SUPPORTING INFORMATION

A divergent SAR study drives a potent 5-HT_{2c} inhibitor to a promising antimalarial scaffold.

Félix Calderón,*,[†] Jaume Vidal-Mas,*,[†] Jeremy Burrows,[‡] Juan Carlos de la Rosa,[†] María Belén Jiménez-Díaz,[†] Teresa Mulet,[†] Sara Prats,[†] Jorge Solana,[†,¶] Michael Witty,[‡] Francisco Javier Gamo,[†] Esther Fernández[†]

[†]Tres Cantos Medicines Development Campus, DDW, GlaxoSmithKline, Severo Ochoa, 2. 28760 Tres Cantos, Madrid, Spain

[‡]Medicines for Malaria Venture (MMV), 20, route de Pré-Bois-PO Box 1826, 1215 Geneva 15, Switzerland

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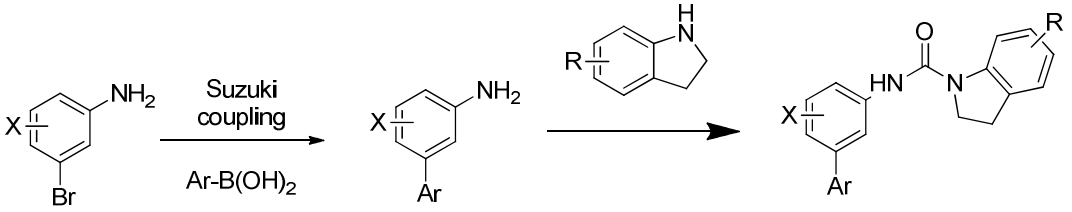
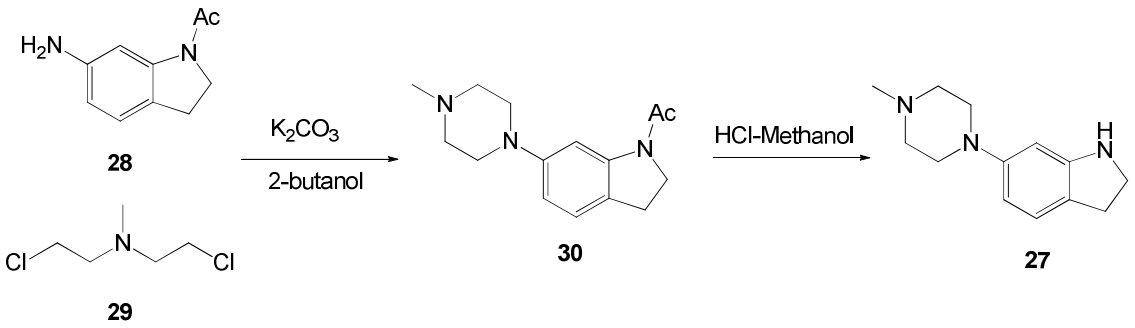
1.General synthetical route



2. General procedure for indole reduction

A solution of the corresponding indole (1.6 mmol), sodium cyanoborohydride (1.59 mmol) in ACOH (7.9 mL) was stirred at r.t. overnight. Afterwards, the mixture was diluted with AcOEt (5 mL) and washed with NaOH (1N) (3 x 5 mL) and brine (2 x 5 mL). The organic phase was dried with MgSO4 anh,, filtered and evaporated. The residue was purified by flash chromatography.

3.Synthesis of indoline 26



To a solution of **28** (300 mg, 1.70 mmol) in 2-butanol (17 mL), **29** (983 mg, 5.11 mmol) and K_2CO_3 (941 mg, 6.81 mmol) were added. The mixture was refluxed overnight. Next morning no SM was detected. The reaction was quenched with water (20 mL). The phases were separated and the aqueous phase was extracted with AcOEt (2 x 20 mL). and dried with MgSO4 anh.. The mixture was filtrated and the solvent evaporated. The crude was purified by flash chromatography affording 295 mg of the desired piperazine.

HNMR (DMSO-*d6*) □ 7.55 (2H, s), 7.0 (1H, d), 6.55 (1H, d), 4.04 (2H, t), 3.4-3.3 (2H, m), 3.0-2.9 (6H, m), 2.4-2.3 (4H, m), 2.19 (3H, s), 2.12 (3H, s). MS: m/e 260 (MH+).

30 (200 mg, 0.771 mmol) was dissolved in etanol-HCl (2M) (25 mL) and the mixture was stirred at reflux for 3 h. After this time no SM was detected by TLC and the solvent was evaporated. The residue was dissolved in MEOH (2 mL) and this solution was applied to an SCX column (5 G, CVs: 25 mL) (preconditioned with 2 CVs of methanol; 50 mL). The column was then eluted, under gravity, using (i) methanol (2x20mL), (ii) 2M ammonia in methanol (20 mL). The ammonia elution was evaporated affording 40 mg of **26**.

HNMR (DMSO-*d6*) □ 6.83 (1H, d), 6.1 (2H, m), 3.3 (2H, m), 2.98 (4H, t), 2.76 (2H, t), 2.41 (4H, t), 2.20 (3H, s). MS: m/e 218 (MH+).

4. General procedure for urea formation

To a solution of the corresponding aniline (1 mmol) in THF anh. (38 mL) at 0 ºC under Nitrogen atmosphere, triphosgene (0.3 mmol) was added. The solution was stirred at this temperature for 45 min. After this time indoline/isoindoline derivative (1.7 mmol) was dissolved in 9 mL of THF anh. and added to the mixture. Next, the mixture was stirred at 0 ºC for 15 min. After this

time anhydrous triethylamine (2 mmol) was added dropwise and the mixture was allowed to react 30 min at 0º C and 2-12 h at room temperature. Once the reaction is finished the mixture was diluted with AcOEt (3 x 40 mL) and washed with brine (3 x 40 mL) and water (3 x 10 mL). The organic phase was dried with MgSO4 and the solvent evaporated. The crude was purified by flash chromatography or preparative HPLC.

5.General procedure for Suzuki copling

Corresponding aniline (1.5 mmol) was disolved in DME (13.3 mL) in a 20 mL-microwave vessel. Then, the corresponding boronic acid (3.0 mmol), Na2CO3 (2.34 mL, 2M solution) and bis(triphenylphosphine)palladium (II) chloride (0.15 mmol) were added. The vessel was sealed and heated in a Biotage Initiator at 120 ºC, Pmax=16 bar, 60 min. After 1 H reacting the reaction was diluted with AcOEt (10 mL) and washed with brine (2 x 5 mL) and NaOH 2N (2 x 5 mL). The organic phase was dried with MgSO4 anh and the solvent evaporated.The reside was purified by flash chromatography/preparative HPLC .

6.Spectroscopical/purity data

1 HNMR (DMSO-*d6*) 8.91 (1H, s), 8.88 (1H, s), 8.63 (1H, d), 8.11 (1H, s), 8.0 (1H, d), 7.94 (1H, s), 7.7 (1H, m), 7.5 (1H, m), 7.36 (1H, m), 7.22 (1H, s), 4.20 (2H, t), 3.84 (3H, s), 3.2 (2H, m). MS: m/e 498 (MH+). Purity was determined as > 95 % by HPLC (269 nm), Rt: 3.27 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

4 HNMR (DMSO-*d6*) 8.85 (1H, s), 8.66 (1H, s), 8.58 (1H, d), 8.12 (1H, s), 8.0 (1H, d), 7.90 (1H, s), 7.63 (1H, d), 7.5 (1H, m), 7.4-7.3 (2H, m), 7.20 (1H, s), 4.19 (2H, t), 3.83 (3H, s), 3.2 (2H, m). MS: m/e 414 (MH+). Purity was determined as > 95 % by HPLC (267 nm), Rt: 3.31 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

5 HNMR (DMSO-*d6*) 8.97 (1H, s), 8.91 (1H, s), 8.63 (1H, s), 8.20 (1H, s), 8.1 (3H, m), 7.68 (1H, s), 7.5 (1H, m), 7.22 (1H, s), 4.21 (2H, t), 3.83 (3H, s), 3.2 (2H, m). MS: m/e 482 (MH+). Purity was determined as > 95 % by HPLC (269 nm), Rt: 3.21 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

6 HNMR (DMSO-*d6*) 8.86 (1H, s), 8.80 (1H, s), 8.61 (1H, d), 8.11 (1H, s), 8.0 (1H, m), 7.9 (1H, m), 7.9 (1H, m), 7.56 (1H, m), 7.5-7.4 (1H, m), 7.25 (1H, s), 4.18 (2H, t), 3.84 (3H, s), 3.2 (2H, m). MS: m/e 492 (MH+). Purity was determined as > 95 % by HPLC (260 nm), Rt: 3.16 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

7 HNMR (DMSO-*d6*) 8.80 (1H, s), 8.10 (1H, s), 7.69 (1H, s), 7.56 (1H, d), 7.40 (1H, t), 7.20 (1H, s), 6.99 (1H, d), 4.20 (2H, t), 3.83 (3H, s), 3.2 (2H, m). MS: m/e 421 (MH+). Purity was determined as > 95 % by HPLC (267 nm), Rt: 3.31 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

8 HNMR (DMSO-*d6*) 8.70 (1H, s), 8.09 (1H, s), 7.48 (1H, s), 7.43 (1H, s), 7.20 (1H, s), 6.80 (1H, s), 4.18 (2H, t), 3.83 (3H, s), 3.2 (2H, m), 2.31 (3H, s). MS: m/e 435 (MH+). Purity was determined as > 95 % by HPLC (271 nm), Rt: 3.54 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

9 HNMR (DMSO-*d6*) 8.92 (1H, s), 8.09 (1H, s), 7.91 (1H, s), 7.72 (1H, s), 7.25 (1H, s), 7.21 (1H, s), 4.18 (2H, t), 3.84 (3H, s), 3.2 (2H, m). MS: m/e 499 (MH+). Purity was determined as > 95 % by HPLC (270 nm), Rt: 3.73 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

10 HNMR (DMSO-*d6*) 8.88 (1H, s), 8.11 (1H, s), 7.92 (1H, s), 7.73 (1H, s), 7.66 (2H, d), 7.50 (2H, t), 7.4 (1H, m), 7.25 (1H, s), 7.22 (1H, s), 4.20 (2H, t), 3.84 (3H, s), 3.2 (3H, m). MS: m/e 497 (MH+). Purity was determined as > 95 % by HPLC (267 nm), Rt: 3.81 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

11 HNMR (DMSO-d6) 9.23 (1H, s), 9.12 (1H, s), 8.96 (1H, s), 8.11 (1H, s), 7.97 (1H, s), 7.83 (1H, s), 7.48 (1H, s), 7.22 (1H, s), 4.20 (2H, t), 3.84 (3H, s), 3.28 (3H, t). MS: m/e 499 (MH+). Purity was determined as > 95 % by HPLC (266 nm), Rt: 3.23 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

12 HNMR (DMSO-d6) 8.90 (1H, s), 8.74 (1H, s), 8.11 (1H, s), 7.95 (1H, d), 7.91 (1H, s), 7.77 (1H, s), 7.39 (1H, d), 7.32 (1H, s), 7.22 (1H, s), 4.20 (2H, t), 3.84 (3H, s), 3.2 (3H, t), 2.52 (3H, s). MS: m/e 512 (MH+). Purity was determined as > 95 % by HPLC (269 nm), Rt: 3.48 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

13 HNMR (DMSO-d6) 11.4 (1H, s), 8.76 (1H, s), 8.11 (1H, s), 7.80 (1H, s), 7.45 (1H, s), 7.25 (1H, s), 7.21 (1H, s), 6.88 (1H, s), 6.51 (1H, s), 6.14 (1H, s), 4.19 (2H, t), 3.84 (3H, s), 3.2 (3H, t), 2.52 (3H, s). MS: m/e 486 (MH+). Purity was determined as > 95 % by HPLC (273 nm), Rt: 3.48 (Waters Sunfire 3.5 u C18 3 x 30 mm, CH3COO NH4 50mM / Acetonitrile, pH 7.0).

14 HNMR (DMSO-*d6*) 8.89 (2H, s), 8.62 (1H, dd), 8.09 (1H, dd), 7.95 (1H, d), 7.88 (1H, d), 7.81 (1H, s), 7.4 (1H, dd), 7.36 (1H, s), 7.21 (1H, d), 7.13 (1H, t), 6.92 (1H, t), 4.15 (2H, t), 3.19 (2H, t). MS: m/e 400 (MH+). Purity was determined as > 95 % by HPLC (264 nm), Rt: 1.50 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).

15 HNMR (DMSO-*d6*) 8.95 (1H, s), 8.87 (1H, d), 8.61 (1H, dd), 8.07 (1H, d), 7.95 (1H, s), 7.79 (1H, s), 7.64 (1H, dd), 7.52 (1H, dd), 7.38 (1H, s), 7.20 (1H, t), 6.7 (1H, m), 4.21 (2H, t), 3.16 (2H, t). MS: m/e 418 (MH+). Purity was determined as > 95 % by HPLC (261 nm), Rt: 1.52 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).

16 HNMR (DMSO-*d6*) 9.01 (1H, s), 8.96 (1H, d), 8.69 (1H, dd), 8.23 (1H, d), 8.01 (1H, s), 7.91 (1H, s), 7.80 (1H, s), 7.66 (1H, dd), 7.42 (1H, s), 7.23 (1H, d), 6.98 (1H, dd), 4.21 (2H, t), 3.18 (2H, t). MS: m/e 434 (MH+). Purity was determined as > 95 % by HPLC (256 nm), Rt: 1.53 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, Formic acid 0.1 % 25mM + 5% Acetonitrile, pH 2.4).

17 HNMR (DMSO-*d6*) 8.98 (1H, s), 8.95 (1H, d), 8.69 (1H, d), 8.23 (1H, d), 8.06 (1H, s), 7.99 (1H, s), 7.80 (1H, s), 7.65 (1H, dd), 7.42 (1H, s), 7.16 (1H, d), 7.10 (1H, dd), 4.19 (2H, t), 3.16 (2H, t). MS: m/e 478 (MH+). Purity was determined as > 95 % by HPLC (260 nm), Rt: 1.79 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 50mM + 5% Acetonitrile, pH 7.4).

18 HNMR (DMSO-*d6*) 8.88 (1H, s), 8.86 (1H, d), 8.62 (1H, d), 8.08 (1H, dd), 7.95 (1H, s), 7.81 (1H, s), 7.75 (1H, s), 7.53 (1H, dd), 7.35 (1H, s), 7.09 (1H, dd), 6.75 (1H, d), 4.14 (2H, t), 3.13 (2H, t), 2.26 (3H, s). MS: m/e 414 (MH+). Purity was determined as > 95 % by HPLC (265 nm), Rt: 1.55 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25 mM + 5% Acetonitrile, pH 6.6).

19 HNMR (DMSO-*d6*) 8.93 (1H, s), 8.67 (1H, d), 8.19 (1H, d), 7.98 (1H, s), 7.89 (1H, s), 7.79 (1H, s), 7.63 (1H, dd), 7.40 (1H, s), 7.18 (1H, dd), 4.17 (2H, t), 3.16 (2H, t). MS: m/e 448 (MH+). Purity was determined as > 95 % by HPLC (266 nm), Rt: 1.69 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 50 mM + 5% Acetonitrile, pH 6.6).

20 HNMR (DMSO-*d6*) 8.89 (1H, s), 8.87 (1H, d), 8.61 (1H, dd), 8.08 (1H, dd), 7.9 (1H, m), 7.85 (1H, dd), 7.80 (1H, s), 7.52 (1H, dd), 7.36 (1H, s), 7.09 (1H, dd), 6.95 (1H, dt), 4.18 (2H, t), 3.20 (2H, t). MS: m/e 418 (MH+). Purity was determined as > 95 % by HPLC (259 nm), Rt: 1.55 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).

21 HNMR (DMSO-*d6*) 8.88 (1H, s), 8.83 (1H, s), 8.62 (1H, dd), 8.07 (1H, d), 7.94 (1H, s), 7.80 (1H, s), 7.75 (1H, d), 7.53 (1H, dd), 7.34 (1H, s), 7.02 (1H, s), 6.93 (1H, d), 4.13 (2H, t), 3.15 (2H, t), 2.23 (3H, s). MS: m/e 414 (MH+). Purity was determined as > 95 % by HPLC (262 nm), Rt: 1.55 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).

22 HNMR (DMSO-*d6*) 9.05 (1H, s), 8.9 (1H, m), 8.67 (1H, d), 8.20 (1H, d), 8.0 (2H, m), 7.82 (1H, s), 7.6 (1H, m), 7.56 (1H, s), 7.5 (1H, m), 7.42 (1H, s), 4.24 (2H, t), 3.27 (2H, t). MS: m/e 468 (MH+). Purity was determined as > 95 % by HPLC (270 nm), Rt: 1.61 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).

23 *HNMR (DMSO-d6) δ 9.58 (1H, s), 8.86 (1H, d), 8.61 (1H, dd), 8.0 (1H, m), 7.8 (1H, m), 7.73 (1H, s), 7.51 (1H, dd), 7.34 (1H, s), 7.1-7.0 (1H, m), 7.0 (2H, m), 4.15 (2H, t), 3.16 (2H, t). MS: m/e 418 (MH+). Purity was determined as > 95 % by HPLC (260 nm), Rt: 1.41 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).*

24 HNMR (DMSO-*d6*) 8.8 (2H, m), 8.82 (1H, dd), 8.07 (1H, m), 7.9 (1H, m), 7.79 (1H, s), 7.5 (2H, m), 7.36 (1H, s), 7.09 (1H, d), 6.50 (1H, dd), 4.16 (2H, t), 3.71 (3H, s), 3.10 (2H, t). MS: m/e 430 (MH+). Purity was determined as > 95 % by HPLC (261 nm), Rt: 1.46 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).

25 *HNMR (DMSO-d6) δ 8.8 (2H, m), 8.82 (1H, dd), 8.07 (1H, m), 7.9 (1H, m), 7.89 (1H, s), 7.82 (1H, s), 7.5 (1H, m), 7.35 (1H, s), 7.14 (1H, d), 6.86 (1H, d), 5.13 (1H, t), 4.43 (2H, d), 4.16 (2H, t), 3.16 (2H, t). MS: m/e 430 (MH+). Purity was determined as > 95 % by HPLC (265 nm), Rt:*

1.26 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).

26 *HNMR (DMSO-d6) 8.88 (1H, d), 8.83 (1H, s), 8.61 (1H, dd), 8.0 (1H, m), 7.94 (1H, t), 7.79 (1H, s), 7.59 (1H, d), 7.52 (1H, dd), 7.36 (1H, s), 7.02 (1H, d), 6.51 (1H, dd), 4.14 (2H, t), 3.0 (6, m), 2.4 (4H, m), 2.20 (3H, s). MS: m/e 498 (MH+). Purity was determined as > 95 % by HPLC (251 nm), Rt: 1.26 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).*

27 *HNMR (DMSO-d6) 8.90 (1H, d), 8.88 (1H, d), 8.62 (1H, dd), 8.0 (1H, m), 7.9 (1H, m), 7.78 (1H, s), 7.76 (1H, d), 7.52 (1H, dd), 7.37 (1H, s), 7.23 (1H, s), 4.17 (2H, t), 3.2 (4H, m), 3.13 (2H, t), 2.9 (4H, m), 2.23 (3H, s). MS: m/e 532 (MH+). Purity was determined as > 95 % by HPLC (271 nm), Rt: 1.39 (ACQuity UPLC BEH C18 1.7u 2.1x50mm, CH3COO NH4 25mM + 5% Acetonitrile, pH 6.6).*

7. Parasites and cell lines

P. falciparum strains (3D7A, HB3, and Dd2) were obtained from MR4 Resource Center (ATCC). All strains were maintained in continuous culture using standard methods (Trager, W.; Jensen, J. B.; Human Malaria Parasites in Continuous Culture. *Science* **1976**, *193*, 673-675). Cell lines HepG2, from human caucasian hepatocellular carcinoma liver; H9c2(2-1), from myocardium heart rat, MDCK, from Canine Cocker Spaniel kidney; L1210, from Mouse DBA/2 lymphocytic leukaemia and Neuro 2a from Mouse Albino neuroblastoma were supplied by ECACC. Cells were maintained as described in Herreros E., M. J. Almela, S. Lozano, F. Gómez de la Heras and D. Gargallo-Viola.. Antifungal activities and cytotoxicity studies of six new azasordarins. *Antimicrob. Agents Chemother*. **2001** *45*, 3132-3139.

8.Antimalarial activity determination

Antimalarial activity of compounds against *P. falciparum* infected erythrocytes was determined using a modification of the in vitro ^{3}H-hypoxanthine incorporation method (see Desjardins, R.E., Canfield, C. J., Haynes, J.D. and Chulay J.D. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique *Antimicrob. Agents Chemother* **1979**, *16*, 710-718), described on Rueda et al (Ref. 9). Chloroquine, Artemisinin and Pyrimethamine were used as controls.

9.Cell citotoxicity assays

To determine cytotoxic effects, cells were exposed to serial dilutions of test compounds for 48 h at 37ºC. The culture medium was as described above, but supplemented with 5% fetal calf serum. Following the 48h exposure period, a 0.004% resazurin solution was prepared by adding 60 mL Dulbecco's PBS to each tablet of resazurin (VWR International, ref. 330884Y). The tablet was allowed to dissolve by placing the container in a bath set at 37ºC, protected from light, for approximately 30 minutes. Medium was removed and 200 µL of fresh culture medium and 50 µL of resazurin solution were added to each well. Plates were incubated for a further 1.5 hours. The fluorescence was stabilized at room temperature for 15 minutes protected from light. Fluorescence was measured using a fluorescence plate reader (Victor V, Perkin Elmer) at an excitation wavelength of 515 nm and an emission wavelength of 590 nm. The percentages of inhibition were calculated relatively to the control wells.

10.Phenotypic assay

In order to determine which was the effect of compounds within the parasite, cultures at 2% hematocrite value and 1% of initial parasitemia (rings as a predomintant form of parasite) were exposed to the bioequivalent concentration of 10 times IC50 value. Samples were taken at 24, 48 and 72 hours post exposure and smears were performed. Slides were stained during 10 minutes using Giemsa Stain at 5% in PBS and pictures from representative forms of parasite were taken. Artemisinin, Chloroquine and Atovaquone were used as control compounds and DMSO was used as non-drug control.

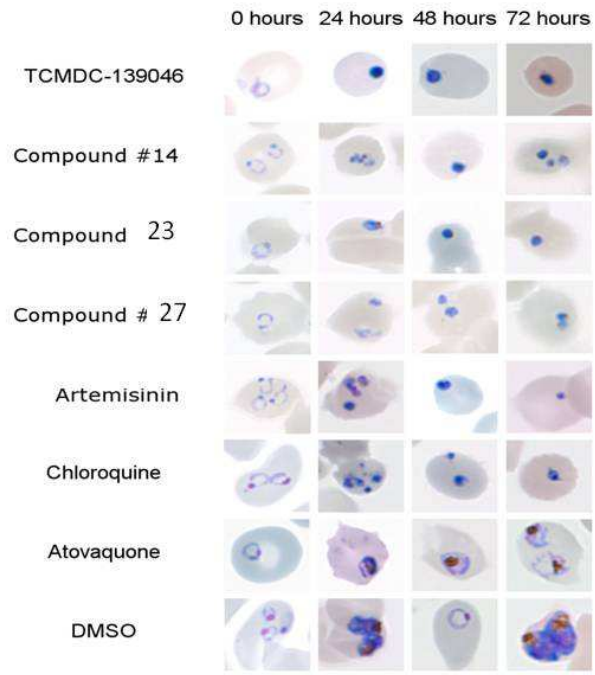


Figure 1. Phenotypic effect of indolines in *P. falciparum* cultures. Picknotics forms appear in all the derivatives. Atovaquone, Artemisinin and Chloroquine were used as control drugs and DMSO as a positive control.

11.Onset of action assay

In order to determine the reduction of number of viable parasites caused by indoline derivatives were measured using a clonal dilution technique.The methodology is based on described by Young et al[Young, R.D. et al. Antimicrobial Agents and chemotheraphy, 1993; 37:1102]. Cultures were exposed at bioequivalent concentration of 10 times IC50 value during 72 hours. After the exposure time, samples of treated and untreated cultures were taken and diluted with uninfected erythrocytes to reach 0.1 parasite/well and then further incubated up to 28 days. Growth of the cultures were folloewd by 3H-hypoxanthine incorporation on day 13 and 28. For each treated culture, the highest dilution in which parasite development was detected is marked as positive and compared to the control cultures to assess the decrease of parasitemia (in logarithms).

12.5HT2c determination

5HT2c inhibition were carried out using aequorin intracellular calcium assay. The aequorin intracellular calcium assay uses frozen CHO-K1 cells stably expressing the 5HT2C receptor. Serotonin is used as the standard agonist in antagonist format assays as described in
Differences between human wild-type and C23S variant 5-HT2C receptors in inverse agonist-induced resensitization Jutta Walstab, Folkert Steinhagen, Michael Brüss, Manfred Göthert, Heinz Bönisch *Pharmacologycal reports* **2011**, *63*,45

13 hERG Dofetilide Binding Assay
The FP assay uses membranes from CHO cells stably expressing hERG and measures the binding of a fluorescent ligand (Cy3b dofetilide). This is a high-throughput 384 well plate-based

assay. Test compounds that bind to hERG compete with the fluorescent compound and to decrease the FP signal.

14.In vitro DMPK methods

Cytochrome p450, Permeability and CLND Solubility Assays
Experimental detail for these assays is described in: Ward, S.E.; Harries, M.; Aldegheri, L.; Austin, N.E.; Ballantine, S.; Ballini, E.; Dradley, D.M.; Bax, B.D.; Clarke, B.P.; Harris, A.J.; Harrison, S.A.; Melarange, R.A.; Mookerherjee, C.; Mosely, J.; Negro, G.D.; Oliosi, B.; Smith, K.J.; Thewlis, K.M.; Woollard, P.M.; Yusaf, S.P. Integration of lead optimisation with crystallography for a memebrane-bound ino channel target: discovery of a new class of AMPA receptor positive allosteric modulators *J. Med. Chem*. **2011**, *54*, 78-94.

Microsome Assay
930 uL of microsomes 0.645mg/mL(0.5mg/mL), 6uL of compound at 100uM (0.5uM), 150uL of NADPH (2mM) and 108uL MgCl (55mg/mL) were incubated at 37oC. Remove 100 uL aliquots at 0, 5, 10, 15, 20, 25, and 30 minutes and place into a 96-deepwell plate containing 200 uL (2 volumes) of Stop Solution with Internal Standard. Precipitate protein by centrifugation (37000 rpm, 15minutes) and analyse by HPLC.

Human Serum Albumin Binding by HPLC
The assay uses an Agilent 1100 HPLC, a Chromtech HSA column 50x3.0mm 5 micron, 50mM ammonium acetate (pH7.4) as mobile phase A and 2-propanol as mobile phase B. 10uL of 10mM DMSO stock solution of sample is diluted with 990uL of 50:50 mobile phases A and B. The flow rate is 1.8mL/min at 30oC with gradient of 0-3min 0-30%B, 3-5min 30%B, 5-5.1min 30-0% B, 5.1-6min 0% B. Injection volume 10uL monitoring at 215 and 254nm. Retention time is then related to % HSA binding by relation to a set of 9 control standards with known binding affinities.

Plasma Protein Binding Assays
Experimental detail for these assays is described in: Ward, S.E.; Harries, M.; Aldegheri, L.; Andreotti, D.; Ballantine, S.; Bax, B.D.; Harris, A.J.; Harker, A.J.; Lund, J.; Melarange, R.; Mingardi, A.; Mookherjee, C.; Mosley, J.; Neve, M.; Olioso, B.; Profeta, R.; Smith, K.J.; Smith, P.W.; Spada, S.; Thewlis, K.; Yusaf, S.P. Discovery of N- [(2S)-5-(6-fluoro-3-pyridinyl)-2,3-dihydro-1H-inden-2-yl]-2-propanesulfonamide, a novel clinical APMA receptor positive modulator J. Med. Chem. **2010**, *53*, 5801-5812.

15.Physicochemical methods

Solvents and buffers
Organic solvents of HPLC grade were used. Ultra pure water (Milli-Q grade) was used. Buffers were prepared with ultra pure water and filtered using 0.45 □ nylon filters. The compositions of each solvent employed in this assay are described below (part III).

I. Procedure.

Determination of equilibrium solubility (assuming chemical stability in the desired solvent is not a problem).

a) 1 mg of solid compound was weighted in one 4 mL glass vial and 2 mL of the corresponding medium freshly prepared (SGF, FaSSIF, FeSSIF or PBS) were added. All these samples were prepared by duplicate.
b) The samples were stirred (rolling mixing)for 24 hr at room temperature. If required, additional solid compound (0.1 mg) was added to maintain excess of it (saturated solutions).
c) After 24 hours, the samples were filtered (Millipore Milex filters nylon 0.45 □m) and the filtrates were analysed by LC-MS.

d) The pH of the final solution in each sample was measured with a pH-meter (WTW pH330i and a pH-electrode Sentix 41).

LC-MS assay for analytical quantification

All filtered aliquots were analysed by LC-MS. Quantification of those samples was carried out against calibration curves obtained from 1 mg/mL DMSO (Aldrich *cat. ref.*: 27685-5) stock solutions, by dilution with the mobile phase used in the chromatography.
Depending on the solubility range, U.V. (1 □g/mL to 100 □g/mL) or MS (1 □g/mL to 1 ng/mL) detector were used in the quantification.

Analysis of data

The analysis of all LC-MS data was performed with MassLynx 3.4 software and Analyst 1.4.2. Statistical and graphic analysis of data was performed using Microsoft Excel. The concentration (□M) and solubility (□g/ml) for each compound was calculated using the peak areas from the sample and those from the calibration curve.

II. Compositions of solvents used in these assays.

A) FaSSIF is a solvent which simulates the Fasted State of the Intestinal Fluid.
(FaSSIF: Fasted State Simulated Intestinal Fluid). Its composition is as given in the table below.

Composition of FaSSIF and the pH 6.8 buffer used in FaSSIF

Composition of FaSSIF		
	Conc.	Quantity per 100 ml
NaTaurochol.	5mM	269 mg
Lecithin	1.5 mM	114 mg
pH 6.8 Buffer	Qs	Qs 100 ml
Composition of pH 6.8 Buffer		
	Conc.	Quantity per L
KH_2PO_4	0.029 M	3.947g
KCl	0.22 M	16.401 g
NaOH	Qs pH 6.8	
Water	N/A	Qs 1 L

FaSSIF Preparation procedure.

1. <u>Preparation of 1 L of pH 6.8 buffer solution</u>

1.a. 3.947 g potassium phosphate and 16.401 g potassium chloride were dissolved in approx. 900 ml of water.
1.b. The pH was adjusted to 6.8 by slow addition of 0.1N sodium hydroxide (Scharlau SO 0441010C) under magnetic stirring.
1.c The mixture was diluted to a volume of 1000ml with water.

2. <u>Preparation of 100 ml of FaSSIF</u>

2.a. 269 mg NaTaurochol. (Aldrich T-4009) was dissolved in approx. 80 ml of pH 6.8 buffer.
2.b. 114 mg lecithin (Sigma P-7318) was dissolved in this NaTaurochol/buffer solution (this was carried out with a nitrogen filled glove bag).
2.c. The resulting mixture was diluted to a volume of 100 ml with further pH 6.8 buffer
2.d. The final solution was covered with a layer of nitrogen or alternative inert gas. The bottle was sealed with parafilm and stored at 4ºC.

B) FeSSIF is a solvent which simulates the Fed State of the Intestinal Fluid.
(FeSSIF: Fed State Simulated Intestinal Fluid). Its composition is as given in the table below.

Composition of FeSSIF and the pH 5.0 buffer used in FeSSIF

Composition of FeSSIF		
	Conc.	Quantity per 100 ml
NaTaurochol.	15mM	806.5 mg
Lecithin	3.8 mM	288 mg
pH 5.0 Buffer	Qs	Qs 100 ml
Composition of pH 5.0 Buffer		
	Conc.	Quantity per L
Glacial Acetic acid	0.137 M	8.250ml
KCl	0.20 M	15.2 g
NaOH	Qs pH 5.0	
Water	N/A	Qs 1 L

Reference(s): Galia, Nicolaides, Horter, Lobenberg, Reppas, and Dressman - Pharmaceutical Research, Vol. 15, No. 5, 1998

FeSSIF Preparation procedure.

1. Prepare 1 L of pH 5 buffer solution

1.a. 15.2 g potassium chloride and 8.25 ml glacial acetic acid were dissolved in approx. 900 ml of water.
1.b. The pH was adjusted to 5 by slow addition of NaOH 0.1N (Scharlau SO 0441010C) under magnetic stirring.
1.c The mixture was diluted to a volume of 1000 ml with water.

2. Preparation of 100 mL of FeSSIF

2.a. 806.5 mg NaTaurochol (Aldrich T-4009) was dissolved in 80 ml of pH 5 buffer.
2.b. 288 mg lecithin (Sigma P-7318) was dissolved in this NaTaurochol/buffer solution (carried out with a nitrogen filled glove bag).
2.c. The resulting solution was diluted to a volume of 100 ml with pH 5 buffer.
2.d. The final solution was covered with a layer of nitrogen or alternative inert gas. The bottle was sealed with parafilm and stored at 4ºC.

C) Solubility at **pH 7.4** was determined in phosphate buffered saline (PBS) (Fluka *cat. ref.:* 79383)

Preparation of Simulated Gastric Fluid

Dissolve 2.0 g of Sodium Chloride and 7 mL of Concentrated Hydrochloric Acid into approximately 500 mL of purified water. Check the pH and adjust if necessary to pH 1.2 with a few drops of Concentrated HCL. Add 5g of Sodium Lauryl Sulfate and stir to complete dissolution. Make up to 1 Litre volume and record pH. Some frothing is apparent when preparing these solutions.

16. *In vivo* assay.

The *in vivo* efficacy of compound 27 was tested in a murine model of *Plasmodium berghei* ANKA. Briefly, CD1 mice were infected with 10^7 *P. berghei*-infected erythrocytes by intravenous route. Two days after infection each mouse received one dose of compound every 24 hours for two consecutive days by oral route. A vehicle-treated group was used as control of infection. Compound 26 was formulated in 20% captisol and administered at 50 mg/K in a volume of 20 ml/Kg of bodyweight.

The effect of treatment on the parasite was assessed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson) and data were analyzed with CellQuest-Pro software. Samples from peripheral blood (2 microliters) of each mouse were taken at day 4 after infection. The fluorescent dye YOYO-1 was used to measure the percentage of parasitemia as described in Cytometry Part A; 67A:27-36. 2005. The effect of treatments was assessed by measuring reduction of parasitemia with respect to vehicle control group 24 hours after last dose.