SUPPORTING INFORMATION

Hybrid Inhibitors of Malarial Dihydrofolate Reductase with Dual Binding Modes That Can Forestall Resistance

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RESULT

Co-crystal Structures of the Hybrid Inhibitors with hDHFR. Compounds **BT1** and **BT2** co-crystal structures with hDHFR were determined in order to visualize the difference in the binding modes of the hybrid inhibitors. For hDHFR, the enzyme accommodates **BT1** and **BT2** in a similar binding mode to those observed in the wild-type PfDHFR. The rigid part of **BT1** binds deep in the pocket interacting with E30, equivalent to PfDHFR D54 (**Figure S1A**). In order to avoid steric clash with T56 (equivalent to PfDHFR S108), the flexible end of **BT2** is docked at the E30 site and the phenyl ring of the rigid end forms a tilted π-π interaction with the F31 side chain, equivalent to PfDHFR M55 (**Figure S1B**). In addition, subtle movement (1 Å) of the hydrophobic cleft at residues 60-64 (IPEKN) was observed in the binding of **BT2**. However, the pyrimidine rings of both **BT1** and **BT2** protrude out of the pocket space, resulting in weaker inhibition in the human enzyme (**Figure S1C**). In contrast, all three hybrid inhibitors stay within PfDHFR enzyme space and the van der Waals interaction of F116 (Figure 2).

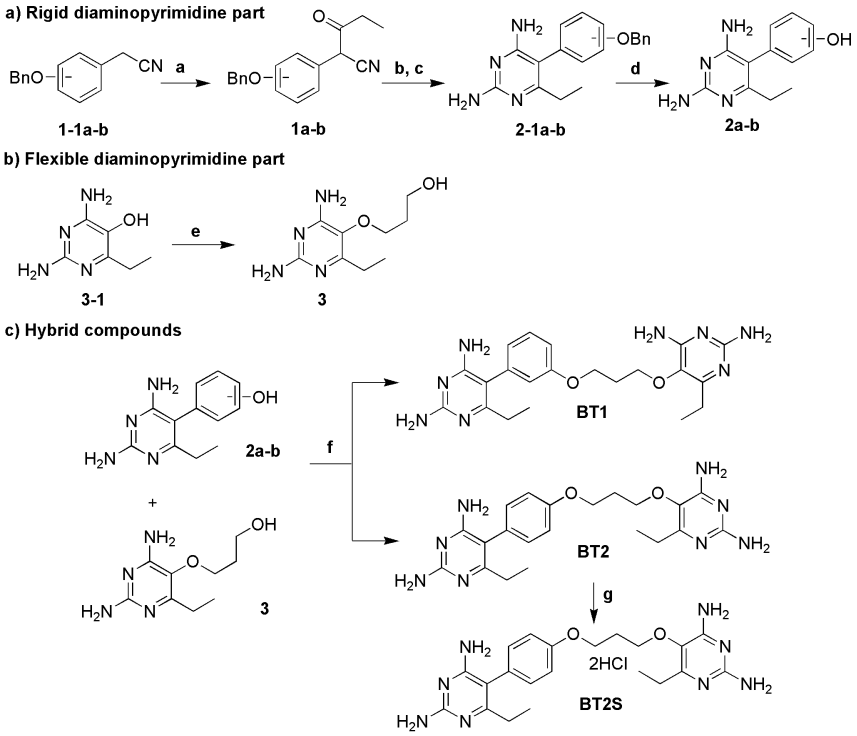
MATERIALS AND METHODS

Chemistry Experimental Section

General Procedure: All reagents were purchased from Aldrich, Fluka and Merck, and used without further purification, unless otherwise indicated. Solvents were purchased from Aldrich, Fluka, Fisher and RCI Labscan, and where necessary dried immediately prior to use by distillation from standard drying reagents. Melting points were recorded using an Electrothermal IA9100 apparatus and are uncorrected. ^{1}H NMR spectra were recorded using a Bruker Avance III 400 (400 MHz), or a Bruker Avance III HD (500 MHz) spectrometer. ^{13}C NMR spectra were recorded using a Bruker Avance III 400 (100 MHz), or a Bruker Avance III HD (125 MHz) spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) and are referenced to the appropriate residual solvent peak. Electrospray ionization high resolution mass spectra were measured on a Bruker microTOF mass spectrometer in positive ion mode.

I. Chemical Synthesis of Rigid-Flexible Hybrid Compounds

Scheme S1 Synthetic strategy employed for hybrid compound



Reagents and conditions: (a) NaH, THF, -78 °C then EtCOOMe, 90 °C; (b) CH_2N_2, dioxane/MeOH; (c) guanidine, DMSO/MeOH, 90-100 °C; (d) cyclohexene, 10% Pd/C, 65 °C; (e) $LiOH.H_2O$, DMF, 3-bromo-1-propanol, rt; (f) Ph_3P, DIAD, DMF, rt; (g) HCl, H_2O

A. Rigid Diaminopyrimidine Part

1. Synthesis of *β*-ketonitrile (**1**)

A solution of phenylacetonitrile **1-1**[1] (781 mg, 3.50 mmol) in THF (15 mL) was slowly added into a cooled suspension of sodium hydride (153 mg, 3.50 mmol, 55% dispersion in mineral oil) in THF (5 mL) at -78 °C and stirred under N_2 for 30 min. A reaction mixture was then heated at 90 °C for 10 min followed by an addition of methyl propionate (1.7 mL, 17.50 mmol) in one portion. The reaction mixture was allowed to stir at 90 °C for 20 min and then quenched with saturated NH_4Cl aqueous solution (50 mL) at 0 °C. The product was extracted with CH_2Cl_2 (3×100 mL) and the organic layer was washed with brine (50 mL), dried ($MgSO_4$), and evaporated to

dryness under reduced pressure. Purification of the crude product by silica gel column chromatography (hexanes/CH_2Cl_2 45:55) afforded the pure compound.

2-(3-(Benzyloxy)phenyl)-3-oxopentanenitrile (1a): a white solid, 40%; mp 69-71 ^oC (CH_2Cl_2/hexanes). ^1H NMR (400 MHz, $CDCl_3$, δ): 7.45-7.30 (m, 6H), 6.99 (m, 3H), 5.08 (s, 2H), 4.65 (s, 1H), 2.68-2.48 (m, 2H), 1.02 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 199.4, 159.5, 136.3, 131.2, 130.7, 128.7, 128.2, 127.6, 120.4, 116.3, 115.7, 114.5, 70.2, 50.7, 33.1, 7.6. HRMS-ESI (*m/z*): $[M+Na]^+$ calcd for $C_{18}H_{17}NNaO_2$, 302.1151; found: 302.1154.

2-(4-(Benzyloxy)phenyl)-3-oxopentanenitrile (1b): a white solid, 45%; mp 134-135 ^oC (CH_2Cl_2/hexanes). ^1H NMR (400 MHz, $CDCl_3$, δ): 7.43-7.34 (m, 5H), 7.29 (d, J = 8.6 Hz, 2H), 7.01 (d, J = 8.6 Hz, 2H), 5.07 (s, 2H), 4.63 (s, 1H), 2.72-2.51 (m, 2H), 1.03 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 199.7, 159.4, 136.3, 130.1, 129.2, 128.7, 128.2, 127.5, 122.0, 116.5, 115.8, 70.1, 49.8, 33.0, 7.6. HRMS-ESI (*m/z*): $[M+Na]^+$ calcd for $C_{18}H_{17}NNaO_2$, 302.1151; found, 302.1156.

2. Synthesis of rigid diaminopyrimidine (2-1)

Diazomethane gas was passed into a cold solution of β-ketonitrile **1** (886 mg, 3.00 mmol) in dioxane/MeOH (6:1, 35 mL) and the reaction mixture was stirred at rt for 8 h. Removal of solvent under reduced pressure gave the desired enol-ether, which was dried under vacuum and used in the next step without purification. A mixture of enol-ether and guanidine (195 mg, 3.30 mmol) in DMSO/EtOH (8:2, 25 mL) was heated at 90-100 ^oC under N_2 for 6 h. After removal of solvents under vacuum, water (100 mL) was added to a residue and extracted with CH_2Cl_2 (3×100 mL). The combined organic extracts were washed with water (50 mL), brine (50 mL), dried ($MgSO_4$) and evaporated under reduced pressure. Purification of the residue by silica gel column chromatography (CH_2Cl_2/MeOH 94:6) yielded the pure product **2-1**.

5-(3-(Benzyloxy)phenyl)-6-ethylpyrimidine-2,4-diamine (2-1a): a white solid, 75%; mp 201-202 ^oC (MeOH). ^1H NMR (400 MHz, DMSO-d_6, δ): 7.45 (d, J = 7.2 Hz, 2H), 7.38 (dd, J = 7.6, 7.1 Hz, 2H), 7.34-7.30 (m, 2H), 6.99 (dd, J = 8.2, 2.2 Hz, 1H), 6.80 (s, 1H), 6.75 (d, J = 7.5 Hz, 1H), 5.90 (s, 2H, NH_2), 5.54 (br s, 2H, NH_2), 5.11 (s, 2H), 2.09 (q, J = 7.5 Hz, 2H), 0.93 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 166.3, 162.0, 161.9, 158.6, 137.4, 137.1, 130.0, 128.4, 127.8, 127.7, 123.0, 116.7, 113.8, 106.4, 69.2, 27.4, 13.2. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{19}H_{21}N_4O$, 321.1710; found, 321.1711.

5-(4-(Benzyloxy)phenyl)-6-ethylpyrimidine-2,4-diamine (2-1b): a white solid, 75%; mp 229-230 °C (MeOH). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.48 (d, J = 7.1 Hz, 2H), 7.42 (m, 2H), 7.36-7.33 (m, 1H), 7.08 (m, 4H), 5.80 (s, 2H, NH_2), 5.39 (br s, 2H, NH_2), 5.11 (s, 2H), 2.10 (q, J = 7.6 Hz, 2H), 0.95 (t, J = 7.6 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 167.4, 163.0, 162.6, 158.2, 137.8, 132.4, 129.1, 128.8, 128.6, 128.5, 115.8, 106.8, 70.0, 28.2, 13.9. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{19}H_{21}N_4O$, 321.1710; found, 321.1716.

3. Synthesis of rigid diaminopyrimidine (2)

A mixture of diaminopyrimidine **2-1** (320 mg, 1.00 mmol), 10% Pd/C (100 mg) and cyclohexene (2 mL) in dioxane/MeOH (1:1, 10 mL) was heated at 65 °C under N_2 for 6 h. The Pd/C was removed by Celite filtration and the filtrate was concentrated under reduced pressure to give a solid, which was then recrystallized from dioxane/MeOH to yield a white solid **3**.

5-(3'-Hydroxyphenyl)-6-ethylpyrimidine-2,4-diamine (2a): a white solid; 85%, mp 248-249 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 9.47 (s, 1H, OH), 7.21 (t, J = 7.8 Hz, 1H), 6.72 (dd, J = 8.0, 1.8 Hz, 1H), 6.59-6.54 (m, 2H), 5.83 (s, 2H, NH_2), 5.41 (br s, 2H, NH_2), 2.12 (q, J = 7.5 Hz, 2H), 0.97 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 166.2, 162.0, 161.9, 157.6, 137.2, 129.9, 121.0, 117.2, 114.2, 106.7, 27.4, 13.3. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{12}H_{15}N_4O$, 231.1240; found, 231.1243.

5-(4'-Hydroxyphenyl)-6-ethylpyrimidine-2,4-diamine (2b): a white solid; 80%, mp 247-249 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 9.44 (s, 1H, OH), 6.95 (d, J = 8.4 Hz, 2H), 6.81 (d, J = 8.4 Hz, 2H), 5.79 (s, 2H, NH_2), 5.38 (br s, 2H, NH_2), 2.10 (q, J = 7.5 Hz, 2H), 0.94 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 166.6, 162.4, 161.8, 156.4, 131.5, 126.0, 115.8, 106.4, 27.4, 13.2. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{12}H_{15}N_4O$, 231.1240; found, 231.1234.

B. Flexible Diaminopyrimidine Part

4. Synthesis of flexible diaminopyrimidine (3)

A suspension of 5-hydroxy-6-ethyl-2,4-diaminopyrimidine (**3-1**) (771 mg, 5.00 mmol) and lithium hydroxide monohydrate (629 mg, 15.00 mmol) in DMF (5 mL) was stirred at rt for 30 min. 3-Bromo-1-propanol (0.45 mL, 5.00 mmol) was then added, and the reaction mixture was continued to stir at rt for 8 h. Removal of DMF under vacuum gave crude product, which was purified by crystallization from water to yield pure 5-(3'-hydroxypropyloxy)-6-ethyl-2,4-

diaminopyrimidine (**3**) as a white solid (690 mg; 65%, mp 182-183 ^{o}C). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 6.13 (s, 2H, NH_2), 5.55 (s, 2H, NH_2), 4.65 (br s, 1H, OH), 3.66 (t, J = 6.1 Hz, 2H), 3.56 (t, J = 6.1 Hz, 2H after D_2O exchange), 2.36 (q, J = 7.5 Hz, 2H), 1.82 (quin, J = 6.1 Hz, 2H), 1.06 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 160.4, 159.7, 158.9, 129.0, 70.0, 58.1, 33.0, 24.2, 13.4. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_9H_{17}N_4O_2$, 213.1346; found, 213.1345.

C. Rigid-Fexible Hybrid Compounds

5. Synthesis of rigid-flexible hybrid compounds

To a solution of compound **2** (230 mg, 1.00 mmol), compound **3** (265 mg, 1.25 mmol) and triphenylphosphine (302 mg, 1.15 mmol) in DMF (3 mL) was added diisopropyl azodicarboxylate (0.23 mL, 1.15 mmol) at rt and stirred under N_2 for 8 h. Removal of solvents under vacuum gave an off-white solid, which was then recrystallized from water, hexanes/MeOH, MeOH/water, and dioxane/MeOH systems, respectively to yield a white solid. Flash silica gel column chromatography (CH_2Cl_2/MeOH 90:10) gave the pure compound.

5-[3-[3-[(2,4-Diamino-6-ethyl-5-pyrimidinyl)oxy]propoxy]phenyl]-6-ethylpyrimidine-2,4-diamine (BT1): a white solid; 30%, mp 208-210 ^{o}C. ^{1}H NMR (500 MHz, DMSO-d_6, δ): 7.35 (t, J = 8.1 Hz, 1H), 6.94 (dd, J = 8.3, 1.6Hz, 1H), 6.75-6.73 (m, 2H), 6.11 (s, 2H, NH_2), 5.86 (s, 2H, NH_2), 5.57 (s, 2H, NH_2), 5.44 (br s, 2H, NH_2), 4.16 (m, 2H), 3.76 (t, J = 6.1 Hz, 2H), 2.32 (q, J = 7.5 Hz, 2H), 2.14-2.10 (m, 4H), 0.99 (t, J = 7.5 Hz, 3H), 0.96 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 166.1, 161.7, 161.6, 159.6, 158.6, 157.9, 137.1, 129.6, 128.4, 122.4, 116.3, 113.3, 106.5, 68.8, 64.2, 29.0, 27.0, 23.3, 12.5, 11.9. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{21}H_{29}N_8O_2$, 425.2408; found, 425.2409.

5-[4-[3-[(2,4-Diamino-6-ethyl-5-pyrimidinyl)oxy]propoxy]phenyl]-6-ethylpyrimidine-2,4-diamine (BT2): a white solid; 35%, mp 257-258 ^{o}C. ^{1}H NMR (500 MHz, DMSO-d_6, δ): 7.09 (d, J = 8.3 Hz, 2H), 7.01 (d, J = 8.3 Hz, 2H), 6.11 (s, 2H, NH_2), 5.81 (s, 2H, NH_2), 5.56 (s, 2H, NH_2), 5.39 (br s, 2H, NH_2), 4.19 (t, J = 5.8 Hz, 2H), 3.79 (t, J = 5.8 Hz, 2H), 2.35 (q, J = 7.5 Hz, 2H), 2.16 (m, 2H), 2.11 (q, J = 7.5 Hz, 2H), 1.03 (t, J = 7.5 Hz, 3H), 0.95 (t, J = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6, δ): 166.6, 162.3, 161.9, 159.8, 159.0, 158.1, 157.5, 131.6, 128.7, 127.8,

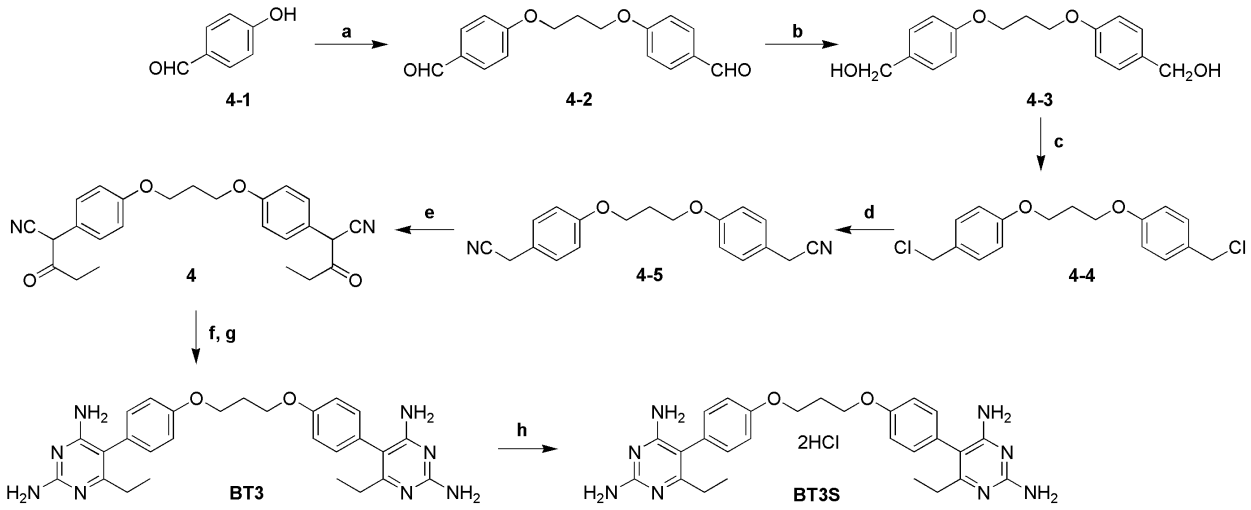
114.9, 106.1, 68.9, 64.2, 29.2, 27.4, 23.5, 13.1, 12.6. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{21}H_{29}N_8O_2$, 425.2408; found, 425.2418.

6. Synthesis of 5-[4-[3-[(2,4-Diamino-6-ethyl-5-pyrimidinyl)oxy]propoxy]phenyl]-6-ethylpyrimidine-2,4-diamine dihydrochloride (BT2S)

A mixture of **BT2** (100 mg, 0.24 mmol) and concentrated hydrochloric acid (0.04 mL, 0.48 mmol) in water (1 mL) was stirred at rt for 1 h. The resulting precipitates were collected by filtration and washed with water to yield pure **BT2S** as a white solid (105 mg, 95%). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 12.76 (s, 1H, NH^+), 12.60 (s, 1H, NH^+), 8.35 (s, 1H, NH), 8.14 (s, 1H, NH), 7.87 (s, 1H, NH), 7.48 (br s, 4H, 2×NH_2), 7.18 (d, *J* = 8.6 Hz, 2H), 7.07 (d, *J* = 8.6 Hz, 2H), 6.70 (s, 1H, NH), 4.18 (t, *J* = 5.9 Hz, 2H), 3.89 (t, *J* = 6.0 Hz, 2H), 2.53 (q, *J* = 7.5 Hz, 2H), 2.24-2.19 (m, 4H), 1.13 (t, *J* = 7.6 Hz, 3H), 1.05 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 164.3, 160.9, 158.4, 154.7, 153.8, 153.2, 147.1, 131.4, 127.4, 122.8, 115.2, 107.7, 70.5, 64.1, 28.7, 23.3, 19.7, 12.0, 11.5. HRMS-ESI (*m/z*): $[M-2HCl+H]^+$ calcd for $C_{21}H_{29}N_8O_2$, 425.2408; found, 425.2403.

II. Chemical Synthesis of Rigid-Rigid Hybrid Compounds

Scheme S2 Synthesis of rigid-rigid hybrid compound



Reagents and conditions: (a) K_2CO_3, 1,3-dibromopropane, DMF; (b) $NaBH_4$, MeOH; (c) $SOCl_2$, CH_2Cl_2; (d) NaCN, DMF; (e) NaH, THF, -78 ^{o}C then EtCOOMe, 90 ^{o}C; (f) CH_2N_2, dioxane/MeOH; (g) guanidine, DMSO/MeOH, 90-100 ^{o}C; (h) HCl, H_2O

1. Synthesis of 4,4-[1,3-propanediylbis(oxy)]bisbenzaldehyde (4-2)

To a mixture of 4-hydroxybenzaldehyde (**4-1**) (1.83 g, 15.00 mmol) and anhydrous potassium carbonate (4.15 g, 30.00 mmol) at 0 °C, dry DMF (50 mL) was added and continued stirring at 0 °C for 3 h. The reaction mixture was then heated to 90 °C for 1 h and followed by an addition of 1,3-dibromopropane (1.5 mL, 7.50 mmol) in one portion. After stirring for 1 h and removal of DMF under vacuum, water (100 mL) was added and extracted with CH_2Cl_2 (3×100 mL). Drying ($MgSO_4$) and evaporation under reduced pressure yielded crude product, which was purified by silica gel column chromatography (hexanes/CH_2Cl_2 70:30) to give the desired product as a white solid (3.20 g; 75%, mp 137-139 °C, lit.[2] mp 134-135 °C). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 9.89 (s, 2H, CHO), 7.84 (d, J = 8.7 Hz, 4H), 7.02 (d, J = 8.7 Hz, 4H), 4.26 (t, J = 6.0 Hz, 4H), 2.35 (quin, J = 6.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 190.9, 164.0, 132.2, 130.4, 115.0, 64.8, 29.2. HRMS-ESI (*m/z*): $[M+Na]^+$ calcd for $C_{17}H_{16}NaO_4$, 307.0941; found, 307.0948.

2. Synthesis of 4,4-[1,3-propanediylbis(oxy)]bisbenzyl alcohol (4-3)

To a suspension of aldehyde **4-2** (2.84 g, 10.00 mmol) in THF/MeOH (25 mL, 4:1), $NaBH_4$ (1.14 g, 30.00 mmol) was added portionwise at 0 °C and then left stirring at rt for 6 h. The resulting reaction mixture was concentrated under reduced pressure and then cooled to 0 °C, ice-water was carefully added dropwise followed by acidification with dil. HCl to provide a white solid. Recrystallization with water and THF gave the pure alcohol **4-3** as white solid (2.60 g; 90%, mp 154-155 °C, lit.[3] mp 145-146 °C). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.21 (d, J = 8.3 Hz, 4H), 6.89 (d, J = 8.4 Hz, 4H), 5.05 (t, J = 5.6 Hz, 2H, 2×OH), 4.40 (d, J = 5.6 Hz, 4H), 4.10 (t, J = 6.2 Hz, 4H) 2.14 (quin, J = 6.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6, δ)*:* 157.4, 134.6, 127.9, 114.1, 64.2, 62.5, 28.7. HRMS-ESI (*m/z*): $[M+Na]^+$ calcd for $C_{17}H_{20}NaO_4$, 311.1254; found, 311.1257.

3. Synthesis of 4,4-[1,3-propanediylbis(oxy)]bisbenzyl chloride (4-4)

To a solution of benzyl alcohol **4-3** (1.50 g, 5.20 mmol) in CH_2Cl_2 (15 mL) was added dropwise $SOCl_2$ (1.1 mL, 15.60 mmol) at 0 °C under N_2, and the mixture was then heated at reflux for 5 h. Neutralization with saturated solution of $NaHCO_3$, extraction with CH_2Cl_2 (3×100 mL) and evaporation to dryness yielded a solid which was recrystallized with diethyl ether to give the desired product **4-4** as an off-white solid with 90% purity as determined by ^{1}H NMR and used directly in the next step (1.06 g; 65%, mp 128-130 °C, lit.[4] mp 126-127 °C). ^{1}H NMR (400 MHz,

$CDCl_3$, δ): 7.30 (d, J = 8.5 Hz, 4H), 6.88 (d, J = 8.5 Hz, 4H), 4.56 (s, 4H), 4.16 (t, J = 6.0 Hz, 4H), 2.29-2.22 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 158.9, 130.1, 114.5, 64.4, 46.3, 29.2.

4. Synthesis of 4,4-[1,3-Propanediylbis(oxy)]bisbenzeneacetonitrile (4-5)

A mixture of benzyl chloride **4-4** (1.63 g, 5.00 mmol) and sodium cyanide (490 mg, 10.00 mmol) in DMF (5 mL) was heated at 100 ^{o}C for 5 h. After removal of DMF, water (100 mL) was added to a residue and followed by extraction with CH_2Cl_2 (3×100 mL). Drying ($MgSO_4$) and evaporation under reduced pressure gave crude product, which was purified by flash chromatography (hexanes/CH_2Cl_2 65:35) to afford **4-5** as a white solid (1.15 g; 75%, mp 119-121 ^{o}C, lit.[4] mp 112-113 ^{o}C). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.22 (d, J = 8.5 Hz, 4H), 6.90 (d, J = 8.5 Hz, 4H), 4.15 (t, J = 6.0 Hz, 4H) 3.68 (s, 4H), 2.26 (quin, J = 6.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 158.5, 129.1, 121.9, 118.2, 115.0, 64.4, 29.1, 22.8. HRMS-ESI (m/z): $[M+Na]^+$ calcd for $C_{19}H_{18}N_2NaO_2$, 329.1260; found, 329.1265.

5. Synthesis of 1,3-Di(4-(1-cyano-2-oxobutyl)phenoxy)propane (4)

Following the procedure as described for compound **1**, compound **4-5** (1.07 g, 3.50 mmol) was alkylated with methyl propionate (1.7 mL, 17.50 mmol) to produce **4** as a white solid (659 mg, 45%; mp 126-128 ^{o}C). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.27 (d, J = 8.5 Hz, 4H), 6.94 (d, J = 8.5 Hz, 4H), 4.62 (s, 2H), 4.16 (t, J = 5.9 Hz, 4H), 2.70-2.50 (m, 4H), 2.27 (quin, J = 5.9 Hz, 2H), 1.02 (t, J = 7 .2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 199.7, 159.4, 129.2, 121.9, 116.5, 115.4, 64.3, 49.8, 33.0, 29.0, 7.6. HRMS-ESI (m/z): $[M+Na]^+$ calcd for $C_{25}H_{26}N_2NaO_4$, 441.1785; found, 441.1793.

6. Synthesis of 5,5-[1,3-Propanediylbis(oxy-4,1-phenylene)]-bis[6-ethyl]-pyrimidine-2,4-diamine (BT3)

Following the procedure as described for compound **2-1**, compound **4** (627 mg, 1.50 mmol) was converted to the corresponding enol-ether, which was then condensed with guanidine (222 mg, 3.75 mmol). Crystallization with hot water and dioxane/MeOH yielded the pure hybrid **BT3** as a white solid (488 mg, 65%, mp 270-272 ^{o}C (dec)). ^{1}H NMR (500 MHz, DMSO-d_6, δ): 7.07 (d, J = 8.6 Hz, 4H), 7.01 (d, J = 8.6 Hz, 4H), 5.82 (s, 4H, 2×NH_2), 5.40 (br s, 4H, 2×NH_2), 4.17 (t, J = 6.0 Hz, 4H), 2.21 (quin, J = 6.1 Hz, 2H), 2.09 (q, J = 7.5 Hz, 4H), 0.94 (t, J = 7.5 Hz, 6H). ^{13}C

NMR (125 MHz, DMSO-d_6, δ): 167.0, 162.8, 162.3, 158.0, 132.1, 128.3, 115.4, 106.6, 64.5, 29.2, 27.9, 13.6. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{27}H_{33}N_8O_2$, 501.2721; found, 501.2723.

7. Synthesis of 5,5-[1,3-Propanediylbis(oxy-4,1-phenylene)]-bis[6-ethyl]-pyrimidine-2,4-diamine dihydrochloride (BT3S)

Following the procedure as described for **BT2S**, **BT3S** was obtained as a white solid (108 mg; 94%, mp >300 ^oC (dec)). ^1H NMR (500 MHz, DMSO-d_6, δ): 12.72 (s, 2H, 2×NH^+), 8.12 (s, 2H, NH_2), 7.57 (br s, 4H, 2×NH_2), 7.18 (d, J = 8.6 Hz, 4H), 7.08 (d, J = 8.6 Hz, 4H), 6.71 (s, 2H, 2XNH_2), 4.19 (t, J = 6.0 Hz, 4H), 2.25-2.18 (m, 6H), 1.04 (t, J = 7.6 Hz, 6H). ^{13}C NMR (125 MHz, DMSO-d_6, δ): 164.0, 158.4, 156.1, 131.6, 123.8, 115.2, 107.5, 64.0, 28.6, 24.2, 12.5. HRMS-ESI (*m/z*): $[M-2HCl+H]^+$ calcd for $C_{27}H_{33}N_8O_2$, 501.2721; found, 501.2736.

Biological Experimental Section

Kinetic Analysis. DHFR activity was determined spectrophotometrically by measuring the rate of reduction of NADPH at 340 nm using ε_{340} of 12,300 M^{-1} cm^{-1}. Kinetics studies were performed as described previously.[5]

***In vitro* Antimalarial and Cytotoxicity Analysis.** *P. falciparum* strains TM4/8.2, K1CB1, W2, CSL-2 and V1/S carrying DHFR wild-type, C59R+S108N, N51I+C59R+S108N, C59R+S108N+I164L and N51I+C59R+S108N+I164L, respectively were maintained continuously in human erythrocytes in RPMI1640 supplemented with 25 mM HEPES, pH 7.4, 0.2% $NaHCO_3$, 40 µg mL^{-1} gentamicin and 8% human serum at 37 °C under 3% CO_2. *In vitro* antimalarial activity was determined using a modified Microdilution Radioisotope Technique.[5] Cytotoxicity tests against African green monkey kidney fibroblast (Vero cells) were performed using the sulforhodamine B (SRB) assay.[6]

Assessment of Possible PfDHFR Resistance Mutations against BT1. PfDHFR genes were PCR-amplified using error-prone PCR conditions as described in Chusacultanachai et al.[7] Plasmids containing the pET17b backbone and cloned synthetic genes for expression of PfDHFR bearing wild-type; S108N single; C59R+S108N double; N51I+C59R+S108N triple and N51I+C59R+S108N+I164L quadruple pyrimethamine-resistance mutations as described in Sirawarporn et al[8] were used as templates for error-prone PCR. The error-prone PCR products

obtained from each plasmid template were combined in a DNA shuffling reaction.[9] The mutagenized, DNA-shuffled PCR product was then cloned into the pET17b plasmid via unique *Hin*dIII and *Nde*I restriction sites and transformed into BL21(DE3) *Escherichia coli* by electroporation. The transformed cells were plated out on 20 plates of M9 minimal medium agar plates supplemented with ampicillin (100 μg mL^{-1}) and trimethoprim (2 μM). Approximately 1.5×10^5 colonies were obtained. Twenty colonies were randomly picked and plasmid DNA purified by alkaline lysis.[10] Plasmid DNA was sequenced using the Single Pass DNA Sequencing service (First BASE Laboratories, Sdn Bhd, Malaysia). Each of the plasmid sequences had unique nucleotide variations, thus demonstrating that the original library has a complexity of approximately 1.5×10^5 PfDHFR variants. Furthermore, each variant possesses sufficient DHFR activity to support the growth of the surrogate cell in which the endogenous DHFR is inhibited by trimethoprim.[7] Plasmid DNA was extracted and purified from the pooled bacterial colonies to generate library DNA. Library DNA was re-transformed into *E. coli* for drug selection. Selection of antifolate-resistant variants was done as follows. Approximately 10 ng of library DNA was transformed into BL21(DE3) *E. coli* by electroporation. The transformed cells were plated out on M9 minimal medium agar plates supplemented with ampicillin (100 μg mL^{-1}), trimethoprim (2 μM) and **BT1** (100 or 200 μM). Previous testing of surrogate cells transformed with plasmids expressing wild-type or N51I+C59R+S108N+I164L quadruple mutant PfDHFR showed that 100 μM **BT1** is completely inhibitory to the growth of these variants.

Five **BT1** resistant colonies on the plate with 100 μM **BT1** and nine resistant colonies from the plate with 200 μM **BT1** were picked and plasmid DNA extracted for sequencing. All plasmids shared the same nucleotide sequence, in which novel resistance mutations K97N, S108T and E199V were identified in addition to the pyrimethamine-resistance mutations N51I, C59R and I164L.

Structural Biology Experimental Section

Crystallization, Structure Determination and Analysis. PfDHFR-TS enzymes were expressed, purified and crystallized as described previously.[11,12] Co-crystals of **BT1**, **BT2** and **BT3** with wild-type and quadruple mutant PfDHFR-TS were obtained from a microbatch method. Diffraction data for the flash cooled co-complex crystals were cryo-collected at the NSRRC beamline 13B1

of Taiwan, ROC. Data processing was performed with the HKL-2000 software package[13] and programs of the CCP4 suite.[14] Structural phases were solved by molecular replacement using PHASER[15] in the CCP4 suite with PfDHFR-TS pdb codes 1J3I and 1J3K. Structure refinement and adjustment were performed with REFMAC5[16] and COOT.[17, 18] Parameter files of inhibitors **BT1**, **BT2** and **BT3** for refinement was generated with LIBCHECK. The structures were validated in PROCHECK.[19] PYMOL (http://www.pymol.org) was used for molecular graphics.

Human DHFR enzyme was expressed, purified and crystallized as described previously.[20] Co-crystals of **BT1** and **BT2** with hDHFR were obtained from hanging drop method at 24 °C. Paratone-N oil (Hampton Research) was used as a cryoprotectant. Diffraction data of **BT1**/hDHFR was collected using Bruker MicroSTAR X-ray generator equipped with marccd detector at Synchrotron Light Research Institute (SLRI, Public Organization) and processed with automar suite. Data of **BT2**/hDHFR was collected using a Bruker-Nonius FR591 X-ray generator equipped with a κCCD detector and processed with HKL2000.[13] Molecular replacement with pdb code 4DDR as template using MOLREP[21] and refinement using REFMAC5[16] were performed in CCP4 suite.[14] The model was built using COOT[17,18] and validated using RAMPAGE.[22] Crystallographic statistics of hDHFR were shown in **Table S2**.

Figure S1. Binding modes of BT1 and BT2 with hDHFR. (A) **BT1** and (B) **BT2** binding with hDHFR. (C) Overlay of co-complex **BT1** (orange) and **BT2** (green) structures of hDHFR. The flexible end of **BT1** binds in the pocket by expulsion of three water molecules of **BT2**/hDHFR structure, while only one water molecule of **BT1**/hDHFR structure is displaced by the rigid end of **BT2**. Water molecules are shown as spheres. Hydrogen bonding interactions are highlighted as dashed lines in black and π-π interaction is drawn as a double dashed line.

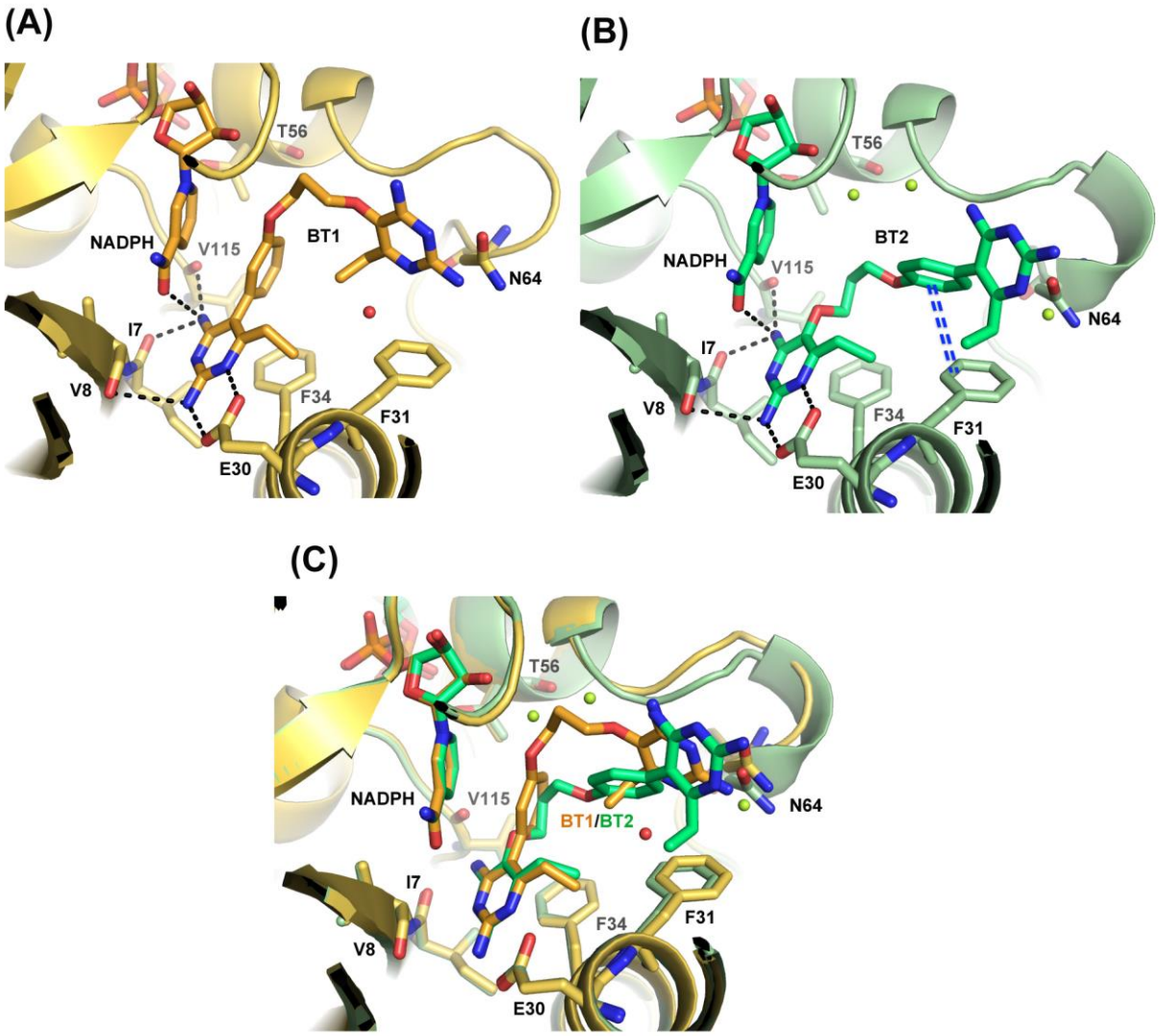


Table S1. Data collection and refinement statistics of PfDHFR-TS wild-type (TM4) and quadruple mutant (V1/S) in complex with hybrid inhibitors BT1, BT2 and BT3

Parameters	TM4-**BT1**	TM4-**BT2**	TM4-**BT3**	V1/S-**BT1**	V1/S-**BT2**	V1/S-**BT3**
Data Collection						
X-ray source	BL13B1,NSRRC	BL13B1,NSRRC	BL13B1,NSRRC	BL13B1,NSRRC	BL13B1,NSRRC	BL13B1,NSRRC
Wavelength (Å)	1	1	1	1	1	
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-Cell Parameters						
a, b, c (Å)	*a*=56.29 *b*=154.25 *c*=163.62	*a*=58.48 *b*=157.28 *c*=165.74	*a*=57.56 *b*=157.17 *c*=165.61	*a*=58.45 *b*=157.17 *c*=165.53	*a*=59.71 *b*=157.11 *c*=166.43	*a*=59.24 *b*=158.90 *c*=167.60
resolution (Å)[a]	30.0-2.38 (2.47-2.38)	30.0-2.20 (2.28-2.20)	30.0-2.35 (2.43-2.35)	30.0-2.38 (2.47-2.38)	30.0-2.45 (2.54-2.45)	30.0-2.60 (2.69-2.60)
total reflections	305,891	336,942	323,077	228,473	247,095	148,231
unique reflections	57,573	77,583	61,346	58,781	54,414	46,152
completeness (%)	98.4 (98.4)	98.8 (90.6)	96.8 (84.7)	94.5 (97.1)	93.4 (90.3)	93.8 (93.1)
redundancy	5.3 (5.0)	4.4 (3.4)	5.3 (4.2)	4.0 (3.8)	4.6 (4.3)	3.3 (3.1)
$<I/\sigma(I)>$	24.5 (5.0)	38.7 (4.0)	31.5 (2.9)	28.9 (4.4)	24.7 (2.7)	14.7 (3.1)
R_{merge} (%)[b]	6.6 (33.7)	3.4 (28.8)	4.4 (39.0)	4.5 (25.3)	5.0 (40.5)	7.8 (39.6)
Refinement						
R_f/R_{free} (%)[c]	19.2/24.8	19.1/24.3	19.7/24.3	19.6/25.5	20.3/29.1	21.1/26.6
No. of Atoms/Average B-factors (Å^2)	9,429/53.6	9,807/40.5	9,888/35.7	9,241/51.6	9,958/36.4	9,492/65.1
Protein	8,880/53.5	8,917/40.3	9,044/35.5	8,762/51.4	9,102/36.4	9,132/65.3
Ligand **BT1**/**BT2**/**BT3**	62/76.7	62/70.9	74/58.6	62/66.5	62/57.3	74/65.9
Rmsd from Ideal						
bond length (Å)	0.013	0.011	0.011	0.012	0.012	0.016
bond angle (deg)	1.696	1.508	1.542	1.580	1.604	1.770
Ramachadran Plot						
favored (%)	87.8	90.1	88.6	87.8	87.9	87.4
allowed (%)	12.0	9.9	11.2	12.2	12.1	12.7
outlier (%)	0.2	0	0.2	0	0	0
PDB ID code	6A2K	6A2M	6A2O	6A2L	6A2N	6A2P

[a]Values in parentheses are for the highest resolution shells. [b]$R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual reflection and $\langle I(hkl)\rangle$ is the mean intensity of symmetry-equivalent reflections. [c]$R_f = \Sigma_{hkl}||F_{obs}| - |F_{calc}||/\Sigma_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively. R_{free} was calculated in the same manner as R_f but using only a 10% unrefined subset of the reflection data.

Table S2. Data collection and refinement statistics of hDHFR in complex with BT1 and BT2

	hDHFR–**BT1**	hDHFR–**BT2**
Data collection		
X-ray source	MicroSTAR	FR591
Wavelength (Å)	1.5418	1.5418
Space group	H_3	H_3
Unit-Cell Parameters		
a, *b*, *c* (Å)	*a*=b=82.942	*a*=b=84.755
	c=77.489	*c*=77.726
Resolution[a] (Å)	30-2.06 (2.13-2.06)	50-1.85 (1.92-1.85)
Total reflections	44,185	44,797
Unique reflections	12,165	17,677
Completeness (%)	99.6 (96.6)	99.3 (99.7)
Redundancy	3.62 (3.27)	2.5 (2.5)
$\langle I/\sigma(I)\rangle$	7.8 (2.4)	17.3 (3.9)
R_{merge}[b] (%)	4.82 (19.46)	4.7 (25.8)
Refinement		
R_f/R_{free} (%)[c]	17.17 (24.27)	18.97 (23.75)
No. of Atoms/Average	1752/28.35	1742/22.81
B-factors (Å^2)		
Protein	1502/27.52	1502/22.50
BT1	31/30.93	-
BT2	-	31/20.02
NAP	48/27.61	48/16.49
SO_4	15/60.08	-
Water	156/33.01	161/28.10
R.m.s. deviation		
Bond lengths (Å)	0.0099	0.0097
Bond angles (°)	1.490	1.533
Ramachadran Plot		
favored (%)	98.4	98.4
allowed (%)	1.6	1.6
outlier (%)	0.0	0.0
PDB ID code	6A7C	6A7E

[a]Values in parentheses are for the highest resolution shell. [b]$R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual reflection and $\langle I(hkl)\rangle$ is the mean intensity of symmetry-equivalent reflections. [c]$R_f = \Sigma_{hkl}||F_{obs}| - |F_{calc}||/\Sigma_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively. R_{free} was calculated in the same manner as R_f but using only a 10% unrefined subset of the reflection data.

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