 2.89 (8 H, m, H-12, H-14, H-17 and H-18), 2.37 (4 H, m, H-16, H-17 and

FIG 11 Synthesis of chlorpromazine-related hybrid compound 42. Reagents and conditions: (a) 33, NaH, THF, DMSO, 0°C to RT, 5 h, 58%; (b) 13, NaHB(OAc)₃, DCM, 0°C to RT, 3 h, 82%; (c) piperidine, DCM, RT, 86%; (d) K_2CO_3 , MeOH, reflux, 4 d, 10 to 12%.

FIG 12 Results for the IC₅₀ assay for the synthesized hybrid compounds, performed on chloroquine-sensitive (CQS) strain 3D7 and chloroquine-resistant (CQR) strain K1 (see Table S1 in the supplemental material for statistics). Values are means plus standard errors of the means (SEM) (error bars) for three or more replicates. Values that are significantly different from the value for CQ by two-tailed *t* test are shown by asterisks as follows: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. Values that are not significantly different from the value for CQ have no asterisk.

H-18), 1.68 (2 H, m, H-15), 1.08 (1 H, t, *J* = 14.2 Hz, H-13); ¹³C NMR (400 MHz, CDCl₃) δ (ppm) = 152.3 (C-3), 150.0 (C-1), 149.3 (C-4), 142.5 (C-25), 142.3 (C-22), 136.4 (C-26), 154.0 (C-31), 133.8 (C-6), 133.2 (C-20), 132.8 (C-28), 132.2 (C-23), 131.4 (C-30), 129.7 (C-29), 129.0 (C-21), 129.0 (C-5), 127.0 (C-24), 126.6 (C-27), 125.4 (C-8), 121.3 (C-7), 117.6 (C-9), 99.5 (C-2), 65.6 (C-19), 56.7 (C-10), 53.9 (C-11), 51.4 (C-14), 51.0 (C-16), 48.7 (C-17), 47.1 (C-12), 46.1 (C-18), 39.9 (C-10), 32.8 (C-32), 24.8 (C-15), 12.1 (C-13); MS (ESI) *m/z* = 620.2 [M+H]⁺; HRMS (ESI) *m/z* = 620.2377 [M+H]⁺, calculated: 620.2376, Diff.: 0.2 ppm.

Biology. (i) Compound preparation. Synthesized compounds for biological testing were diluted in dimethyl sulfoxide (DMSO) to create stock solutions that were stored in aliquots in the dark at −20°C. All other drugs were purchased from Sigma-Aldrich, including chloroquine diphosphate, desipramine hydrochloride, loperamide hydrochloride, and L703-606 oxalate salt hydrate. Chloroquine and desipramine were dissolved in phosphate-buffered saline (PBS), filter sterilized, and stored at −20°C for up to a month. All individually purchased compounds were dissolved in DMSO, stored in aliquots at −20°C, and diluted to working concentrations with PBS. All compounds were protected from light.

(ii) Inhibitory concentration (IC₅₀) determination for hybrid compounds. Parasitized cultures, synchronized to the ring stage, and diluted with fresh erythrocytes and malaria culture medium (MCM) to 1% parasitemia and 1.25% hematocrit, were incubated with various concentrations of hybrid compounds for 48 h. Appropriate controls consist of vehicle control (PBS or DMSO), CQ, and CQ in combination with a known chemosensitizer. To determine parasitemia, cultures were stained with Hoechst 33342 and analyzed by flow cytometry. Data obtained from flow analysis were used to plot the sigmoidal dose-response curve (i.e., percent parasitemia against concentration). The 50% inhibitory concentrations (IC₅₀s), which were the concentrations required to inhibit 50% growth could then be determined from the plot.

(iii) Hoechst staining. Hoechst 33342 stain (Life Technologies) is a DNA-binding fluorescent stain that has an excitation wavelength of 350 nm (UV range) and an emission wavelength of 461 nm (blue fluorescence). After drug treatment, 1 μg/ml of Hoechst stain was added per well for 30 min at 37°C. Cells were then washed twice and resuspended in PBS before flow cytometry analysis.

(iv) Flow cytometric analyses. Flow cytometry (Dako Cytomation CyAn ADP, Fort Collins, CO, USA) was used for Hoechst-stained cells. Both Hoechst-stained cells and CM-CQ-stained cells were excited with a 405-nm violet laser prior to 450/50 nm bandpass (BP) (±25) filter. For the

determination of the proportion of parasites positive for CM-CQ (8), Hoechst-stained duplicate wells were used to determine parasitemia. To detect both infected erythrocytes and liberated parasites, forward and side scattering adjustments were made. At least three independent experiments were performed, unless otherwise stated, to take into account any interassay variability. All results are saved in FCS 2.0 format and analyzed using the Flowjo version X (Tree Star) software.

(v) Determination of the digestive vacuole disruption potency of the hybrid compounds. Parasitized cultures, synchronized to the trophozoite stage and diluted with fresh erythrocytes and MCM to 8 to 10% parasitemia and 2.5% hematocrit, were incubated with either 10 μM or 1 μM hybrid compounds for 4 h. Appropriate controls consist of vehicle control (PBS with 0.1% DMSO) and 3 μM CQ with 0.1% DMSO. After drug treatment, 1 μg/ml of Hoechst stain and 1 μM Fluo-4 AM (Molecular Probes) diluted in MCM were added per well for 30 min at 37°C. The cells were then washed twice and resuspended in PBS to 5% hematocrit before

TABLE 1 IC₅₀ assay data for the synthesized hybrid compounds, performed on chloroquine-sensitive (CQS) strain 3D7 and chloroquine-resistant (CQR) strain K1

Compound	3D7		K1	
	Mean IC ₅₀ (nM)	SEM (nM)	Mean IC ₅₀ (nM)	SEM (nM)
1 (CQ)	28.6	5.37	513.6	29.7
24	129.1	13.8	362.7	52.7
26	262.4	37.2	408.1	91.8
27a	98.6	13.1	110.3	11.6
27b	199.6	51.4	196.9	28.0
28	456.8	5.55	221.5	28.3
29	513.3	73.2	508.6	107.6
30	351.8	36.3	303.9	75.0
31	113.6	41.9	127.5	23.3
32	282.4	63.7	280.1	33.4
35	32.4	2.08	189.5	6.53
36	101.9	3.07	337.3	28.9
37	150.9	18.0	172.3	21.3
38a	289.0	77.5	433.5	69.1
38b	149.7	59.8	229.1	49.8
42	603.3	40.4	492.7	54.1

FIG 13 Results for the IC_{50} assay for compounds 1 (CQ), 27a, 31, and 35, performed on various strains, including chloroquine-sensitive (CQS), chloroquine-resistant (CQR), and artemisinin-resistant (ArtR) strains as well as 7G8 showing intermediate chloroquine resistance (see Table S2 in the supplemental material for SEM and statistics). Values are means plus SEM (error bars) for three or more replicates. Values that are significantly different from the value for CQ by two-tailed t test are shown by asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Values that are not significantly different from the value for CQ have no asterisk.

Imagestream X MkII (AMNIS) analysis. Four independent experiments were performed to take into account any interassay variability.

Graphical data plots. All histograms were generated using Microsoft Excel Starter 2010. All other regression plots were plotted using Graphpad Prism version 5.0.

Quantitative analysis. All assays were done independently at least three times to account for interassay variability. Statistical analyses were then conducted on the data obtained before coming to any conclusions.

Statistical analysis. All data were presented as means $\pm$ standard errors of the means (SEM). Statistical differences were measured using univariate two-tailed t test. Conclusions were made based on no difference between independent runs (i.e., P value more than 0.05), whereas significant results were indicated as a P value of less than 0.05.

Ethics statement. The blood collection protocol used for *in vitro* malaria parasite culture was approved by the National University of Singapore Institutional Review Board (NUS IRB; reference code 11-383, approval number NUS-1475). Written informed consent was obtained from all of the participants involved in this study. The clinical isolates used in this study were collected in accordance with the ethical guidelines in the approved protocols (OXTREC reference number 29-09; Center for Clinical Vaccinology and Tropical Medicine, University of Oxford, Oxford, United Kingdom). The use of field isolates for work done at the NUS was in accordance with the NUS IRB (reference code 12-369E).

RESULTS AND DISCUSSION

Synthesis of hybrid compounds. Simplified analogs of CRA hit compounds 4 to 6 were used as precursor starting materials for hybrid compound synthesis (compounds 7 to 12; Fig. 4). For compounds 8 and 10, a linker was attached in order to study the influence of the distance of the two attached scaffolds (Fig. 5). After optimization to identify the most suitable protecting group (tert-butoxycarbonyl [Boc] and benzyloxycarbonyl [Cbz] groups were tested for C_2 and C_3 linkers, but failed due to difficult deprotection), the Fmoc-protected C_3 linker compound 13 could be installed onto CRA compounds 8 and 10 via reductive amination in good yield. Fmoc deprotection with piperidine yielded the free amine compounds 15 and 17.

Preserving only the key elements of the 4-amino-quinoline scaffold, analogues of compounds 1, 19, and 20 were obtained by refluxing of the neat amines with 4,7-dichloroquinoline (compound 18; Fig. 6) (15–17). The alcohol compound 21 was transformed to mesylate compound 22, possessing better leaving groups for subsequent substitution (15–17). New hybrid com-

pounds were obtained by reacting amine compounds 6, 7, 9, 12, 15, and 17 with compound 22 via a microwave-mediated (Reaction times with thermal heating were 7 days or more, which could be significantly reduced to 4 to 10 h by microwave-induced heating.) displacement reaction (Fig. 7). This afforded access to hybrid compounds with features of various CRAs in moderate yields: compounds 27a, 27b, 28, 29, and 30 from analogues of compound 4, as well as compounds 31 and 32 derived from compound 6.

In order to explore the structural features necessary to retain chemoreversal activity in the hybrid compounds, some analogues linking relatively simple precursors of the CRAs were synthesized. Joining quinuclidinone compound 23 via reductive amination to amine compound 19c, the hybrid compound 24—with only the quinuclidine core of compound 4 attached to the CQ scaffold—was obtained albeit in very poor yield (Fig. 8). Substitution of bromide compound 25 with amine compound 21a gave hybrid compound 26, possessing the *gem*-diphenylbutanenitrile feature of compound 5 (Fig. 9).

A further series of hybrid compounds retaining the tertiary ethylamine from compound 1 was prepared from intermediate compound 21a. It has been shown previously that a tertiary amine in this position impacts the activity of CQ analogues, as it influences the diffusion of the compounds in the digestive vacuole (19, 20). Ethyl amine compound 21a was first connected to a C_3 linker, using chloriodopropane (compound 33), leading to chloropro-

TABLE 2 IC_{50} assay data for selected hybrid compounds, performed on CQS, CQR, and ArtR strains^a

Strain	Mean IC_{50} (nM) of compound:			
	1 (CQ)	27a	31	35
Hb3 (CQS)	35.0	75.9	368.2	97.3
Dd2 (CQR)	331.9	113.8	408.4	108.8
ARS-233 (CQR ArtR)	357.8	140.6	499.2	113.4
ARS-272 (CQR ArtR)	321.1	123.2	216.7	110.0
NHP-04559 (CQR)	450.1	88.3	503.3	148.5
NHP-04773 (CQR ArtR)	468.0	64.4	644.8	146.5
7G8 (CQR, diff. PfCRT haplotype)	224.7	183.8	42.6	68.2

^a Abbreviations: CQS, chloroquine sensitive; CQR, chloroquine resistant; ArtR, artemisinin resistant; diff., different (referring to the different haplotype present in 7G8 versus K1); PfCRT, *P. falciparum* chloroquine resistance transporter.

FIG 14 Response modification index for compounds 27a, 31, and 35 compared to compound 1 (CQ) tested on CQR (A) and CQS (B) strains. The response modification index (RMI) is the potency of compound in each strain divided by potency of compound 1, and the error bars show the SEM of the compound in each strain divided by the potency of compound 1.

pylamine compound 34 (Fig. 10). Chloride compound 34 was then attached to various amines via a microwave-mediated Finkelstein reaction, yielding hybrid compounds in moderate to good yields: compounds 35, 36, and 37 with features of compound 4, as well as compounds 38a and 38b with features of CRA compound 6.

Additionally, a hybrid compound combining compound 3 with the scaffold of known chemosensitizer chlorpromazine was synthesized. Phenothiazine (compound 39) was transformed to chloride compound 40 using chloriodopropane (compound 33) in moderate yield (21). Chloroquine fragment compound 21a was attached to a C₃ linker with the same method used previously, yielding primary amine compound 41. Chloride compound 40 then underwent a substitution with amine compound 41, giving hybrid compound 42 in moderate yield (Fig. 11).

Antiparasitic testing in CQ/Art-sensitive and -resistant strains. Mechanisms of drug resistance in malaria depend on the specific drug and are not yet completely understood. In the case of chloroquine (compound 1) resistance (CQR), it originates in mutations in the PfCRT (*P. falciparum* chloroquine resistance transporter) (22, 23). The antimalarial mechanism of action of compound 1 is to hinder heme detoxification of the parasite in the digestive vacuole. In resistant strains, the modified transporters are able to remove compound 1 from this vacuole. The mutated PfCRTa have altered amino acids in positions 72 to 76 (CVMNK in the wild type), depending on the geographical origin: CVIET for African and most Southeast Asian resistant strains versus SVMNT for the South American resistant strains (24, 25). The hybrid compounds were initially tested in an IC₅₀ assay, determin-

ing the concentration required to inhibit the survival of 50% of parasites. The chloroquine-sensitive (CQS) parasite strain 3D7 and the resistant (CQR) strain of the CVIET haplotype, K1, were used for initial studies (Fig. 12 and Table 1). Compound 1 was used as a reference compound in all assays, with an IC₅₀ of 28.6 nM for 3D7 and a higher IC₅₀ of 514 nM for resistant strain K1 (18-fold resistance), both in line with reported data for these strains (8; Boudhar et al., submitted).

Hybrid compounds 42 and 29 showed the weakest effects of the tested compounds but were similarly active for both strains. All other hybrid compounds had IC₅₀s below 500 nM for both strains. Gratifyingly, approximately half of the compounds eradicated any effects of resistance, having very similar IC₅₀s against both 3D7 and K1 strains. Compounds with IC₅₀s of about 200 nM or lower for both strains are compounds 27a, 27b, 31, 35, 37, and 38b. Hybrid compound 35, linking CQ with the most active CRA analogue compound 4, shows an IC₅₀ of 190 nM for K1, more potent than compound 1, and retains an excellent antimalarial effect on 3D7 with an IC₅₀ of 32.4 nM. However, although exhibiting reduced resistance of about sixfold, hybrid compound 35 still has a similar pattern to compound 1 and significant resistance. More in line with our goal to identify compounds with minimal resistance, compounds 27a and 31 both stand out, as they show similarly good effects on both strains, albeit less potent against 3D7: compound 27a, combining the chloroquine pharmacophore with model compound 7, possessing the major features of the CRA pharmacophore, shows IC₅₀s of 98.6 nM and 110 nM for 3D7 and K1, respectively; compound 31 has IC₅₀s of 114 nM for 3D7 and 127 nM for K1, the best activity for compounds linking the chloroquine pharmacophore with a CRA analogue of series 6 compounds based on octoclotheptin. Compounds 27b, 37, and 38b were similar but less potent.

TABLE 3 Response modification index for compounds 27a, 31, and 35 compared to compound 1 (CQ) tested on CQS and CQR strains

Strain	RMI for compound:		
	27a	31	35
3D7 (CQS)	3.44	3.97	1.13
Hb3 (CQS)	2.17	10.52	2.78
7G8 (CQR, diff. PfCRT haplotype)	0.82	0.19	0.30
K1 (CQR)	0.21	0.25	0.37
Dd2 (CQR)	0.34	1.23	0.33
ARS-233 (CQR ArtR)	0.39	1.40	0.32
ARS-272 (CQR ArtR)	0.38	0.68	0.34
NHP-04559 (CQR ArtS)	0.20	1.12	0.33
NHP-04773 (CQR ArtR)	0.14	1.38	0.31

TABLE 4 Toxicity of three hybrid compounds in two healthy cell lines with their therapeutic windows^a

Compound	K1 IC ₅₀ (nM)	TAMH IC ₅₀ (μM)	AC10 IC ₅₀ (μM)	Therapeutic window	
				TAMH/K1	AC10/K1
1	514	85.3 ± 3.0	ND	166	ND
27a	110	72.0 ± 3.5	24.1 ± 1.0	655	219
35	190	19.4 ± 1.3	71.7 ± 3.3	102	378
37	172	2.84 ± 0.03	2.84 ± 0.27	16	16

^a TAMH and AC10 IC₅₀s were determined by CellTiter-Glo cell viability assay (Promega Corporation) and are the means of three or four independent determinations. ND, not determined.

TABLE 5 Solubility and permeability properties for the best-performing hybrid compound, compound 35

Compound	Structure	Mol wt (g/mol)	cLogD (pH 7.4) ^a	Aqueous solubility (μg/ml) (pH 7.4, 24 h) ^b	<i>P_e</i> ^c (10 ⁻⁶ cm/s, pH 7.4)
35		596	2.0	115 ± 2 at 3 h (192 ± 3 μM) 108 ± 3 at 24 h (181 ± 5 μM)	1.28 ± 0.06 at 6 h 1.10 ± 0.04 at 16 h

^a cLogD, calculated LogD using the properties viewer by Chemaxon (www.chemicalize.org).

^b Aqueous solubility values are means ± standard deviations (SD) (*n* = 3). The solubilities at the 3-h time point were also recorded and were in good agreement with the reported values at 24 h. Values in parentheses are micromolar.

^c *P_e*, effective permeability determined by the parallel artificial membrane permeation assay (PAMPA). Values are means ± SD (*n* = 3).

Based on these results, three hybrid compounds representing the most potent hybrids without any resistance to strain K1 (compounds 27a and 31) and the improved compound with good potency against strain 3D7 (compound 35) were chosen for further testing on multiple lab strains and field isolates. Strains selected include chloroquine-sensitive or -resistant strains as well as artemisinin-resistant strains (ArtR) and the intermediate chloroquine-resistant strain with the SVMNT haplotype, 7G8 (Fig. 13 with data presented in Table 2). Compound 31 shows a slightly lower IC₅₀ than compound 1 for three strains, K1, ARS-272, and 7G8. However, compounds 27a and 35 proved to be efficient on all tested strains. This indicates that aromatic rings, present in compounds 35 and 31, but not compound 27a, are not strictly required for broad activity.

These trends can be seen clearly in Fig. 14 (data presented in Table 3), illustrating the response modification index (RMI) (the ratio of IC₅₀ for new compounds divided by the IC₅₀ for compound 1) for CQR and CQS strains. Compound 31 is 4 to 5 times more effective against K1 and 7G8 CQR strains (RMIs of 0.25 and 0.19, respectively), whereas it is 4- to 10-fold less effective than compound 1 against both CQS strains. On the other hand, compounds 27a and 35 show RMIs of 0.2 to 0.4 for most CQR strains while retaining acceptable activity for CQS strains (≤100 nM). Hybrid compound 35 was particularly significant, given its consistent broad spectrum activity for all the resistant strains studied. These strains include not only CQR strains but also artemisinin-resistant strains, further evidence that the chemosensitizing approach has general applicability in tackling some of the latest most concerning field isolates. Furthermore, this conclusion is supported by the fact that the CRA component of compound 35, i.e., compound 4, is the most promising CRA from our earlier studies of combinations of CRAs with compound 1. We next carried out preliminary absorption, distribution, metabolism, and excretion (ADME) and toxicity profiling of both of the most promising molecules, compounds 27a and 35.

Digestive vacuole disruption efficacy of hybrid compounds. The mechanisms of action of chloroquine have been previously elucidated. Apart from heme polymerization (26), recent studies have shown that chloroquine at micromolar concentrations disrupts the digestive vacuole of the parasite, triggering a cascade of programmed cell death (27, 28). We performed a high-content phenotypic assay using the imaging flow cytometer (Imagstream X MkII) to detect for digestive vacuole disruption after drug treatment. We observed that there was disruption of the parasite's

digestive vacuole upon treatment with all the hybrid compounds, and the largest amount of disruption occurred after treatment with compound 35 (see Fig. S1 in the supplemental material). The digestive-vacuole-disrupting activities of all compounds tested were not as potent as chloroquine, suggesting that they probably work in a similar manner to chloroquine, but the potency was reduced, possibly due to an increase in the size of the compound.

Cytotoxicity. The cytotoxicity of compound 1 and selected hybrids was investigated with a transforming growth factor alpha (TGFα)-transfected mouse hepatocyte (TAMH) cell line, determining the IC₅₀ via an ATP luminescence assay (29). Compounds 27a and 1 had little effect on cell viability, requiring high concentrations, hence very high therapeutic windows. Compound 35 was only weakly toxic with an IC₅₀ of 19.4 μM in TAMH cells, representing a therapeutic window of more than 100-fold. However, compound 37, selected for testing due to it being a biaryl pharmacophore analogue of compound 35 but without the quinuclidine, was more toxic with an IC₅₀ of 2.84 μM and lower therapeutic window of 16 (Table 4).

In addition to TAMH cells, the cardiomyocyte cell line, AC10, was selected to study the potential cardiotoxic properties of the hybrid compounds (30). Interestingly, in AC10 cells, compound 37 had the same toxicity as in TAMH cells, confirming its lower therapeutic window. Compound 27a was about 10-fold-less toxic than compound 37 with a therapeutic window of more than 200. Encouragingly, compound 35 was only weakly toxic, with an IC₅₀ of 71.7 μM equating to a high therapeutic window of 378 in AC10 cells.

Solubility and permeability. In a kinetic solubility assay with HPLC quantification, compound 35 had solubility of 108 μg/ml at pH 7.4 following 24 h of incubation, similar to the data at 3 h. In a parallel artificial membrane permeability assay (PAMPA) (31–33) compound 35 was permeable with an effective permeability (*P_e*) of 1.10 × 10⁻⁶ cm⁻¹ after a 16-h equilibration (Table 5). Its molecular weight is below 600, and the calculated LogD (pH 7.4) value of 2.0 is suitable for an orally administered drug.

Concluding remarks. In this study, we have designed and synthesized new hybrid compounds merging the antimalarial activity of chloroquine (compound 1) with the chemoreversal activity of selected chemoreversal agents (CRAs) into a single agent. Linkers of three carbons were effective in joining the CRA to a minimum pharmacophore chloroquine moiety. Hybrid compounds 27a, 31, and 35 were tested in dose-response studies against a panel of malaria parasites both sensitive and resistant to compound 1 and artemisinin. The most promising hybrid, compound 35, presents

a good solubility, permeability, and *in vitro* toxicity profile. *In vivo* safety studies will be conducted before nominating compound 35 as a clinical candidate. Good laboratory practice (GLP) toxicology studies would involve dosing compound 35 to two species at high, medium, and low doses to establish a no-effect level dose and to identify the starting dose in humans. This preliminary study provides support for potent hybrid CQ-CRA compounds as promising potential therapy for the treatment of CQR malaria.

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