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Synthesis and evaluation of the antiplasmodial activity of tryptanthrin derivatives

Liliane Abodo Onambele [a], Herbert Riepl [b], Rainer Fischer [a], Gabriele Pradel [a], Aram Prokop [c,1,*], Makoah Nigel Aminake [a,d,1,**]

[a] Institute of Molecular Biotechnology, RWTH Aachen University, 52074 Aachen, Germany
[b] HochschuleWeihenstephan-Triesdorf, Organic-analytical Chemistry, 94315 Straubing, Germany
[c] Department of Paediatric Oncology, Children's Hospital Cologne, 50735 Köln, Germany
[d] Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa

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ABSTRACT

Malaria remains one of the most deadly diseases threatening humankind and is still affecting a significant proportion of the world population, especially in Africa. Chemotherapy is a vital component of the fight against the disease and new antimalarial agents are urgently needed to curb the spread of malaria parasites that are resistant to existing drugs. The natural product tryptanthrin is known for its wide range of activities, including antiplasmodial activity, but its poor solubility has undermined its development as potent antimicrobial and antiprotozoan agent. The aim of this work was to synthesize analogues of tryptanthrin and to evaluate their antiplasmodial activity against the asexual and sexual blood stages of *Plasmodium falciparum*. Our results suggest that most tryptanthrin analogues retained their antiplasmodial activity against chloroquine-sensitive and chloroquine-resistant malaria parasites in the nanomolar range (30–100 nM). The antiplasmodial activity of the most active compound NT1 (IC_{50}: 30 nM; SI: 155.9) was similar in both strains and close to that of chloroquine (IC_{50}: 20 nM) on the sensitive strain. The antiplasmodial activity was improved with derivatization, thus pointing out the necessity to explore tryptanthrin using medicinal chemistry approaches. Ten (10) of the tested derivatives met the criteria, allowing for advancement to animal testing, i.e., SI > 100 and IC_{50} < 100 nM. In addition to their activity on the asexual stages, tryptanthrin and two selected derivatives (NT1 and T8) prevented the maturation of gametocytes at their IC_{90} concentrations, indicating a transmission-blocking potential. Moreover, NT1 was able to impair gametogenesis by reducing the exflagellation of microgametes by 20% at IC_{90}, while tryptanthrin and T8 had no influence on exflagellation. The results of this study confirm that tryptanthrin and its derivatives are potential antimalarial candidates with abilities to kill the intraerythrocytic asexual stages and prevent the formation of sexual stages of the parasite.

1. Introduction

Malaria, a disease caused by protozoan parasites of the genus *Plasmodium*, remains one of the most devastating diseases in the world. Despite encouraging progress in the control of malaria, there was an estimated 627,000 malaria-related deaths worldwide in 2012. Most of the estimated cases occurred in sub-Saharan Africa (90%), with children under 5 years of age representing the most affected group (77%). Out of the five human pathogenic *Plasmodium* species, *Plasmodium falciparum* is the most virulent strain, responsible for almost all malaria-related death cases in Sub-Saharan Africa (World Health Organization, 2013). In the absence of an effective vaccine, the use of chemotherapy represents the most important pillar of malaria eradication besides vector control and prophylaxis. The World Health Organization now advises to apply artemisinin combination therapies (ACTs) as a new first line treatment, which shows very good clinical efficacy, especially towards *P. falciparum* malaria (World Health Organization, 2011). Despite the reduction of the parasite's sensitivity to artemisinins that has been reported in South East Asia, ACTs continue to cure patients as long as the partner drug is still effective. Nevertheless, resistance has been reported against both components of multiple ACTs in a province of Cambodia (World Health Organization, 2013). To maintain strict control of the disease, it is therefore crucial for humans to remain a step ahead of the

* Corresponding author. Department of Paediatric Oncology, Children's Hospital Cologne, 50735 Köln, Germany. Tel.: 0027-214066389; fax: 0027-216505195.
E-mail address: prokopa@kliniken-koeln.de (A. Prokop).
** Corresponding author. Institute of Molecular Biotechnology, RWTH Aachen University, 52074 Aachen, Germany. Tel.: 0027-214066389; fax: 0027-216505195.
E-mail address: nigel.makoah@uct.ac.za (M.N. Aminake).
[1] These authors contributed equally.

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malaria parasite by continually seeking for novel antimalarial compounds to overcome drug-resistant malaria parasites. In this regard, *in vitro* screens for compound activity constitute a key component of antimalarial drug discovery programs. To properly treat malaria, the new hit must be highly potent against the asexual blood stages of both drug-sensitive and drug-resistant parasites and be able to prevent parasite transmission to the mosquito.

Tryptanthrin (indolo-[2, 1-*b*]-quinazoline-6, 12-Dione) is an alkaloid originally isolated from *Isatis tinctoria*, a medicinal plant in temperate climate zones which has been used since antiquity as a source for indigo dye production (Honda et al., 1980; Seifert and Unger, 1994; Lee et al., 2007). Tryptanthrin was also identified as the active principle of a traditional Japanese herbal remedy used to treat fungal infections (Honda and Tabata, 1979). Following these discoveries, a number of biological activities have been reported for tryptanthrin and its derivatives, including antibacterial activity (Kataoka et al., 2001), particularly against *Mycobacterium tuberculosis* (Mitscher and Baker, 1998; Lee et al., 2007; Hwang et al., 2013), and activities against the protozoan parasites *Plasmodium falciparum* (Bhattacharjee et al., 2004), *Leishmania donovani* (Bhattacharjee et al., 2002), *Trypanosoma brucei* (Scovill et al., 2002), and *Toxoplasma gondii* (Krivogorsky et al., 2008). In addition, the inhibitory activities of tryptanthrin against cyclooxygenase (COX)-2 (IC_{50} = 64 nM), 5-lipoxygenase (LOX) (IC_{50} = 0.15 μM), nitric oxide synthase (NOS), and prostaglandin E(2) expression at the cellular level opened a vista for a possible lead for anti-inflammatory agents (Jahng, 2013). Activity against tumor and cancer cells has also been reported (Yu et al., 2007, 2010; Yang et al., 2013) and tryptanthrin derivatives were recently reported to inhibit indoleamine 2,3-dioxygenase with therapeutic activity in Lewis Lung cancer (LLC) tumor-bearing mice (Yang et al., 2013). However, tryptanthrin has not been extensively taken into consideration for pharmaceutical development because of its poor solubility. The aim of this study was to generate and synthesize tryptanthrin analogues with improved solubility and to evaluate their antiplasmodial activity against the asexual and sexual blood stages of *P. falciparum in vitro*.

2. Experimental section

2.1. Procedures for the synthesis of tryptanthrin derivatives

2.1.1. General synthesis of 8-substituted tryptanthrins from 5-R-isatins (R = Me, F, Cl, Br, NO_2)

Method A: 8-R-isatin (0.011 mol) was suspended in 10–15 ml dry pyridine. After addition of 5 drops of triethylamine and 2.2 g isatoic acid anhydride (MW = 163.13, 0.013 mol), the solution was heated to approximately 60 °C for 15 min. Then, 1.5 ml diisopropylcarbodiimide was added, and the solution was heated to reflux for an additional 15–20 min. During refluxing, the dark solution gave a crop of yellow crystals. These were isolated by suction and washed with cold methanol.

8-Bromotryptanthrin, 8-Bromo-indolo [2,1-b] quinazoline-6,12-dione (T2)

Synthesis according to method A: From 2.9 g of 5-bromoisatin (MW = 226, 0.013 mol).

Yield: 1.1 g, 51%, yellow crystals recrystallized from chloroform.

Mp.: 276 °C.

MS (Cl): 328.8 (15%), 327.8 (96.8), 326.8 (14.5), 325.8 (100), 299.8 (24.35), 297.8 (23.75), 247.8 (10.7), 218. 8 (18.9), 190.9 (44.1), 163.8 (15.6).

IR (KBr, cm^{-1}): 3067.9 (w), 1731 (m), 1674 (vs), 1592 (m), 1458 (m), 1429 (w), 1338 (m), 1300 (m), 1262 (w), 1181 (m), 1127 (w), 1041 (w), 872 (w), 772 (m), 687 (w).

^{1}H-NMR ($CDCl_3$, ppm): 8.28 (d, J = 8.23 Hz, 1H), 8. 18 (dd, J = 8.23 Hz, 1.5 Hz, 1H), 7.7 ("m", 2H), 7.6 ("q", 2H), 7.4 ("t", 1H).

^{13}C-NMR (d_6-DMSO, ppm): 181.56, 157.98, 146.45, 145.15, 140.04, 135.7, 130.33, 130.3, 127.45, 127.43, 124.6, 123.47, 119.47, 119.28.

$C_{15}H_7O_2N_2Br$: calc.: C: 55.0, H: 2.1, O: 10, N: 8.8, Br: 24; found: C: 54.73, H: 2.2, N: 8.52, Br: 23.44.

8-Chlorotryptanthrin, 8-Chloro-indolo [2,1-b] quinazoline-6,12-dione (T7)

Synthesis according to method A: From 2.2 g of 5-chloroisatin (MW = 181, 0.012 mol).

Yield: 1.3 g, 38%, yellow crystals recrystallized from acetone.

Mp.: 287 °C.

MS (Cl): 282 (100), 283 (35.85), 284 (34.12), 285 (11.84), 254 (11.24), 226 (4.95), 191 (24.52), 164 (2.81).

IR (KBr, cm^{-1}): 3069 (w), 1730 (s), 1674 (vs), 1594 (s), 1558 (m), 1459 (s), 1450 (w), 1341 (s), 1300 (s), 1263 (w), 1218 (w), 1182 (m), 1125 (m), 1042 (w), 915 (w), 877 (w), 843 (w), 772 (m), 742 (w), 787 (w). ^{1}H-NMR ($CDCl_3$, ppm): 8.55 (d, J = 8.98 Hz, 1H), 8.41 (dd, J = 8.23 Hz/1.5 Hz, 1H), 8.01 (d, J = 8.23 Hz, 1H), 7.84 (dt, J = 6.73 Hz/1.5 Hz, 2H), 7.72 (dd, J = 8.98 Hz/2.25 Hz, 1H), 7.67 (t, J = 6.5 Hz, 1H).

$C_{15}H_7O_2N_2Cl$: calc.: C: 63.8, H: 2.5, O: 11.3, N: 9.9, Cl: 12.4; found: C: 63.8, H: 2.5, N: 9.9, Cl: 12.5.

8-Fluorotryptanthrin, 8-Fluoro-indolo [2,1-b] quinazoline-6,12-dione (T6)

Synthesis according to method A: From 2.14 g of 5-fluoroisatin (MW = 165, 0.013 mol).

Yield: 1.9 g, 54%, yellow crystals recrystallized from 80 ml THF.

Mp.: 271.5 °C.

MS (CI, e/m): 267 (37.18), 266 (100), 238 (14.11), 210 (7.53), 148 (3.75), 130 (10), 120 (4. 82), 108 (21.93).

IR (KBr, cm^{-1}): 1722 (s), 1687 (s), 1592 (w), 1558 (w), 1484 (s), 1456 (w), 1351 (m), 1305 (w), 1269 (w), 1232 (w), 1185 (w), 1135 (w), 1116 (w), 1040 (w), 890 (w), 836 (m), 770 (s), 703 (w), 684 (w).

^{1}H-NMR ($CDC1_3$, ppm): 8.6 (dd, J = 8.98Hz/3.74 Hz, 1H), 8.4 (dd, J = 8.22Hz/1.5 Hz, 1H), 7.99 (d, J = 8.2 Hz, 1H), 7.82 (dt, J = 8.23 Hz/1.5 Hz, 1H), 7.65 (t, 7.48 Hz, 1H), 7.54 (dd, 6.73 Hz/2.99 Hz, 1H), 7.44 (dt, 8.97 Hz/2.99 Hz, 1H).

^{13}C-NMR ($CDCl_3$, ppm): 181.7, 162.35, 159.86, 157.87, 146.5, 144.28, 142.5, 135.22, 130.67 (d, 2JCF = 34.9 Hz), 127.54, 124.81 (d, $^2J_{CF}$ = 24.3 Hz), 124.15, 123.68, 123.33 (d, $^2J_{CF}$ = 7.7 Hz), 119.67 (d, $^2J_{CF}$ = 6.8 Hz), 112.05 (d, $^2J_{CF}$ = 25.26 Hz).

$C_{15}H_7O_2N_2F$: calc.: C: 67.66, H: 2.63, O: 12.03, N: 10.52, F: 7.14; found: C: 67.86, H: 2.77, N: 10.71, F: 6.44

8-Methyltryptanthrin, 8-Methyl-indolo [2,1-b] quinazoline-6,12-dione (T1)

Synthesis according to method A: From 2.1 g of 5-methylisatin (MW = 161.3, 0.013 mol).

Yield: 2.4 g, 70%, yellow crystals recrystallized from acetone.

Mp.: 270.5 °C.

MS (CI, m/e): 261.9 (100%), 233.8 (24.14), 204.8 (15.5), 130.8 (3.33), 129.8 (3.22), 101.8 (6.15)

IR (KBr, cm^{-1}): 3061 (w), 3029 (w), 2933 (w), 1724 (s), 1684 (vs), 1613 (w), 1593 (m), 1558 (w), 1482 (m), 1458 (m), 1340 (m), 1309 (m), 1297 (w), 1261 (w), 1227 (m), 1186 (w), 1154 (w), 1138 (m), 1041 (m), 947 (w), 886 (w), 830 (m), 796 (w), 774 (s), 705 (w), 688 (m), 659 (w).

^{1}H-NMR (d_6-DMSO, ppm): 8.33 (d, J = 8.23 Hz, 1H), 8.3 (d, J = 7.5 Hz, 1H), 7.93 (s, 1H), 7.92 (s, 1H), 7.7 ("m", 3H), 2.4 (s, 3H).

^{13}C-NMR (d_6-DMSO, ppm): 205.67, 182.6, 157.61, 146.55, 145.31, 144.05, 138.2, 136.75, 135.13, 129.87, 126.95, 124.85, 123.43, 122.4, 116.87, 20.55.

$C_{16}H_{10}O_2N_2$: calc.: C: 73.1, H: 4.0, O: 12.0, N: 11.1; found: C: 73.15, H: 3.78, N: 10.64.

8-Nitrotryptanthrin, 8-Nitro-indolo [2,1-b] quinazoline-6,12-dione (T4)

Synthesis according to method A: From 2.2 g of 5-nitroisatin (MW = 192, 0.011 mol).

The solution was kept overnight in a refrigerator and the solid was isolated by suction.

Yield: 0.7 g, 23%, crystals recrystallized from 100 ml THF.

Mp.: 298 °C.

MS (CI, e/m): 293.8 (21.55%), 292.7(100), 264.8 (21.6), 262.9 (24.31), 218.8 (32.82), 190.8 (16.22), 164.8 (7.34).

IR (KBr, cm^{-1}): 3089 (w), 3029 (w), 1734 (s), 1682 (vs), 1607 (s), 1590 (s), 1522 (s), 4660 (m), 1444 (m), 1341 (vs), 1324 (s), 1309 (m), 1300 (s), 1278 (vs), 1218 (w), 1194 (w), 1156 (w), 1125 (m), 1090), 1062 (w), 1039 (w), 951 (w), 923 (w), 889 (w), 853 (m), 839 (w), 803 (w), 784 (m), 747 (w), 726 (w), 690 (w), 660 (w), 626 (w).

^{1}H-NMR ($CDCl_3$, ppm): 8.85 (d, J = 8.98 Hz, 1H), 8.75 (d, J = 2.24 Hz, 1H), 8.69 (dd, J = 8.98 Hz, 2.24 Hz, 1H), 8.47 (dd, J = 7.48 Hz, 1.49 Hz, 1H), 8.06 (d, J = 7.48 Hz, 1H), 7.9 (t, J = 1.49 Hz, 1H), 7.73 (t, J = 7.6 Hz, 1H).

$C_{15}H_7O_4N_3$: calc.: C: 61.1, H: 2.0, O: 22.0, N: 14.1; found: C: 61.52, H: 2.58, N: 14.13.

2.1.2. General synthesis of 8-substituted tryptanthrins from 2-chloro-3H-indol-3-one

Method B: 5-nitro-2-chloro-3H-indol-3-one (according to Grimshaw and Begley, 1974).

1.9 g of 5-nitroisatin (MW = 192, 0.01 mol) and 2.4 g (MW = 206, 0.012 mol) phosphorus pentachloride were refluxed in 10 ml anhydrous benzene for several hours. The yellow solid slowly dissolved to become a red liquid. After 3–4 h, the liquid was filtrated over a fine glass frit under exclusion of moisture and left to crystallize. After 1 day at room temperature, deep red crystals of the precipitate appeared. Those were filtrated via suction and dried in high vacuum for 3–5 h. Yield: 1.5 g, 75%.

Synthesis of 5-bromo 2-chloro-3H-indol-3-one

5-bromo 2-chloro-3H-indol-3-one was synthesized analogously.

General procedure of the synthesis of tryptanthrins by ring-closing reaction of 5-nitro-2-chloro-3H-indol-3-one with several anthranilic acids:

The 5-nitro-2-chloro-3H-indol-3-one or 5-bromo-2-chloro-3H-indol-3-one resulting from the above procedure was suspended in dry dimethylformamide and combined with a solution of 4-chloro-2-aminobenzoic acid and 5-chloro-2-aminobenzoic acid or 2-aminoterephtalic acid or others in DMF. The darkening mixture was stirred at room temperature. Then, the mixture was heated to 100–120 °C for 10–15 min and a ring-closing reaction occurred, identified by decolorization and crystallization. The reaction was monitored until completeness, which was controlled via thin layer chromatography. After cooling, the crystals were separated by suction. Depending on the different solubility of the compounds, the addition of a second solvent such as acetic acid may be required.

2-Chloro-8-nitro-tryptanthrin, 2-Chloro-8-nitro-indolo [2,1-b] quinazoline-6,12-dione (NT2)

Synthesis according to method B: 1.7 g of 2-amino-5-chlorobenzoic acid (MW = 172, 0.01 mol) in 2.5 ml DMF and 1.92 g (0.01 mol) 5-nitro-2-chloro-3H-indol-3-one in 2.5 ml DMF.

Yield: 0.96 g, recrystallization from 20 to 25 ml DMF. From the mother liquor, a further 0.5 g of product can be obtained after cooling. Total yield: 39%.

Mp./decomp.: 282 °C.

MS (CI, e/m): 329.0 (33.07), 328.1 (19.17), 327.1 (100), 299.2 (2.03), 283.2 (2.03), 282.1 (3.13).

IR (KBr, cm^{-1}): 3336 (br), 3093 (w), 1730 (s), 1700 (s), 1615 (m), 1577 (w), 1523 (m), 1459 (w), 1414 (w), 1382 (w), 1344 (m), 1325 (w), 1284 (m), 1245 (m), 1128 (m), 1093 (w), 1064 (w), 1042 (w), 952 (w), 944 (w), 905 (w), 863 (w), 844 (w), 777 (w), 742 (w), 640 (w), 554 (w), 526 (w).

^{1}H-NMR ($CDCl_3$, ppm): 8.43 (d, J = 8.9 Hz, 1H), 8.75 (d, J = 2.25 Hz, 1H), 8.68 (dd, J = 2.24/8.9 Hz, 1H), 8.41 (d, J = 2.24 Hz, 1H), 7.99 (d, J = 8.23, 1H), 7.83 (dd, J = 2.24/8.9 Hz, 1H).

3-Chloro-8-nitro-tryptanthrin, 3-Chloro-8-nitro-indolo [2,1-b] quinazoline-6,12-dione (NT1)

Synthesis according to method B: 1.7 g of 2-amino-4-chlorobenzoic acid (MW = 172, 0.01 mol) in 2.5 ml DMF and 1.92 g (0.01 mol) 5-nitro-2-chloro-3H-indol-3-one in 2.5 ml DMF.

0.83 g, 25%, recrystallized from THF, brownish powder. When the DMF mother liquor was allowed to stand at –18 °C, an additional 800 mg was isolated as crystals. Total yield: 50%.

Mp./decomp.: 228 °C.

IR (KBr, cm^{-1}): 1729 (s), 1691 (vs), 1641 (w), 1604 (s), 1586 (s), 1527 (s), 1462 (s), 1415 (m), 1342 (s), 1313 (s), 1285 (s), 1215 (w), 1183 (m), 1128 (m), 1115 (m), 1094 (m), 1067 (s), 1044 (m), 951 (m), 905 (m), 842 (m), 780 (m), 743 (w), 705 (w), 682 (w), 641 (m), 626 (w), 554 (w).

MS (CI, e/m): 329 (1.08), 328 (1.28), 327 (1.9), 326 (2.67), 252 (0.82), 185 (2.89), 146.1 (6.73), 145.1 (100), 144.1 (16.84), 129.1 (7.89).

IR (KBr, cm^{-1}): 1729 (s), 1691 (vs), 1641 (w), 1604 (s), 1586 (s), 1527 (s), 1462 (s), 1415 (m), 1342 (s), 1313 (s), 1285 (s), 1215 (w), 1183 (m), 1128 (m), 1115 (m), 1094 (m), 1067 (s), 1044 (m), 951 (m), 905 (m), 842 (m), 780 (m), 743 (w), 705 (w), 682 (w), 641 (m), 626 (w), 554 (w).

^{1}H-NMR ($CDCl_3$, ppm): 8.82 (d, J = 8.85 Hz, 1H), 8.74 (d, J = 2.25 Hz, 1H), 8.67 (ddt, J = 8.8 Hz/2.4 Hz, 1H), 8.37 (d, J = 8.4 Hz, 1H), 8.02 (d, J = 2.0 Hz, 1H), 7.66 (dd, J = 8.4 Hz/2.0 Hz, 1H).

$C_{15}H_6ClN_3O_4$: calc.: C: 54.7; H: 1.82; N: 12.76; Cl: 10.6; found: C: 55.0; H: 1.9; N: 13.0; Cl: 10.8.

2,3-Dimethoxy-8-nitro-tryptanthrin, 2,3-Dimethoxy-8-nitro-indolo [2,1-b] quinazoline-6,12-dione (NT3)

Synthesis according to method B: 3.7 g of 5-nitro-2-chloro-3H-indol-3-one (MW = 210, 0.0176 mol) in 15 ml DMF and 3.47 g 4,5-dimethoxy-2-aminobenzoic acid (M = 197, 0.0176 mol) in 5 ml DMF.

Yield: 3.5 g, 56%, fine needles. Purification was performed by decoction of the crude product with acetone or THF, then the filtrate was crystallized by allowing to stand exposed to air.

Mp./decomp.: 328 °C.

MS (CI, e/m): 354 (30.73), 353 (100), 338 (4.82), 337 (5.49), 191 (3.07)

IR (KBr, cm^{-1}): 3459 (br), 3090 (w), 1739 (s), 1686 (vs), 1636 (m), 1601 (m), 1585 (s), 1534 (m), 1503 (vs), 1453 (m), 1437 (w), 1421 (w), 1376 (m), 1344 (vs), 1301 (w), 1293 (s), 1250 (w), 1214 (m), 1186 (w), 1153 (w), 1117 (w), 1078 (w), 1042 (w), 990 (w), 927 (w), 872 (w), 851 (w), 779 (w), 771 (w), 745 (w), 667 (w).

^{1}H-NMR ($CDCl_3$, ppm): 8.81 (d, J = 8.65 Hz, 1H), 8.7 (d, J = 2.1 Hz, 1H), 8.64 (dd, J = 8.65 Hz/2.1 Hz, 1H), 7.7 (s, 1H), 7.42 (s, 1H), 4.06 (s, 3H), 4.02 (s, 3H).

$C_{17}H_{11}O_6N_3$: calc.: C: 57.7; H: 3.11, N: 11.9, found: C: 47.9; H: 3.0; N: 12.1.

2-Chloro-8-bromo-tryptanthrin, 8-Bromo-2-chloro-indolo [2,1-b] quinazoline-6,12-dione (BT2)

Synthesis according to method B: 0.5 g of 5-bromo-2-chloro-3H-indol-3-one (M = 276, 0.00181 mol) in 7 ml DMF and 310 mg 4-chloro-anthranilic acid (MW = 171.6, 0.00181 mol). Purification was performed by washing with acetone and recrystallizing from 200 ml THF.

Yield: 390 mg, 59%, yellow crystals.

Mp.: 304 °C.

MS (CI, e/m): 364 (20.9), 363 (24.65), 362 (100), 361 (18.82), 360 (69.31), 334 (10.07), 332 (7.75), 225 (4.24).

IR (KBr, cm^{-1}): 3349 (br), 3069 (w), 1731 (vs), 1674 (vs), 1648 (m), 1587 (m), 1456 (s), 1431 (w), 1332 (m), 1298 (m), 1262 (m), 1211 (m), 1181 (m), 1131 (w), 1084 (w), 1036 (w), 943 (w), 905 (w), 845 (s), 782 (w), 748 (m), 707 (w), 645 (w), 616 (w), 560 (w).

$C_{15}H_6O_2N_2BrCl$: calc.: C: 49. 85, H: 1.6, N: 7.8, Br: 22.0, Cl: 9.7, found: C: 49.6; H: 1.8; N: 7.4; Br 21.8; Cl: 9.7.

3-Chloro-8-bromo-tryptanthrin, 8-Bromo-3-chloro-indolo [2,1-b] quinazoline-6,12-dione (BT1)

Synthesis according to method B: 0.5 g of 5-bromo-2-chloro-3H-indol-3-one (M = 276, 0.00181 mol) in 7 ml DMF and 0.310 g 3-chloro-anthranilic acid (MW = 171.6, 0.00181 mol). Recrystallization from 100 ml dioxane.

Yield: 390 mg, 59%, yellow crystals.

Mp.: 303 °C.

MS (CI, e/m): 363.6 (20.92), 363.6 (21.15), 362.6 (100), 360.6 (16.74), 359.6 (69.05), 333.6 (16.56), 331.7 (12.74), 252.9 (5.15), 224.7 (13.49).

IR (KBr, cm^{-1}): 3000 (w), 3100 (w), 1734 (s), 1675 (vs), 1584 (s), 1455 (m), 1431 (w), 1419 (w), 1338 (m), 1300 (m), 1274 (w), 1212 (w), 1181 (s), 1133 (w), 1117 (w), 1072 (w), 1038 (w), 943 (w), 900 (w), 888 (w), 842 (w), 778 (w), 741 (w), 702 (w), 688 (w), 634 (w).

$C_{15}H_6O_2N_2BrCl$: calc.: C: 49.85, H: 1.6, N: 7.8, Br: 22.0, Cl: 9.7, found C: 49.9; H: 1.9; N: 8.4; Br: 21.16; Cl: 9. 8.

2,3-dimethoxy-8-bromo-tryptantrin, 8-Bromo-2,3-dimethoxy-indolo[2,1-b]quinazoline-6,12-dione (BT3)

Synthesis according to method B: 0.5 g 5-bromo-2-chloro-3H-indol-3-one (MW = 276, 0.00181 mol) in 4 ml benzene and 0.36 g of 4,5-dimethoxyanthranilic acid in 2 ml DMF.

Yield: 310 mg, 44%, orange needles obtained by recrystallization from DMF (relatively low solubility, 150 mg in 20 ml).

Mp.: 332 °C

MS (CI, e/m): 389 (21.8), 388 (100), 387 (24.9), 386 (86. 78), 373 (17.03), 345 (6.96), 308 (5.17).

IR (KBr, cm^{-1}): 3000 (w), 3100 (w), 1720 (s), 1681 (s), 1627 (w), 1586 (m), 1547 (w), 1502 (s), 1473 (w), 1456 (s), 1437 (s), 1422 (s), 1375 (s), 1338 (m), 1309 (s), 1279 (m), 1249 (m), 1216 (s), 1183 (s), 1119 (m), 1075 (w), 1051 (w), 1041 (w), 1022 (w), 994 (s), 869 (m), 838 (m), 786 (m), 770 (m), 661 (w), 600 (w), 538 (w), 509 (m), 418 (m).

^{1}H-NMR ($CDCl_3$, ppm): 8.49 (d, J = 8.5 Hz, 1H), 7.97 (d, J = 2.0 Hz, 1H), 7.8 (dd, J = 8.5 Hz/2.0 Hz, 1H), 7.73 (s, 1H), 7.4 (s, 1H), 4.04 (s, 3H), 4.0 (s, 3H).

8-Nitro-tryptanthrin-3-carboxylic acid, 8-Nitro-6,12-dioxo-6,12-dihydro-indolo[2,1-b]quinazoline-3-carboxylic acid (NT4)

Synthesis according to method B: 5-nitro-2-chloro-3H-indol-3-one was synthesized from 3.84 g of 5-nitroisatin (MW = 192, 0.02 mol) in 20 ml benzene and 4.25 g phosphorous pentachloride (MW = 206, 0.02 mol + 15%) and was then totally evaporated and dried in high vacuum. The total mass of crystalline 5-nitro-2-chloro-3H-indol-3-one was dissolved in 30 ml DMF, and 3.62 g aminoterephthalic acid (MW = 181, 0.02 mol) was added.

Yield: 2.9 g, 43%, a yellow crystalline powder was obtained by recrystallization from DMF. This powder then was washed with acetone by decoction and filtered until small yellow cubes were formed.

Mp.: > 340 °C.

MS (CI, e/m): 338.0 (20.94), 337.1 (100), 309.1 (10.28), 307.1 (17.41), 263.0 (18.53).

IR (KBr, cm^{-1}): 3441 (br), 3111 (w), 2924 (w), 1749 (m), 1739 (m), 1689 (s), 1654 (w), 1636 (m), 1600 (s), 1533 (m), 1465 (w), 1437 (w), 1417 (w), 1383 (w), 1346 (m), 1281 (s), 1259 (m), 1233 (m), 1183 (w), 1129 (w), 1111 (w), 1078 (w), 1057 (w), 1040 (w), 932 (w), 868 (w), 765 (m), 686 (w), 676 (w).

^{1}H-NMR (DMSO, ppm): 8.73 (dd, J = 8.9 Hz/2.25 Hz, 1H), 8.68 (d, J = 8.23 Hz, 1H), 8.57 (d, J = 2.25 Hz, 1H), 8.45 (d, J = 8.22 Hz, 1H), 8.38 (1H), 8.22 (dd, J = 1.5 Hz/8.23 Hz, 1H) $C_{16}H_7O_6N_3$: calc.: C: 56.9, H: 2.07, N: 12.46, found: C: 56.9; H: 2.0; N: 12.0.

8-Bromo-tryptanthrin-3-carboxylic acid, 8-Bromo-6,12-dioxo-6,12-dihydro-indolo[2,1-b] quinazoline-3-carboxylic acid (BT4)

Synthesis according to method B: 1 g 5-bromo-2-chloro-3H-indol-3-one (MW = 243, 0.0041 mol) in 7 ml DMF and 0.745 g 2-aminoterepthalic acid (MW = 181.15, 0.0041 mol).

Yield: 0.64 g, 42%, yellow crystals obtained by recrystallization of 1.3 g in 30 ml DMF, then purified by decoction in acetone.

MS (CI, e/m): 373.1 (34.03), 372 (100), 371.1 (4.65), 370 (90.3), 292.2 (20.79), 170.0 (22.2), 168.0 (53.4).

IR (KBr, cm^{-1}): 3412 (br, m), 1737 (m), 1684 (vs), 1595 (m), 1558 (w), 1458 (m), 1429 (w), 1334 (w), 1300 (s), 1255 (w), 1219 (w), 1186 (m), 1133 (w), 1051 (w), 933 (w), 854 (w), 780 (w), 761 (w), 683 (w), 632 (w).

^{1}H-NMR (DMSO, ppm): 8.41 ("t", J = 1.5 Hz, 2H), 8.34 (s, 1H), 8.18 (d, J = 8.23 Hz, 1H), 8.08 (s, 1H), 8.04 (dd, J = 8.18 Hz/1.5 Hz, 1H).

^{13}C-NMR (DMSO, ppm): 181.6, 166.7, 157.8, 147.0, 146.0, 145.2, 140.5, 137.5, 131.14, 130.4, 128.32, 127.87, 126.7, 124.8, 120.1, 119.66.

2.1.3. Synthesis of esters and amides

8-Nitro-tryptanthrin-3-carboxylic acid methylester, 8-Nitro-6,12-dioxo-6,12-dihydro-indolo[2,1-b] quinazoline-3-carboxylic acid methyl ester (NT5)

0.34 g 8-nitro-tryptanthrin-3-carboxylic acid (MW = 338, 1 mmol) was suspended in 20 ml anhydrous DMF. 0.36 g oxalyl chloride (MW = 126, 2.85 mmol, 2.85 eq.) was dissolved in anhydrous diethylether and added dropwise to the DMF solution. Foaming and gas formation was observed. After 45 min stirring at room temperature, the mixture was heated to 70–80 °C, whereby ether was evaporated. After 1 h at this temperature, a clear solution has formed. Then, 10 ml anhydrous methanol and 3 ml pyridine were added. Under heat generation, precipitation can be observed. The precipitate was filtrated by suction and recrystallized from acetone. Hereby, only about half of the yellow solid dissolves, the rest being hardly soluble. The suspension was then filtrated and the acetone extract was further concentrated *via* rotary evaporator.

Yield: 280 mg, 80%, yellow flake-like crystals.

MS (CI, e/m): 351.8 (23.72), 350.8 (100), 320.9 (50.91), 319.8 (80.63), 291.9 (19.47), 276.9 (10.1)

IR (KBr, cm^{-1}): 3100 (w), 3087 (w), 3077 (w), 1735 (s), 1683 (vs), 1600 (s), 1586 (s), 1464 (s), 1447 (m), 1423 (m), 1347 (s), 1311 (m), 1286 (vs), 1263 (m), 1236 (w), 1219(w), 1184 (w), 1119 (w), 1101 (w), 1082 (w), 1067 (w), 1043 (w), 955 (w), 933 (w), 869 (w), 762 (m), 747 (m), 708 (w), 685 (w).

^{1}H-NMR (d_6-Acetone, ppm): 8.82 ("dq", 2.3 Hz/8.8 Hz, 2H), 8.65 (d, J = 2.3 Hz, 1H), 8.52 ("dt", J = 1.5 Hz, 2H), 8.32 ("dt", J 1.5 Hz, 1H), 3.99 (s, 3H).

8-Bromo-tryptanthrin-3-carboxylic acid methylester, 8-Bromo-6,12-dioxo-6,12-dihydro-indolo[2,1-b] quinazoline-3-carboxylic acid methylester (BT5)

0.37 g 8-bromo-tryptantrhin-3-carboxylic acid (MW = 372, 1 mmol) was suspended in 20 ml anhydrous DMF and 40 ml methylene chloride. Upon cooling in an ice bath, 0.26 g oxalyl chloride (MW = 162, 2 mmol) was added dropwise, upon which foaming and gas generation was observed. The ice bath was removed, and the mixture was allowed to reach room temperature. After 30 min, the methylene chloride was removed *via* rotary evaporation. After stirring overnight, 10 ml methanol and 2 ml pyridine were added, and the solution was stirred for a further 24 h. Then, the mixture was poured in 10 ml of diluted NaOH solution. The resulting precipitate was filtrated over a suction filter and recrystallized from acetone.

Yield: 100 mg, 30%, fine yellow powder.

MS (CI, e/m): 387.7 (3.56), 386.7 (17.9), 385.7 (100), 384.7 (18.9), 383.7 (94.90), 355.8 (12.88), 354.8 (40.53), 353.8 (7.42), 352.8 (42.82), 326.8 (13.05), 325.8 (5.84), 324.8 (12.9), 305.9 (25.2), 274.9 (13.43).

IR (KBr, cm^{-1}): 3105 (w), 3056 (w), 2953 (w), 1734 (s), 1667 (vs), 1593 (s), 1558 (w), 1459(s), 1430 (m), 1338 (m), 1288 (s), 1240 (w), 1217 (w), 1184 (m), 1117 (w), 1097 (w), 1045 (w), 983 (w), 918 (w), 855 (w), 838 (w), 762 (s), 683 (w), 632 (w), 554 (w), 485 (w).

^{1}H-NMR ($CDCl_3$, ppm): 8.65 (d, J = 1.5 Hz, 1H), 8.52 (d, J = 8.3 Hz, 1H), 8.48 (d, J = 8.23 Hz, 1H), 8.27 (dd, J = 1.5 Hz/8.23 Hz, 1H), 8.02 (d, J = 2.5 Hz, 1H), 7.8827 (dd, J = 2.4 Hz/8.23 Hz, 1H), 3.99 (s, 3H).

3-Chloro-indolo [2,1-b] quinazoline-6,12-dione (T8)

Preparation of 7-chloro-1H-3,1-benzoxazine-2,4-dione (7-Chloroisatoic acid anhydride)

Five grams 4-chloro-2-amino-benzoic acid (0.03 mol, MW = 171.58) was dissolved in 150 ml distilled water with 4.15 g K_2CO_3. Within one hour, 4.05 g (3.7 ml) chloroformic acid ethylester was dropped into this solution with intense stirring. Then, the solution containing some solid particles was heated in a water bath set at 50–60 °C for two hours. Then, the solution was acidified with hydrochloric acid, whereby a mass of solid appeared. This solid was isolated by suction. After drying in an exsiccator, this mass of product containing 4-chloro-2-ethyloxycarbonylamino-benzoic acid (5.4 g) was recrystallized from toluene.

^{1}H-NMR ($CDCl_3$, ppm): 10.21 (s, 1H), 8.57 (d, J = 1.05 Hz, 1H), 8.02 (d, J = 8.22 Hz, 1H), 7.017 (dd, J = 2.25 Hz/8.22 Hz, 1H), 4.24 (q, J = 6.73 Hz, 2H), 1.33 (t, J = 7.48 Hz, 3H).

4.0 g of 4-chloro-2-ethyloxycarbonylamino-benzoic acid was treated with 30 ml of thionylchloride and heated to reflux. Initially, all the solids dissolved within 10 min. During refluxing, a mass of crystals, platelets of product, appeared gradually. After 3.5 h, two thirds of thionylchloride was removed by vacuum and the rest was swept over a frit. The filtrate contained less than 10% material. The crystals of 7-chloroisatoic acid anhydride present on the frit were dried *in vacuo*.

IR (KBr, cm^{-1}): 3320 (w), 3181 (m), 2996 (w), 1782 (vs), 1764 (vs), 1708 (w), 1617 (s), 1599 (m), 1489 (w), 1474 (w), 1404 (m), 1346 (s), 1327 (w), 1266 (w), 1244 (m), 1083 (w), 1028 (s), 931 (w), 892 (w), 794 (w), 761 (w), 750 (w), 707 (w), 677 (w).

MS (CI): 198.2 (18.7), 197.2 (15.36), 182.2 (10.04), 180.2 (33.6), 155.2 (31.02), 154.2 (22.14), 153.2 (100), 128.1 (21.9), 126.1 (85.65).

Preparation of 3-chlorotryptanthrin

1.9 g of 4-Chloroisatoic acid (MW = 197, 0.0096 mol) and 1.47 g isatine (MW = 147, 0.007 mol) was suspended in 10 ml dry pyridine. After addition of three drops of triethylamine, the mixture was heated at 50–60 °C for 15 min. Then, 1 ml diisopropylcarbodiimid was added and the mixture was refluxed. All solids gradually dissolved over an hour of heating and crystalline needles appeared. During cooling, the mass became solid. The crystals were isolated by suction, washed with methanol, and dried *in vacuo*. Yield: 1.2 g. Recrystallization was performed with THF.

MS (CI): 285.0 (7.36%), 284.0 (35.04), 283.0 (19.96), 282.0 (100), 254.0 (14.15), 191.1 (21.28)

IR (KBr, cm^{-1}): 3444 (br), 3090 (w), 2996 (w), 1728 (vs), 1696 (vs), 1646 (w), 1587 (s), 1460 (w), 1419 (w), 1350 (m), 1311 (s), 1192 (m), 1107 (w), 1070 (w), 1070 (w), 934 (m), 885 (w), 838 (w), 808 (w), 774 (w), 6751 (s), 682 (w).

^{1}H-NMR ($CDCl_3$, ppm): 8.45(d, J = 8.23 Hz, 1H), 8.3 (d, J = 8.5 Hz, 1H), 8.0 (s, 1H), 7.88 (m, 2H), 7.77 (d, J = 8.22 Hz, 1H), 7.48 (m, 1H)

^{13}C-NMR ($CDCl_3$, ppm): 182.3, 173.7, 157.3, 147.8, 146.2, 145.9, 139.8, 138.0, 130.0, 129.0, 128.9, 127.2, 124.9, 122.3, 117.1

7-Methyl-indolo [2,1-b] quinazoline-6,12-dione (T3)

4-Methyl/6-Methylisatin mixture of isomers: 16 g concentrated H_2SO_4 was diluted by addition into 400 ml distilled water. Then, 30.5 g m-toluidin was added and the mixture was heated until complete dissolution of m-toluidin. Subsequently, 23.5 g chloralhydrate and 31.5 g hydroxylamine were added. This mixture was boiled and a precipitate was obtained, isolated, and dried: 5.2 g, 52%. The dry material was then slowly added into 20 g concentrated H_2SO_4 at 70 °C. During addition, the temperature rose to 85 °C. After cooling, the dark mixture was added to 0.5 kg of ice. The red precipitate was isolated and dried. This material is a mixture of isomers, consisting of 4- and 6-methylisatin in approximately a 1:1 ratio. Yield: 4.5 g, yellow powder. This material was recrystallized several times from 100 ml 8% NaOH and acidified to pH 3by addition of diluted HCl. This material still contained 18% of other isomers. Several of these batches were pooled. After recrystallization from ethanol, a sample of 2.2 g of 4-methylisatin containing at least 10% of 6-methylisatin was obtained.

^{1}H-NMR ($CDCl_3$): 7.3(d, 1H), 7.2("t", 1H), 6.85 (dd, 1H), 6.8 (d, 1H), 6.7 (d, 1H), 6.58 (d, 1H), 1.34 (s, 6H)

Transformation into 7-methyltryptanthrin

2.2 g of 4-methylisatin was condensed with 2.1 isatoic acid anhydride in 10 ml pyridine/5 drops NEt_3, heated to 60 °C for 15 min. Then, 1,5 ml diisopropylcarbodiimide was added, the mixture heated to reflux for 30 min. After cooling, the precipitate was washed with methanol and ether. Yield 1.25 g, 35%.

MS (CI): 261.9 (100%), 262.9 (18.4), 233.9 (25.6), 232.9 (6.77), 205.8 (7.7), 204.8 (15.6)

^{1}H-NMR ($CDCl_3$): 8.2 (m, 1H), 7.55 (m, 2H), 7.4 (m, 2H), 7.35 (d, J = 8.2Hz, 1H), 6,93 (d, J = 8.2HZ, 1H) 2.47 (s, 3H)

3-Bromo-8-nitro-indolo [2,1-b] quinazoline-6,12-dione (NT6)

NT6 was condensed from 8-nitroisatin and the corresponding isatoic acid anhydride, 7-bromo-1H-3,1-benzoxazine-2,4-dione 1 (R′ = 7-Br) according to Method A, itself obtained from 4-bromo-2-amino-benzoic acid *via* 4-bromo-2-(ethoxycarbonylamino) benzoic acid according to T8. Yield (after recrystallization from DMF): 36%.

MS (CI): 373.9 (11.71%), 372.9 (74.21), 371.8 (12.70), 370.8 (73.35), 328.9 (15.84), 327.9 (100), 326.9 (21.29), 325.9 (98.55)

^{1}H-NMR ($CDCl_3$) 8.82 (d, J = 8.98 Hz, 1H), 8.74 (d, J = 2.24 Hz, 1H), 8.67 (dd, J = 8.97 Hz/2.25 Hz, 1H), 8.57 (d, J = 2.25 Hz, 1H), 7.98 (dd, J = 8.23 Hz/2.25 Hz, 1H), 7.90 (d, J = 8.23 Hz, 1H)

2.2. Evaluation of the antiplasmodial activity of tryptanthrin derivatives

Compounds were screened for growth inhibition activity against *P. falciparum* using the Malstat assay as described in the literature (Makler and Hinrichs, 1993; Makler et al., 1993). Synchronized ring stages of *P. falciparum* strains NF54 and Dd2 were plated in triplicate in 96-well plates (200 µl/well) at a parasitemia of 1% in the presence of the compounds dissolved in dimethyl sulfoxide (DMSO). The IC_{50} concentration of each compound was determined as previously described (Aminake et al., 2011). The final concentration of DMSO in the assay was 0.5%.

2.3. Gametocyte production

Asexual cultures of *P. falciparum* NF54 parasites were used to seed gametocyte cultures at 1–2% parasitemia and 5% hematocrit in a total volume of 25 ml. The culture was then incubated at 37 °C under a gas mixture of 5% O_2, 5% CO_2, and 90% N_2. The culture medium (RPMI medium with 25 mM HEPES, 50 mg/l hypoxanthine, 2 g/l sodium bicarbonate, 10% human serum) was replaced daily for at least 14 days, with the medium and work surface heated to 37 °C. Once the parasitemia increases to 5–10% after 3–4 days of culture, the asexual stages became stressed and started to differentiate into stage II gametocytes at day 5, stage III visible at day 9, stage IV visible at day 11, and stage V, with high levels of exflagellation, visible around day 14 or 15.

2.4. Gametocyte toxicity test

P. falciparum NF54 parasites were cultivated at high parasitemia to favor gametocyte formation as described above. Upon the appearance of stage II gametocytes, 1 ml of culture was aliquoted in triplicate in a 24-well plate in the presence of compounds at their respective IC_{50} values. The gametocytes were cultivated for 7 days and the medium was replaced daily. For the first 48 h of cultivation, the gametocytes were treated with the test compounds and afterwards the medium used for feeding was compound free. After 7 days, Giemsa-stained blood smears were prepared and the gametocytemia was evaluated by counting the number of stage IV and V gametocytes in a total number of 1000 erythrocytes.

2.5. Exflagellation assay

From day 13 of culture, exflagellation was tested by withdrawing 100 µl of gametocyte culture and mixing with 10 µl xanthurenic acid (XA) 1 mM to activate the mature gametocytes. The mixture was incubated for 15 min and centrifuged to pellet cells. Most of the supernatant was removed and the cells were resuspended in an equal pellet volume (RPMI medium with 25 mM HEPES, 50 mg/l hypoxanthine, 2 g/l sodium bicarbonate, 100 µM xanthurenic acid, 10% human serum). Then, 10 µl of the solution were placed on a glass slide and covered by a cover slide. Exflagellation was observed at a magnification of 10× and the culture was considered suitable for the assay if at least 4 exflagellation centers per field were counted in the preparation. For the assay, the IC_{50} and IC_{90} concentrations were investigated against a control which was treated with DMSO at a final concentration of 0.5%. Two (2) milliliters of the aliquots of mature gametocyte culture were transferred into two different 25-cm^2 flasks. The compound was added in the first culture flask and only DMSO was added in the second; the final concentration of DMSO in the two cultures was 0.5%. After an incubation time of 15 min at 37 °C, an aliquot of 100 µl per culture flask was collected and the culture flask was returned to the incubator for 24 h incubation. The aliquot was activated with XA and prepared for microscopy as described above. The exflagellation centers were counted in 30 fields. After 24 h incubation, another aliquot of 100 µl per culture flask was collected, activated with XA, and prepared for microscopy as described above. The experiment was repeated once with a different culture seeded on a different day.

2.6. Evaluation of compounds cytotoxicity

The cytotoxicity of the compounds was evaluated using the MTT assay (Mosmann, 1983). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazoliumbromid) is taken up by living cells and converted by mitochondrial dehydrogenases to a violet formazan that cannot diffuse through cell membranes and crystallizes in vital cells. The method was carried out with MCF-7 cells as previously described (Aminake et al., 2011).

2.7. Hemolysis assay

Freshly prepared uninfected erythrocytes were rinsed using culture medium and resuspended in the same medium at a final hematocrit of 5%. They were then plated in triplicates in a 96-well plate. A two-fold serial dilution of the compounds was tested, starting at 10 µM. Medium supplemented with 0.15% saponin was used for the positive control and 0.5% DMSO was used for the negative control. The plates were then placed in a sealed jar, flushed with a gas mixture of 5% O_2, 5% CO_2, and 90% N_2, and incubated at 37 °C for 48 h. After incubation, the plates were centrifuged at 800 *g* for 2 min and an aliquot of 100 µl of the supernatant was transferred to a new 96-well microtiter plate. The absorbance of free hemoglobin in the supernatant was measured at 550 nm.

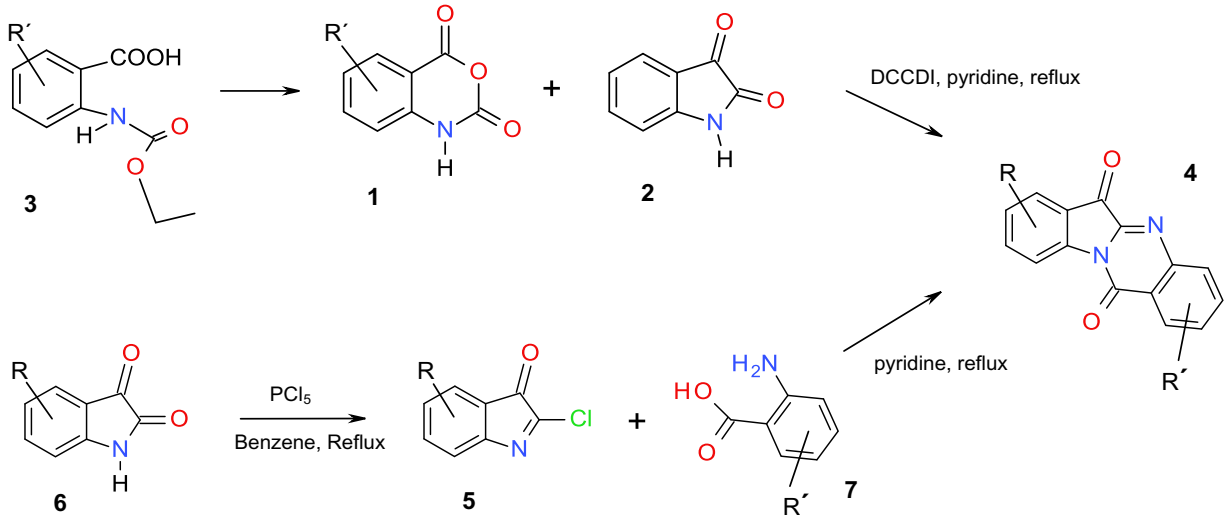
2.8. Statistical analysis

The GraphPad prism software (GraphPad version 5 http://www.graphpad.com/prism/prism.htm) was used for all the analyses. The comparison of the activities of the tested compounds was performed using one way ANOVA followed by a *Tukey's* test to compare the mean gametocytemia of the tested compounds against the mean gametocytemia in the DMSO control as well as to compare the mean relatives exflagellation centers observed after treatment. The OD values obtained after the Malstat and cytotoxicity assays were plotted against logarithmic concentrations of the compounds and a dose–response curve was automatically generated; the IC_{50} values were deduced using GraphPad Prism.

3. Results and discussion

3.1. Synthesis of tryptanthrin derivatives

The synthesis of our panel of substituted tryptanthrins relied on some longer known methods that are complementary because not all derivatives are equally accessible by diverse reactions. Method A (Scheme 1) comprises the reaction of substituted 1H-Benzo[d][1,3]oxazine-2,4-diones **1** (isatoic acid anhydrides) with isatines **2** (Mitscher et al., 1981; Bergman et al., 1985), a route not fully exploited. This was applied to synthesize compounds T1–T7. Isatoic acid anhydrides are generally not available commercially, except for one or two compounds. To obtain 2-nitrotryptanthrin, isatine and 6-Nitro-1H-benzo[d][1,3]oxazine-2,4-dione (=6-nitroisatoic acid anhydride) were reacted in pyridine. For the synthesis of 6-nitroisatoic acid anhydride, we applied a ring-closing procedure of 2-ethoxycarbonylamino-5-nitro-benzoic acid **3** (R′ = 5-NO_2) with the advantage of directly obtaining a



Scheme 1. Synthesis of tryptanthrins.

Table 1
Antiplasmodial activities of tryptanthrin and its derivatives.

Code	Substituents				LogP	Mean IC_{50} ± SD (μM)		Mean CC_{50} ± SD (μM)	Selectivity index (SI)	
	R2	R3	R7	R8		NF54	Dd2	MCF7	SI (NF54)	SI (Dd2)
NT1	H	Cl	H	NO_2	3.2	0.033 ± 0.004	0.031 ± 0.005	5.15 ± 0.11	155.9	168.7
NT2	Cl	H	H	NO_2	3.2	0.129 ± 0.022	0.091 ± 0,013	12.47 ± 0.23	96.7	136.5
NT3	OCH3	OCH3	H	NO_2	2.2	0.283 ± 0.093	0.134 ± 0.047	15.17 ± 4.41	53.6	113.2
NT4	H	COOH	H	NO_2	2.4	NE	NE	>100	ND	ND
NT5	H	COOCH3	H	NO_2	2.7	0.167 ± 0.067	0.166 ± 0.036	11.98 ± 0.50	71.7	72.1
NT6	H	Br	H	NO_2	3.3	0.072 ± 0.023	0.101 ± 0.068	17.52 ± 0.77	243.3	174
T1	H	H	H	CH3	3	0.085 ± 0.037	0.216 ± 0.116	>100	>1000	>400
T2	H	H	H	Br	3.4	0.081 ± 0.008	0.095 ± 0.058	10.47 ± 1.52	129.3	110.2
T3	H	H	CH3	H	3	0.450 ± 0.098	0.602 ± 0.115	17.65 ± 0.30	39.2	29.3
T4	H	H	H	NO_2	2.5	0.106 ± 0.038	0.165 ± 0.055	4.78 ± 0.49	45.1	29
T6	H	H	H	F	2.4	0.057 ± 0.010	0.100 ± 0.015	17.47 ± 0.65	306.5	175.6
T7	H	H	H	Cl	3.3	0.095 ± 0.033	0.172 ± 0.018	27.18 ± 6.68	286.1	158.5
T8	H	Cl	H	H	3.3	0.092 ± 0.003	0.165 ± 0.081	17.12 ± 4.26	186	103.5
BT1	H	Cl	H	Br	4.1	0.036 ± 0.017	0.238 ± 0.080	2.38 ± 0.001	66	10
BT2	Cl	H	H	Br	4.1	0.036 ± 0.024	0.123 ± 0.062	>100	>2000	>800
BT3	OCH3	OCH3	H	Br	3	0.088 ± 0.041	0.761 ± 0.194	>100	>1000	>100
BT4	H	COOH	H	Br	3.3	NE	NE	71 ± 39.73	ND	ND
BT5	H	COOCH3	H	Br	3.5	0.162 ± 0.093	0.287 ± 0.024	11.49 ± 5.42	70.9	40
TRYP	H	H	H	H	2.6	0.288 ± 0.087	0.114 ± 0.017	45.90 ± 0.70	159.4	318
CQ	/	/	/	/	/	0.021 ± 0.002	0.294 ± 0.030	>100	>4000	>4000

TRYP: Tryptanthrin; CQ: Chloroquine; CC_{50}: Cytotoxic Concentration 50; NE: Not effective. SI: Selectivity index (CC50/IC50). LogP of compounds were calculated with Molinspiration (http://www.molinspiration.com).

crystalline material from the solution during the reaction. 2-Nitrotryptanthrin is highly unstable and could not be used for biological experimentation. In several cases, this method gave low yields, e.g., with 8-nitroisatine. Another approach (method B, Scheme 1) was therefore needed and resulted in the application of 2-Chloro-3H-indol-3-ones **5.** These compounds condense to tryptanthrins **4** in the presence of anthranilic acids **7**.

This approach appeared to also be applicable in cases where low yields were achieved with method A because of either the low reactivity of the partners or the lengthy preparation of the isatoic acid anhydrides. All isatines **6** gave 2-Chloro-3H-indol-3-ones by refluxing in benzene solution with phosphorus pentachloride. Condensation with anthranilic acids is most yielding in DMF (formic acid dimethylamide) or acetic acid dimethylamide solution because the product is considerably more soluble than hydrocarbons. There are a number of derivatives that were inaccessible by these two methods. By this approach or with the simpler method of condensing 2-Chloro-3H-indol-3-one with anthranilic acids, no derivatives that were substituted at position 10 could be obtained. This may arise from sterical considerations due to the neighborhood of the oxygen of the amide group. The goal of obtaining derivatives with better solubility was only partially achieved. Even the use of tryptanthrin carboxylic acid and its esters resulted in low solubility substances, prompting the use of DMSO as a co-solvent. In the future, some inclusion complexes might be of relevance (Kumar et al., 2011). The synthesis of esters and amides generally proceeded well by the simple application of the bis-imidazolylcarbonyl-method. Preparation by way of acid chlorides often does not yield much. The application of methods with limited thermal stress seems to be appropriate, e.g., the use of oxalylchloride in the preparation of acid chloride, as seen in the preparation of esters.

3.2. Antiplasmodial activities of tryptanthrin derivatives

The antiplasmodial activity of tryptanthrin and 17 tryptanthrin derivatives was evaluated on chloroquine (CQ)-sensitive and CQ-resistant strains of *P. falciparum* using the Malstat assay. The parasites were exposed to serial dilutions of the compounds at concentrations ranging from 100 μM to 0.01 μM, and the half maximal inhibitory concentrations (IC_{50}) were determined by measuring the activity of the parasite lactate dehydrogenase (Table 1). Tryptanthrin was active against both the CQ-sensitive and CQ-resistant strains of *P. falciparum*, with IC_{50} values of 288 nM and 114 nM, respectively. Sixteen 16 of the nineteen tryptanthrin derivatives were active against both strains of *P. falciparum*, with IC_{50} concentrations in the nanomolar range (between 33 and 602 nM), among which **NT1, T6, BT1,** and **BT2** were at least 5 times more potent than tryptanthrin on the CQ-sensitive strain and **NT1** had the strongest effect on the CQ-resistant strain (3.3 times more active than tryptanthrin). In this study, CQ was used as a reference drug and was 13 times more potent against CQ-sensitive strains when activities were compared against the resistant strain. However, the difference in activity between the two strains was not significant for tryptanthrin and its derivatives. Compounds **NT4** and **BT4** were considered to be not effective (IC_{50} > 50 μM) and are all carboxylic acid derivatives with the acidic moiety at **position 3** (Fig. 1). This result suggests that the addition of the carboxylic moiety at that position is not beneficial for the antiplasmodial activity. The introduction of carboxylic acid into a biologically active compound that does not contain such a group already led to a generally better solubility and to the amelioration of the binding of the derivative to the target. However, carboxylic acids are already highly ionized at physiological pH and cannot cross biological membranes, which are permeable to non-dissociated molecules or to molecules with a very distributed charge (Bazzani and Wermuth, 2008). Therefore, the addition of a carboxylic acid group in a small molecule that needs to cross biological membranes to reach his target will tend to weaken the activity of the parent compound (Bazzani and Wermuth, 2008). Nevertheless, the loss in initial activity of a compound because of the introduction of a carboxylic acid group is often recovered by

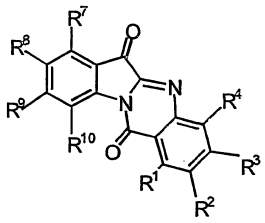


Fig. 1. Structure of tryptanthrin depicting substitution sites.

carboxyl-derived functions such as esters, which protect the acid function and can cross biological membranes (Bazzani and Wermuth, 2008). This may explain the regain of activity that we noted in compounds **NT5** and **BT5**, in which the carboxylic acid group of compounds **NT4** and **BT4** was replaced by a carboxylic acid methyl ester. A mono-derivatization of tryptanthrin with halogens (chlorine, bromine, fluorine) or with nitro and methyl groups in **position 8** (Fig. 1) led to an equal gain in activity of approximately three times than the parent compound. There was no significant difference in the IC_{50} value of the concerned compounds (**T1, T2, T4, T6,** and **T7**). The reason for that behavior might be an increase in the lipophilicity of tryptanthrin (Prokop et al., 2004) and thus a favored passage of the compounds through the biological membranes. By moving a substituent such as the methyl group from **position 8** (**T1**) to **position 7** (**T3**), we noticed a loss of activity of approximately 5-fold compared to that of **T1**. This might suggest that the **position 8** of tryptanthrin is important in improving biological activity. This observation was previously made by Scovill et al. (2002), who showed that 8-halogenated tryptanthrin had better anti-trypanosomal activities. Yu et al. reported a better activity on cancer cells when tryptanthrin was modified at **position 8** (Yu et al., 2010) and recently, modifications at position 8 have retained special attention when evaluating antitubercular derivatives (Hwang et al., 2013). It is interesting to note that moving the chlorine substituent from **position 8** (**T7**) to **position 3** (**T8**) retains the biological activity, suggesting that derivatization at **position 3** could also be of interest. Tri-derivatization with nitro-groups (**NT3**) or bromine (**BT3**) at **position 8** and methoxy-groups at both **positions 2** and **3** did not improve the activity of the 8-mono-derivatives (**T2** and **T4**). A similar observation was made by Yu and colleagues, who found out that dimethoxy derivatization of tryptanthrin did not enhance the growth inhibitory activity in cancer cells (Yu et al., 2010). We further predicted the lipophilicity of tryptanthrin and its derivatives and the predicted LogP values were in the range of 2.2 to 4.1. It is generally accepted that compounds with LogP values between 1 and 4 have a good permeability. It is therefore important to maintain the permeability when further optimization is considered. Based on these data alone, **NT1** emerged as the most suitable compound for further studies: it was the most soluble compound in DMSO with no visible precipitate at a concentration of 10 mM and had an IC_{50} slightly equivalent to the reference drug **CQ**. Our observations and data suggest that tryptanthrin derivatives could be evaluated in *in vivo* mouse models; however, there is a necessity to further improve the solubility of tryptanthrin derivatives *via* either appropriate formulation or chemical modifications.

3.3. Cytotoxicity of tryptanthrin derivatives

Tryptanthrin and its derivatives were subjected to cytotoxicity testing against the human cell line MCF-7 to determine the specificity of their antiplasmodial effect. Therefore, the MTT assay was carried out to measure the mitochondrial activity of the MCF-7 cells after drug exposure as an indicator of cell proliferation, and the cytotoxicity of the compounds was determined by calculating the concentration that leads to *in vitro* 50% cytotoxicity (CC50). The data obtained were used to calculate the selectivity index (SI, Table 1), allowing the identification of compounds with potential therapeutic windows that are not toxic to human or mammalian cells. To further investigate the compounds using the mouse model, it is usually recommended that SI > 100 (Nwaka and Hudson, 2006). Based on this criterion, only compounds **NT1, NT2, NT6, T1, T2, T6, T7, T8, BT2, BT3,** and tryptanthrin can be considered for animal studies (Table 1). Interestingly, compounds **T1** and **BT2** were the safest molecules with SI values higher than 1000. While the antiplasmodial activity of **NT1** was only 1.3 to 3 times higher than that of **BT2** and **T1**, these two compounds were 20 times safer than **NT1** when tested on the sensitive strain. **NT1** showed a strong antiplasmodial activity on both the sensitive and resistant strains, and despite its strong inhibition of MCF-7 cells, the SI was higher than 100 for both strains of *P. falciparum*. The high safety of **BT2** and **T1** is particularly important because it is sometimes necessary to increase the compound's dose to achieve suitable drug plasma concentrations and efficacy *in vivo* during pre-clinical studies. Compounds such as **BT2** and **T1** will have less limitation because a higher dose of the molecule can be administered. Based on the cytotoxicity results, **T1** and **BT2** should be considered alongside **NT1** for optimization and potential pre-clinical studies. We further evaluated the effect of tryptanthrin and its derivatives on red blood cell integrity and we did not observe any signs of membrane disruption at 10 μM (data not shown).

3.4. Transmission blocking activity

Malaria eradication using chemotherapy will be very effective if the transmission of the parasite from human to human *via* mosquito bites is prevented. Considerable efforts are currently devoted to evaluating the early-stage gametocytocidal activity of the MMV Malaria Box (Lucantoni et al., 2013; Sun et al., 2014), which is a collection of 400 compounds with known activity against the asexual stages of *P. falciparum* (Lucantoni et al., 2013; Sun et al., 2014). This result highlights the importance of experimental verification of early-stage gametocytocidal activity in the development of new antimalarial candidates. The effect of tryptanthrin and the derivatives NT1, T8, epoxomicin, and chloroquine was investigated on the development of stage II to stage V gametocytes. The results confirmed that nanomolar concentrations of the positive control epoxomicin were able to fully eliminate the gametocytes in the culture (Fig. 2), which is in accordance with previous findings on the gametocytocidal activity of the proteasome inhibitor epoxomicin (Czesny et al., 2009; Aminake et al., 2011, 2012). Furthermore, it was shown that IC_{50} concentrations of chloroquine did not significantly affect gametocyte maturation, while IC_{90} concentrations led to mature gametocyte reduction of up to 20%. It was further observed that IC_{50} concentrations of tryptanthrin and T8 did not significantly affect the maturation of gametocytes even though the culture treated with T8 had a very slightly reduced number of stage IV/V gametocytes compared to the number of stage IV/V gametocytes in the DMSO control. Interestingly, the IC_{50} concentration of NT1, which was previously determined by the Malstat assay, reduced the amount of mature gametocytes by approximately 40–50% (Fig. 2). At IC_{90} concentrations, all three compounds (tryptanthrin, NT1, and T8) were effective and a 100% killing of gametocytes was observed for NT1 and T8. However, the IC_{90} value of T8 was four times higher than that of NT1. The parent compound tryptanthrin did not achieve a 100% killing of gametocyte with an IC_{90} concentration (Fig. 2) that was more than 10 times higher than that of NT1, showing a clear improvement in gametocytocidal activity due to the derivation.

Another approach of the transmission blocking strategy is the inhibition of exflagellation by mature stage V gametocytes, which are directly responsible for parasite transmission from the human host to the mosquito and are thus a target for transmission-blocking interventions (Delves et al., 2013). Mature stage V gametocytes, which are quiescent in the human blood stream, must be ingested by the mosquito to complete the maturation process and develop into male or female gametes, thereby initiating the mosquito stage of the parasite. However, the mature stage V gametocytes are resistant to most of the antimalarials affecting the asexual blood stages (Kumar and Zheng, 1990; Noedl et al., 2003). The effect of tryptanthrin, T8, and NT1 on the exflagellation of mature gametocytes was investigated, and it was shown that neither tryptanthrin nor T8 were able to significantly inhibit the exflagellation of

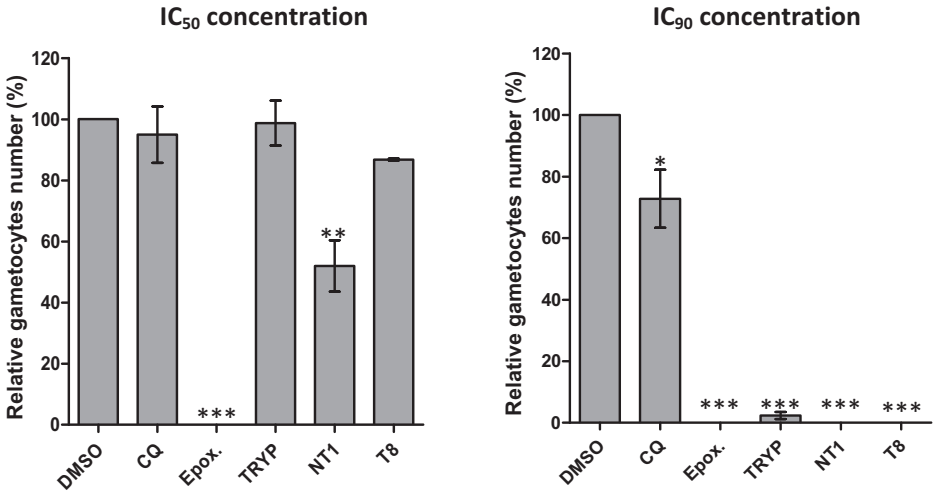


Fig. 2. Inhibition of gametocyte maturation. Stage II gametocytes were incubated with compounds at IC_{50} and IC_{90} concentrations or with a 0.5% vol DMSO for 2 days. The number of stage IV and V gametocytes was counted after 7 days and the gametocytemia of drug-treated cultures was correlated to the gametocytemia of the DMSO control (normalized to 100%). The graphs represent the results of two independent experiments performed in triplicate. Asterisks represent a significant difference between the tested compounds and DMSO control, $^{***}P < 0.001$; $^{**}0.001 < P < 0.01$; $^{*}P < 0.05$. The bar labels represent the standard deviation.

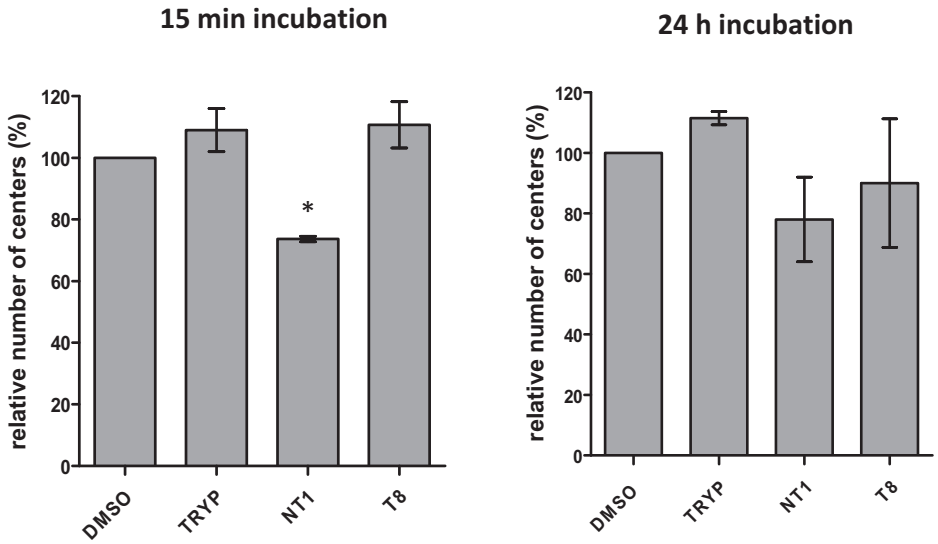


Fig. 3. Effect of selected tryptanthrin-based compounds on exflagellation. Compounds at IC_{50} concentrations or 1% vol DMSO were added to mature gametocyte cultures 24 h or 15 min prior to their activation with xanthurenic acid. The number of exflagellation centers was counted 15 min after activation; the number of centers was recorded and compared to the number of centers in the DMSO control. The graphs represent the results of two independent experiments. Mean ± SEM. $^{*}P < 0.05$. The bar labels represent the standard deviation.

microgametes after 15 min as well as after 24 h of incubation at their IC_{90} concentrations (Fig. 3). Interestingly, it was shown that the IC_{90} concentrations of NT1 could achieve up to 20% reduction of exflagellation, which is again an improvement in the transmission-blocking activity due to the derivation of tryptanthrin to NT1.

4. Conclusion

We report the antiplasmodial activity of tryptanthrin derivatives and their potential transmission-blocking activity. Our data suggest that NT1 is the most interesting antiplasmodial candidate of all the tryptanthrin derivatives that were tested. NT1 showed the strongest activity in the asexual stages in drug-sensitive as well as in drug-resistant *P. falciparum* parasites. Furthermore, NT1 exhibited the potential to prevent the maturation of early- to late-stage gametocytes as well as a potential to inhibit the exflagellation of microgametes, thus making it a transmission-blocking drug candidate. Nevertheless, as with any *in vitro* screening report, the compound activity data produced in this study will benefit from additional studies to specify the activity.

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Conflict of interest

The authors declared that there is no conflict of interest.

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