Supporting Information

Discovery of Triazines Mimetics as potent antileishmanial agents

Kuldeep Chauhan,[†] Moni Sharma,[†] Rahul Shivahare,[‡] Utsab Debnath[†], Suman Gupta, [‡]

Yenamandra S. Prabhakar[†] and Prem M. S. Chauhan[†*]

[†]Medicinal and Process Chemistry Division and [‡]Division of Parasitology, CSIR- Central Drug Research Institute,

Lucknow-226031, U.P., India

*Corresponding author mailing address: Medicinal and Process Chemistry Division, CSIR-Central Drug Research Institute, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow-226031, U.P, India, Phone: +91 522 2771940, 2771942; Fax: +91 522 2771941. Email: prem_chauhan_2000@yahoo.com; premsc58@hotmail.com

Table of content

EXPERIMENTAL SECTION

General: All commercially available starting materials and solvents were reagent grade, and used without further purification. Reactions were carrying out under dry glassware with magnetic stirring. IR spectra were recorded on a FTIR spectrophotometer Shimadzu 8201 PC and are reported in terms of frequency of absorption (cm^{-1}). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Supercon Magnet Avance 400, 300, 200 spectrometers using TMS as an internal reference and the samples were dissolved in suitable deuterated solvents (Chemical shifts (δ) are given in ppm relative to TMS and coupling constants (*J*) in Hz). Electro Spray Ionisation Mass spectra (ESI-MS) were recorded by micromass quattro II instrument. HR-DART MS were recorded on JEOL, JMS T100LC Accu TOF. Melting points were determined in open capillary tubes on an electrically heated block and were uncorrected. Thin-layer chromatography (TLC) was carried out with silica gel plates (silica gel 60 F254), that were visualized by exposure to ultraviolet light. Purity of final compounds was determined by analytical HPLC, which was carried out on a Waters HPLC system (model pump: 515, detector PDA-2998). HPLC analysis conditions: Waters Sunfire^{TM} C18 (5.0µM), 4.6x250 mm column, flow rate 0.5 mL/min, UV detection at 254 nm. All biologically evaluated compounds are ≥ 95% pure.

Representative Procedure for the Synthesis of 4,6-dichloro-*N*-phenyl-1,3,5-triazin-2-amine (2). To a stirred solution of cyanuric chloride (1.93 g, 10.5 mmol) in anhydrous tetrahydrofuran (20 mL) was cooled at 0ºC, aniline **1** (0.8 mL, 8.76 mmol) was added ~30 min drop wise in this solution. The mixture was stirred at 0ºC for 2 h, the resultant precipitate was concentrated under reduced pressure to provide crude product. The crude product was purified by column chromatography over silica gel using 10% ethyl acetate/hexane as eluent. White solid (yield = 96%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 7.57 (d, 2H, *J* = 7.3 Hz), 7.43 (t, 2H, *J* = 5.7 Hz), 7.24–7.21 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 171.3, 170.1, 164.1, 135.7, 129.3, 125.9, 121.4. ESI-MS $C_9H_6Cl_2N_4$ (m/z) : 240.0 $(M + H)^+$.

Representative Procedure for the Synthesis of 5-(4-chloro-6-(phenylamino)-1,3,5-triazin-2-ylamino) pentan-1-ol (3). To a stirred solution of **2** (1.0 g, 4.13 mmol) in anhydrous tetrahydrofuran (20 mL) was treated with 5-aminopentan-1-ol (0.44 mL, 4.13 mmol) and K_2CO_3 (855 mg, 6.20 mmol), the mixture was stirred at room temperature for overnight. The resultant mixture was evaporated in vacuo to provide crude product. The crude product was purified by column chromatography over silica gel using 30% ethyl acetate/hexane as eluent. White solid (Yield = 93%). mp = 152–154°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.59 (d, 2H, *J* = 5.9 Hz), 7.47 (d, 2H, *J* = 5.9 Hz), 7.35–7.32 (m, 1H), 4.41(s, 2H), 4.50 (m, 2H), 3.79 (s, 2H), 3.35–3.28 (m, 2H), 1.28 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 171.0, 169.1, 166.0, 139.2, 133.0, 130.4, 122.8, 122.0, 64.4, 43.0, 32.0, 25.2, 23.1. ESI-MS $C_{14}H_{18}ClN_5O$ (m/z) : 308.0 $(M+H)^+$.

4-(4-chloro-6-(phenylamino)-1,3,5-triazin-2-ylamino) butan-1-ol (4). The above procedure was followed for compound **4**. White solid (Yield = 95%); mp = 148–150°C. ^{1}H NMR (400 MHz, $CDCl_3$): δ: 7.55 (d, 2H, *J* = 5.9 Hz), 7.45 (d, 2H, *J* = 5.7 Hz), 7.32–7.29 (m, 1H), 4.36–4.33 (m, 2H), 3.98 (s, 2H), 3.65–3.56 (m, 2H), 1.53 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 170.3, 168.4, 165.8, 135.4, 134.0, 129.1, 126.5, 121.9, 67.3, 41.5, 25.4, 24.8. ESI-MS $C_{13}H_{16}ClN_5O$ (m/z) : 294.0 $(M+H)^+$.

Representative Procedure for the Synthesis of 6-chloro-N^2-(5-(4-chloro-6-(phenylamino)-1,3,5-triazin-2-yloxy) pentyl)-N^4-phenyl-1,3,5-triazine-2,4-diamine (5). A cooled solution of **3** (500 mg, 1.62 mmol), NaH (42.8 mg, 1.78 mmol) in anhydrous THF (10 mL) was treated with **2** (392 mg, 1.62 mmol) and the mixture was stirred at room temprature for overnight. The resultant mixture was evaporated in vacuo to provide crude product. The crude product was purified by column chromatography over silica gel using 50% ethyl acetate/hexane as eluent. White solid (Yield = 54%); mp = 159–161°C. HPLC–DAD: t_r = 6.23 min (% area = 97.20%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.54 (d, 4H, *J* = 7.0 Hz), 7.36 (d, 4H, *J* = 8.2 Hz), 7.18–7.10 (m, 2H), 4.38 (s, 2H), 3.47 (s, 2H), 1.82 (s, 2H), 1.65 (s, 4H), 1.50 (s, 2H). ^{13}C NMR (75 MHz,

$CDCl_3$ + DMSO–d_6): δ: 170.3, 169.9, 168.8, 168.4, 143.7, 142.7, 133.4, 133.3, 128.8, 127.8, 125.6, 125.4, 125.0, 73.0, 33.7, 33.0, 28.0, 27.8. IR (KBr): 3669, 3108, 2900, 1306, 932 cm^{-1}. ESI–MS $C_{23}H_{23}Cl_2N_9O$ (m/z): 512.0 $(M+H)^+$. HRMS: calc.: 512.1475 (MH^+); Found: 512.1472(MH^+).

6-Chloro-*N*^{2}-(4-(4-chloro-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{4}-phenyl-1,3,5-triazine-2,4-diamine (6). The above procedure was followed for compound **6**. White solid (Yield = 55%); mp = 153–155°C. HPLC–DAD: t_r = 6.0 min (% area = 95.2%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.55 (d, 4H, *J* = 7.3 Hz), 7.33 (d, 4H, *J* = 8.1 Hz), 7.14–7.09 (m, 2H), 4.45 (s, 2H), 3.96 (s, 2H), 3.63 (s, 2H), 2.10 (s, 2H), 1.73 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 166.3, 165.9, 165.2, 163.8, 137.6, 136.6, 128.9, 128.8, 125.0, 124.1, 121.6, 121.1, 120.7, 65.9, 54.9, 37.8, 31.6, 28.4. IR (KBr): 3682, 3020, 2960, 1216, 927 cm^{-1}. ESI–MS $C_{22}H_{21}Cl_2N_9O$ (m/z): 498.0 $(M+H)^+$. HRMS: calc.: 498.1318 (MH^+); Found: 498.1331(MH^+).

Representative Procedure for the Synthesis of 6-morpholino-*N*^{2}-(5-(4-morpholino-6-(phenylamino)-1,3,5-triazin-2-yloxy) pentyl)-*N*^{4}-phenyl-1,3,5-triazine-2,4-diamine (7). A solution of **5** (250 mg, 0.48 mmol) in anhydrous THF (15 mL) was treated with morphilino (0.085 mL, 0.97 mmol) in the presence of potassium carbonate (101 mg, 0.73 mmol) and the mixture was stirred at 60ºC for overnight. The resultant mixture was evaporated in vacuo to provide crude product. The crude product was purified by column chromatography over silica gel using 50% ethyl acetate/hexane as eluent. White solid (Yield = 96%); mp = 142–144°C. HPLC–DAD: t_r = 6.2 min (% area = 98.3%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.54 (d, 4H, *J* = 7.9 Hz), 7.31 (d, 4H, *J* = 8.2 Hz), 7.05–6.97 (m, 2H), 6.79 (s, 2H), 4.98 (s, 1H), 4.33 (t, 2H, *J* = 6.3 Hz), 3.82–3.72 (m, 12H), 3.44–3.37 (m, 2H), 1.83–1.79 (m, 2H), 1.68–1.52 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 170.7, 166.2, 165.3, 164.2, 139.4, 138.7, 128.8, 128.6, 123.1, 122.4, 120.1, 119.8, 66.8, 66.7, 43.9, 43.7, 40.6, 29.5, 28.5, 23.4. IR (KBr): 3681, 3083, 2887, 1241,

922 cm^{-1}. ESI–MS $C_{31}H_{39}N_{11}O_3$ (m/z) : 614.0 $(M+H)^+$. HRMS: calc.: 614.3310 (MH^+); Found: 614.3313 (MH^+).

The above procedure was followed for compound (**8**–**23**).

***N*^{2}-phenyl-*N*^{4}-(5-(4-(phenylamino)-6-(propylamino)-1,3,5-triazin-2-yloxy)pentyl)-*N*^{6}-propyl-1,3,5-triazine-2,4,6-triamine (8).** White solid (Yield = 94%); (mp = 110–112°C). HPLC–DAD: t_r = 5.96 min (% area = 98.0%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.64 (d, 4H, *J* = 7.6 Hz), 7.22 (d, 4H, *J* = 7.6 Hz), 7.13–6.84 (m, 2H), 4.36 (s, 2H), 3.39–3.32 (m, 6H), 1.86–1.78 (m, 6H), 1.61–1.57 (m, 4H), 0.92–0.88 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 170.1, 169.2, 166.0, 165.4, 164.8, 139.1, 138.6, 129.4, 129.1, 123.8, 122.0, 120.7, 120.1, 66.6, 66.1, 43.0, 42.6, 32.0, 27.0, 25.1, 23.4, 22.5, 14.0, 11.2. IR (KBr): 3442, 3025, 2987, 1220, 942 cm^{-1}. ESI–MS $C_{29}H_{39}N_{11}O$ (m/z): 558.0 (M^++H). HRMS: calc.: 558.3411 (MH^+); Found: 558.3414 (MH^+).

6-Morpholino-*N*^{2}-(4-(4-morpholino-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{4}-phenyl-1,3,5-triazine-2,4-diamine (9). White solid (Yield = 88%); (mp = 185–187°C). HPLC–DAD: t_r = 6.0 min (% area = 95.7%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.54 (d, 4H, *J* = 7.8 Hz), 7.33 (d, 4H, *J* = 7.8 Hz), 7.13–6.90 (m, 2H), 4.40 (t, 2H, *J* = 6.2 Hz), 3.94–3.72 (m, 16H), 3.50 (q, 2H, *J* = 12.4 Hz, *J* = 6.3 Hz), 2.00–1.85 (m, 2H), 1.82 (s, 2H). ^{13}C NMR (75 MHz, DMSO–d_6): δ: 170.7, 166.1, 165.5, 165.2, 141.0, 140.1, 128.9, 128.7, 122.6, 121.7, 120.4, 119.8, 66.4, 66.3, 43.9, 43.7, 26.4. IR (KBr): 3421, 3006, 2858, 1274, 888 cm^{-1}. ESI–MS $C_{30}H_{37}N_{11}O_3$ (m/z): 600.0 (M^++H). HRMS: calc.: 600.3153 (MH^+); Found: 600.3164 (MH^+).

***N*^{2}-(2-Morpholinoethyl)-*N*^{4}-(4-(4-(2-morpholinoethylamino)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{6}-phenyl-1,3,5-triazine-2,4,6-triamine (10).** White solid (Yield = 93%); (mp = 136–138°C). HPLC–DAD: t_r = 6.69 min (% area = 95.2%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.58 (s, 4H), 7.32 (d, 4H, *J* = 7.8 Hz), 7.07–6.97 (m, 2H), 4.38 (s, 2H), 3.68 (s, 8H), 3.51 (s, 8H), 2.53 (s, 4H), 1.88 (s, 8H), 1.74 (s, 2H) . ^{13}C NMR (75 MHz, $CDCl_3$): δ: 170.4, 167.0, 165.4, 164.2, 139.3, 138.8, 128.7, 123.1, 122.5, 120.7, 120.3, 119.9, 66.8, 57.3, 57.0, 53.3, 37.4, 37.0,

31.5, 29.7, 26.7, 22.6. IR (KBr): 3682, 3020, 2875, 1216, 927 cm^{-1}. ESI–MS $C_{34}H_{47}N_{13}O_3$ (m/z): 686.0 (M^++H). HRMS: calc.: 686.3997 (MH^+); Found: 686.3999 (MH^+).

***N*^{2}-(3-Morpholinopropyl)-*N*^{4}-(4-(4-(3-morpholinopropylamino)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{6}-phenyl-1,3,5-triazine-2,4,6-triamine (11).** White solid (Yield = 85%); (mp = 139–141°C). HPLC–DAD: t_r = 8.48 min (% area = 96.0%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.55 (s, 4H, *J* = 7.4 Hz), 7.33 (d, 4H, *J* = 7.2 Hz), 7.14–7.09 (m, 2H), 4.45 (s, 2H), 3.96 (s, 10H), 3.63 (s, 8H), 2.54 (s, 4H), 1.89 (s, 8H), 1.65 (s, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 170.5, 166.8, 165.3, 164.1, 140.1, 138.9, 138.6, 128.5, 123.0, 122.2, 121.4, 118.8, 67.0, 63.2, 58.1, 57.6, 57.0, 53.0, 39.8, 37.2, 36.0, 31.0, 29.5, 26.3, 22.5. IR (KBr): 3431, 3019, 2967, 1216, 929 cm^{-1}. ESI–MS $C_{36}H_{51}N_{13}O_3$ (m/z): 714.0 (M^++H). HRMS: calc.: 714.4310 (MH^+); Found: 714.4324 (MH^+).

***N*^{2}-Pentyl-*N*^{4}-(4-(4-(pentylamino)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{6}-phenyl-1,3,5-triazine-2,4,6-triamine (12).** White solid (Yield = 91%); (mp = 102–104°C). HPLC–DAD: t_r = 6.84 min (% area = 98.4%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.62–7.53 (m, 4H), 7.32 (d, 4H, *J* = 7.8 Hz), 7.06–6.96 (m, 2H), 4.37 (s, 2H), 3.45–3.38 (m, 6H), 2.00–1.55 (m, 8H), 1.32 (s, 8H), 0.88 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 170.3, 167.2, 166.1, 165.4, 139.4, 138.9, 128.7, 123.3, 123.0, 122.3, 120.7, 119.7, 66.3, 66.0, 41.1, 40.9, 32.0, 26.6, 22.4, 14.0. IR (KBr): 3431, 3034, 1649, 1557, 1459, 1227, 1114, 779 cm^{-1}. ESI–MS $C_{32}H_{45}N_{11}O$ (m/z): 600.0 (M^++H). HRMS: calc.: 600.3881 (MH^+); Found: 600.3884 (MH^+).

***N*^{2}-Butyl-*N*^{4}-(4-(4-(butylamino)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{6}-phenyl-1,3,5-triazine-2,4,6-triamine (13).** White solid (Yield = 81%); (mp = 108–110°C). HPLC–DAD: t_r = 5.84 min (% area = 95.6%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.60 (s, 4H), 7.31 (d, 4H, *J* = 7.7 Hz), 7.11–6.93 (m, 2H), 4.40 (s, 2H), 3.51–3.42 (m, 6H), 2.00–1.86 (m, 4H), 1.82–1.76 (m, 8H), 0.93 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 169.3, 168.0, 165.3, 164.2, 139.2, 138.9, 128.2, 122.3, 120.5, 120.1, 119.1, 68.0, 66.7, 43.0, 42.7, 32.0, 28.5, 26.0, 23.0, 22.9, 14.0, 13.4.

IR (KBr): 3431, 3034, 1649, 1557, 1459, 1227, 1114, 779 cm^{-1}. ESI–MS $C_{30}H_{41}N_{11}O$ (m/z): 572.0 (M^++H). HRMS: calc.: 572.3568 (MH^+); Found: 572.3574 (MH^+).

***N*^{2}-Phenyl-*N*^{4}-(4-(4-(phenylamino)-6-(propylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{6}-propyl-1,3,5-triazine-2,4,6-triamine (14).** White solid (Yield = 90%); (mp = 106 – 108°C). HPLC–DAD: t_r = 6.84 min (% area = 97.4%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.62 (s, 4H), 7.31 (d, 4H, *J* = 7.8 Hz), 7.07–7.02 (m, 2H), 4.41 (s, 2H), 3.49–3.24 (m, 6H), 1.83–1.75 (m, 8H), 1.61 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 167.3, 166.0, 165.4, 164.3, 139.4, 138.9, 128.7, 123.3, 122.4, 120.8, 120.2, 119.7, 66.3, 66.0, 42.9, 42.6, 31.5, 26.6, 25.2, 23.0, 22.8, 14.1, 11.4. IR (KBr): 3431, 3019, 2967, 1216, 929 cm^{-1}. ESI–MS $C_{28}H_{37}N_{11}O$ (m/z): 544.0 (M^++H). HRMS: calc.: 544.3255 (MH^+); Found: 544.3265 (MH^+).

***N*^{2}-Tert-butyl-*N*^{4}-(4-(4-(tert-butylamino)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{6}-phenyl-1,3,5-triazine-2,4,6-triamine (15).** White solid (Yield = 90%); (mp = 188–190°C). HPLC–DAD: t_r = 5.84 min (% area = 96.9%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.55 (d, 4H, *J* = 7.7 Hz), 7.32 (d, 4H, *J* = 7.8 Hz), 7.09 (s, 2H), 4.43 (s, 2H), 3.98 (s, 2H), 3.51 (s, 2H), 2.10 (s, 2H),1.84 (s, 6H), 1.65 (s, 6H), 1.42 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 171.0, 167.1, 166.2, 164.0, 141.8, 140.2, 129.9, 128.7, 121.5, 121.2, 120.8, 119.2, 66.8, 66.5, 43.8, 43.2, 29.2, 26.0. IR (KBr): 3430, 3024, 2964, 1206, 922 cm^{-1}. ESI–MS $C_{30}H_{41}N_{11}O$ (m/z): 572.0 (M^++H). HRMS: calc.: 572.3568 (MH^+); Found: 572.3573 (MH^+).

***N*^{2}-Isopropyl-*N*^{4}-(4-(4-(isopropylamino)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{6}-phenyl-1,3,5-triazine-2,4,6-triamine (16).** White solid (Yield = 94%); (mp = 183–185°C). HPLC–DAD: t_r = 6.64 min (% area = 95.6%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.58 (d, 4H, *J* = 7.7 Hz), 7.33 (d, 4H, *J* = 7.9 Hz), 7.09–7.03 (m, 2H), 4.40 (s, 2H), 4.22 (s, 2H), 3.96 (s, 2H), 3.52 (s, 2H), 1.86 (s, 6H), 1.64 (s, 6H), 1.42 (s, 2H). ^{13}C NMR (50 MHz, DMSO–d_6): δ: 170.4, 168.0, 166.5, 164.2, 142.0, 141.1, 129.4, 128.8, 122.3, 121.4, 120.1, 119.3, 67.0, 66.1, 44.0, 43.5,

26.0, 24.0. IR (KBr): 3431, 3034, 1649, 1557, 1459, 1227, 1114, 779 cm^{-1}. ESI–MS $C_{28}H_{37}N_{11}O$ (m/z): 544.0 (M^++H). HRMS: calc.: 544.3255 (MH^+); Found: 544.3259 (MH^+).

***N*^{2}-(Cyclopropylmethyl)-*N*^{4}-(4-(4-(cyclopropylmethylamino)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{6}-phenyl-1,3,5-triazine-2,4,6-triamine (17).** White solid (Yield = 88%); (mp = 215–217°C). HPLC–DAD: t_r = 8.28 min (% area = 95.9%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.66 (d, 4H, *J* = 5.5 Hz), 7.44 (s, 2H), 7.37 (d, 4H, *J* = 5.5 Hz), 4.42 (s, 2H), 3.94 (s, 2H), 2.85 (s, 2H), 2.00 (s, 2H), 1.76 (s, 5H), 1.02 (s, 1H), 0.90–0.86 (m, 2H), 0.76 (d, 2H, *J* = 14.1 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 161.7, 161.1, 160.6, 160.0, 129.3, 129.2, 127.6, 127.3, 126.3, 123.6, 122.9, 121.8, 120.4, 116.6, 112.8, 109.1, 70.0, 69.7, 41.5, 31.5, 25.0, 22.6, 10.2. IR (KBr): 3431, 3034, 1649, 1557, 1459, 1227, 1114, 779 cm^{-1}. ESI–MS $C_{28}H_{33}N_{11}O$ (m/z): 540.0 (M^++H). HRMS: calc.: 540.2942 (MH^+); Found: 540.2951 (MH^+).

6-(4-Methylpiperazin-1-yl)-*N*^{2}-(4-(4-(4-methylpiperazin-1-yl)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{4}-phenyl-1,3,5-triazine-2,4-diamine (18). White solid (Yield = 92%); mp = 138–140°C. HPLC–DAD: t_r = 8.2 min (% area = 96.2%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.54 (d, 4H, *J* = 7.8 Hz), 7.29–7.22 (m, 4H), 7.03–6.92 (m, 2H), 4.37 (t, 2H, *J* = 6.4 Hz), 3.84–3.78 (m, 8H), 3.47 (q, 2H, *J* = 12.3 Hz, *J* = 6.1 Hz), 2.40 (s, 8H), 2.28 (s, 6H), 2.03–1.97 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ: 170.6, 169.1, 166.0, 160.6, 138.4, 138.0, 128.6, 128.1, 123.1, 122.5, 119.5, 68.0, 58.0, 57.4, 55.0, 52.1, 46.0, 26.3. IR (KBr): 3683, 3022, 2898, 1224, 929 cm^{-1}. ESI–MS $C_{32}H_{43}N_{13}O$ (m/z): 626.0 $(M+H)^+$. HRMS: calc.: 626.3786 (MH^+); Found: 626.3791 (MH^+).

6-(4-Ethylpiperazin-1-yl)-*N*^{2}-(4-(4-(4-ethylpiperazin-1-yl)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{4}-phenyl-1,3,5-triazine-2,4-diamine (19). White solid (Yield = 90%); (mp = 131–133°C). HPLC–DAD: t_r = 13.5 min (% area = 97.4%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.54 (m, 4H, *J* = 7.7 Hz), 7.42 (s, 4H), 7.04–6.93 (m, 2H), 4.39 (s, 2H), 3.86 (s, 8H), 3.47 (s, 2H), 2.62–2.46 (m, 12H), 1.85 (s, 10H). ^{13}C NMR (50 MHz, $CDCl_3$): δ: 171.0, 168.2, 166.3, 160.3,

138.1, 137.6, 128.9, 128.7, 122.4, 120.5, 119.8, 67.2, 57.8, 55.1, 52.0, 47.1, 29.7, 26.7. IR (KBr): 3683, 3020, 2976, 1216, 929 cm^{-1}. ESI–MS $C_{34}H_{47}N_{13}O$ (m/z): 654.0 (M^++H). HRMS: calc.: 654.4099 (MH^+); Found: 654.4094 (MH^+).

6-(3,4-Dihydroquinolin-1(2H)-yl)-*N*^{2}-(4-(4-(3,4-dihydroquinolin-1(2H)-yl)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{4}-phenyl-1,3,5-triazine-2,4-diamine (20). White solid (Yield = 84%); (mp = 148–150°C). HPLC–DAD: t_r = 9.0 min (% area = 95.0%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.62 (d, 4H, *J* = 7.8 Hz), 7.31 (d, 4H, *J* = 7.8 Hz), 7.18 (s, 8H), 7.06–6.94 (m, 2H), 4.91 (s, 4H), 4.71 (s, 4H), 4.42 (s, 2H), 2.85 (s, 5H), 1.81–1.76 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 172.0, 168.0, 165.0, 160.1, 140.0, 139.3, 132.1, 131.9, 131.5, 128.9, 128.2, 126.2, 126.0, 122.9, 122.3, 120.1, 118.2, 65.3, 44.5, 40.4, 40.1, 40.0, 32.5, 29.0, 27.1, 22.0, 14.2. IR (KBr): 3684, 3031, 2973, 1206, 942 cm^{-1}. ESI–MS $C_{40}H_{41}N_{11}O$ (m/z): 692.0 (M^++H). HRMS: calc.: 692.3568 (MH^+); Found: 692.3573 (MH^+).

6-(3,4-Dihydroisoquinolin-2(1H)-yl)-*N*^{2}-(4-(4-(3,4-dihydroisoquinolin-2(1H)-yl)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{4}-phenyl-1,3,5-triazine-2,4-diamine (21). White solid (Yield = 96%); (mp = 150–152°C). HPLC–DAD: t_r = 6.69 min (% area = 96.8%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.61 (d, 4H, *J* = 7.7 Hz), 7.32–7.28 (d, 4H, *J* = 7.6 Hz), 7.17 (s, 8H), 7.10–7.04 (m, 2H), 4.92 (s, 4H), 4.43 (s, 2H), 4.04 (s, 4H), 2.90 (s, 5H), 1.91–1.89 (m, 2H), 1.78–1.76 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 170.6, 166.0, 165.0, 139.5, 138.9, 135.1, 134.9, 133.6, 128.8, 128.6, 126.5, 126.2, 123.0, 122.3, 120.2, 119.7, 66.3, 45.6, 41.5, 41.2, 41.0, 40.6, 31.6, 28.9, 26.6, 22.7, 14.1. IR (KBr): 3425, 3019, 2980, 1216, 930 cm^{-1}. ESI–MS $C_{40}H_{41}N_{11}O$ (m/z): 692.0 (M^++H). HRMS: calc.: 692.3568 (MH^+); Found: 692.3578 (MH^+).

***N*^{2}-(4-(4-(cyclopropylamino)-6-(phenylamino)-1,3,5-triazin-2-yloxy)butyl)-*N*^{4}-phenyl-*N*^{6}-propyl-1,3,5-triazine-2,4,6-triamine (22).** White solid (Yield = 91%); (mp = 135–137°C). HPLC–DAD: t_r = 8.9 min (% area = 95.4%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.64 (d, 4H, *J* = 7.8 Hz), 7.32 (d, 4H, *J* = 7.8 Hz), 7.16–6.95 (m, 2H), 4.40 (s, 2H), 3.48–3.35 (m, 2H), 2.83 (s,

2H), 1.93 (s, 4H), 1.76 (s, 2H), 1.60 (s, 2H), 0.94 (s, 2H), 0.89 (t, 2H, *J* = 5.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 171.0, 167.3, 166.2, 160.6 141.3, 140.6, 128.9, 128.6, 122.0, 120.7, 119.5, 66.0, 42.3, 40.2, 32.0, 26.6, 23.8, 22.0, 12.0. IR (KBr): 3431, 3034, 1649, 1557, 1459, 1227, 1114, 779 cm^{-1}. ESI–MS $C_{29}H_{35}N_{11}O$ (m/z): 542.0 (M^++H). HRMS: calc.: 542.3098 (MH^+); Found: 542.3108 (MH^+).

***N*^{2}-cyclopropyl-*N*^{4}-phenyl-*N*^{6}-(4-(4-(phenylamino)-6-(propylamino)-1,3,5-triazin-2-yloxy)butyl)-1,3,5-triazine-2,4,6-triamine (23).** White solid (Yield = 85%); (mp = 128–130°C). HPLC–DAD: t_r = 6.80 min (% area = 96.2%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 7.70 (s, 4H), 7.31–7.25 (m, 4H), 7.06–6.97 (m, 2H), 4.38 (s, 2H), 3.46–3.33 (m, 4H), 2.80 (s, 2H), 1.88 (s, 4H), 1.70 (s, 2H), 0.93 (s, 2H), 0.89 (t, 2H, *J* = 5.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 168.5, 165.8, 163.2, 161.7, 139.4, 138.9, 128.7, 128.6, 122.4, 120.1, 119.8, 66.2, 42.6, 40.6, 31.6, 26.7, 23.5, 22.6, 11.4. IR (KBr): 3431, 3034, 1649, 1557, 1459, 1227, 1114, 779 cm^{-1}. ESI–MS $C_{28}H_{35}N_{11}O$ (m/z): 542.0 (M^++H). HRMS: calc.: 542.3098 (MH^+); Found: 542.3093 (MH^+).

Experimental section for biology

***In vitro* antileishmanial assay:**

For assessing the activity of synthetic compounds against the amastigote stage of parasite, mouse macrophage cell line (J-774A.1) infected with WHO reference strain (MHOM/IN/80/Dd8) of promastigote (expressing luciferase firefly reporter gene) was used. Macrophages were seeded in a 96 well plate (4 x 10^4/100µL/well) in RPMI-1640 containing 10% Fetal calf serum and the plates were incubated at 37 ºC in a 5% CO_2 incubator. After 24 hr, the medium was replaced with fresh medium containing stationary phase promastigotes (4 x 10^5/100µL/well). Promastigotes invade the macrophage and are transformed into amastigotes. The test compounds were added at two fold dilutions up to 7 points in complete medium starting from 40 µM conc. after replacing the previous medium and the plates were incubated at 37 ºC in

a CO_2 incubator for 72 hr. After incubation, the drug containing medium was aspirated and 50µL PBS was added in each well and mixed with an equal volume of Steady Glo reagent. After gentle shaking for 1–2 min, the reading was taken in a luminometer. The values are expressed as relative luminescence units (RLU). Data were transformed into a graphic program (Excel). IC_{50} of antileishmanial activity was calculated by nonlinear regression analysis of the concentration response curve using the four parameter of Hill equations.[1]

Cytotoxicity assay:

The cell viability was determined using the MTT assay.[2] As described previously, [3] mammalian fibroblast cells, (vero cell line) (1x 10^5cells/100µL/well) were incubated with test compounds at 7 concentrations starting from 400µM. After 72 hr of incubation, 25 µL of MTT reagent (5 mg/mL) in PBS medium was added to each well and incubated at 37 °C for 2 hr. At the end of the incubation period, the supernatant were removed and 150 µL of pure DMSO was added to each well. After 15 min. of shaking the readings were recorded as absorbance at 544 nm on a micro plate reader. Fifty percent cytotoxic concentration (CC_{50}) values were estimated as described by Huber and Koella. [4] The selectivity index (SI) for each compound was calculated as ratio between, cytotoxicity (CC_{50}) and activity (IC_{50}) against *Leishmania* amastigotes.

***In vivo* trial in *L. donovani* / hamster model:**

The *in vivo* antileishmanial activity was determined in golden hamsters (*Mesocricetus auratus*) infected with MHOM/IN/80/Dd8 strain of *L. donovani*. The method as described by Gupta *et al*[5] was used for *in vivo* evaluation. Golden hamsters (Inbred strain) of either sex weighing 40–45 g were infected intracardiacally with 1 x 10^7 amastigotes per animal. After establishment of infection in 15–20 days, pre-treatment spleen biopsies in all the animals were carried out to assess the degree of infection. The animals with +1 infection (5–10 amastigotes/ 100 spleen cell nuclei) were included in the chemotherapeutic trials. Five to six infected animals were randomized into several groups used for each test sample. Drug treatment by intraperitoneal

(IP) route or oral was initiated after 2 days of biopsy and continued for 5 consecutive days. SSG, pentamidine and miltefosine are used as reference drugs. Post-treatment biopsies were done on day 7 of the last dose administration and amastigote counts are assessed by Giemsa staining. Intensity of infection in both, treated and untreated animals, and also the initial count in treated animals was compared and the efficacy was expressed in terms of percentage inhibition (PI) using the following formula:-

PI = 100 – [(ANAT x 100)/ (INAT x TIUC)]

Where PI is Per cent Inhibition of amastigotes multiplication, ANAT is Actual Number of Amastigotes in Treated animals, INAT is Initial Number of Amastigotes in Treated animals and TIUC is Time Increase of parasites in Untreated Control animals.

***In vitro* cytokine production assay:**

The level of various cytokines in the murine macrophages was measured using a sandwich ELISA kit (OptEIA, BD Biosciences, CA, and USA). The assay was performed as per manufacturer's instructions. Briefly, 1 x 10^6 murine macrophages (J–774A.1) infected with *Leishmania* parasite (1:10) were incubated with compound **14** and standard drug, miltefosine at IC_{50} concentration in 6-well plates. Supernatants were collected after 24 hr and 48 hr for cytokines (IL–12, TNF–α, IL–10 and TGF–β) determination. The absorbance of the test samples was measured at 450 nm in an ELISA reader.

***In vitro* nitric oxide (NO) production assay:**

NO was quantified by the accumulation of nitrite in macrophage culture supernatants and nitrite was detected by the Griess reaction as described by Kar *et al.*[6] Briefly, macrophages (1x 10^6/mL) infected with *Leishmania* parasite (1:10 ratio) were incubated with compound **14** and miltefosine for 24 hr and 48 hr before nitrite assay. Supernatants (500 µL) were collected after 24 hr and 48 hr, mixed with an equal volume of Griess reagent (Sigma, USA), and left for 10

min at room temperature. The absorbance of the test samples was measured at 540 nm in an ELISA reader.

Experimental section for docking analysis

Materials and Method:

Molecular docking study specifies the binding site and residues for biological activity of smost potent and less potent synthesized molecules. Autodock 4 tool [7] is used to identify the probable site of binding to the enzyme for inhibitory activity of our molecules. We utilized protein data bank to collect crystal protein with ligand (**PDB ID**. 1E7W) to perform molecular docking studies.[8] Since there is no PDB is available for standard drug Pentamidine, we used to dock pentamidine at first with ligand to get a docked conformation of that molecule with crystal protein. Before docking study, structure preparation, minimization and alignment studies were done with ligand in the SYBYL program package 7.3 on silicon graphics fuel workstation with IRIX 6.5 operating system.[9] The molecule/ligand and protein structure were prepared by geometry optimization using a combination of the standard Tripos molecular mechanics force field of the SYBYL molecular modeling package with Powell energy minimization algorithm, Gasteiger-Huckel charges, and 0.001 kcal/(mol.Å) energy gradient convergence criterion. Alignment did with ligand through 'Fit Atom' method. We executed the same experiment for our most and less potent molecules by taking docked conformation of pentamidine as a template to see how they differ from each other based on their binding affinity with pocket residues of pteridine reductase (PTR1). During molecular docking studies, we set our parameters by taking 100 docking runs for all molecules including pentamidine. The grid size was set at 60×60×60 distributed around the binding domain with a default grid spacing of 0.375 A˚. The Lamarckian genetic algorithm was used for docking the molecules. MOE and Pymol softwares were applied to scrutinize the protein-ligand complex with their feasible 2D and 3D interactions respectively in between.[10, 11]

Table 1: Docking statistics of the compounds **14**, **17** and pentamidine with their possible hydrophobic interactions

entry	IC_{50}(μM)	binding energy (-kcal/mol)	possible hydrophobic interactions
14	1.99	8.54	Tyr-191,Tyr-194, Phe-113, Tyr-114
17	38.23	6.05	Phe-113, Asp-231, Met-233, Tyr-114, Tyr-194
Pentamidine	13.68	6.95	Tyr-191, Ser-111, Phe-113, Tyr-114, Tyr-194

Statistical analysis: Results were expressed as mean ± SD from two independent experiments. The results were analyzed by one – way ANOVA followed by student's t test using Graph Pad Prism (version 5.0) software.

REFERENCES

1. Gupta, L.; Talwar, A.; Shakya N.; Palne, S.; Gupta, S.; Chauhan, P. M. S. Synthesis of marine alkaloid: 8, 9-Dihydrocoscinamide B and its analogues as Novel class of antileishmanial agents. *Bioorg. Med. Chem. Lett*. **2007**, *17*, 4075–4079.
2. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.
3. Sharma, M.; Chauhan, K.; Shivahare, R.; Vishwakarma, P.; Suthar, M. K.; Sharma, A.; Gupta, S.; Saxena, J. K.; Lal, J.; Chandra, P.; Kumar, B.; Chauhan, P. M. S. Discovery of a New Class of Natural Product–Inspired Quinazolinone Hybrid as Potent Antileishmanial agents. *J. Med. Chem.* **2013**, *56*, 4374–4392.

4. Huber, W.; Koella, J. C. A comparison of three methods of estimating EC_{50} in studies of drug resistance of malaria parasites. *Acta Trop.* **1993**, *55*, 257–261.
5. Gupta, S.; Sharma, R.; Srivastava, S. C. V. M. L. Efficacy of picroliv combination with miltefosine, an orally effective antileishmanial drug against experimental visceral leishmaniasis. *Acta Trop.* **2005**, *94*, 41–47.
6. Kar, S.; Sharma G.; Das, P. K. Fucoidan cures infection with both antimony–susceptible and –resistant strains of Leishmania donovani through Th1 response and macrophage-derived oxidants. *J. Antimicrob. Chemother.* **2011**, *66*, 618–625.
7. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. *J. Comput. Chem.* **2009**, *30*, 2785–2789.
8. Gourley, D. G.; Schuttelkopf, A.; Leonard, G.; Luba, J.; Hardy, L.; Beverley, S.; Hunter, W. N. Pteridine reductase mechanism correlates pterin metabolism with drug resistance in trypanosomatid parasites. *Nat. Struct. Biol.* **2001**, *8*, 521–525.
9. *SYBYL*, Version7.3, Tripos Associates: St. Louis, MO, **2006**.
10. MOE: The Molecular Operating Environment from Chemical Computing Group Inc., 1255 University St., Suite 1600, Montreal, Quebec, Canada H3B 3X3. http://www.chemcomp.com.
11. The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrodinger, LLC.