Supporting information

In vitro cytotoxic activities of 2-alkyl-4,6-diheteroalkyl-1,3,5-triazines: new molecules in anticancer research.

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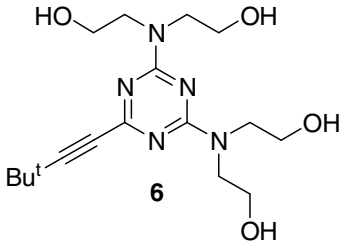
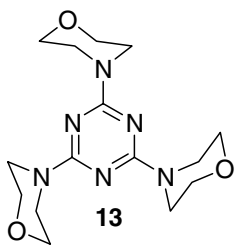
2-(3,3′-dimethylbut-1′-ynyl)-4,6-di(hydroxyethylamino)-1,3,5-triazine (6): A cooled (-20 °C) THF solution of 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) was treated with an ethereal 0.39 M solution (Et_2O/THF 2/3 v/v) of 3,3-dimethylbut-1-ynylmagnesium bromide ([cyanuric chloride]/[RMgBr] = 1/1 molar ratio). The temperature was raised to 5°C, the mixture stirred until the complete conversion of cyanuric chloride into the intermediate 2-(3,3′-dimethylbut-1′-ynyl)-4,6-dichloro-1,3,5-triazine was observed (TLC, glc, gc-mass) and finally treated with a dioxane solution of bis-(2-hydroxyethyl)amine (3 molar equivalents) and diisopropylethylamine (DIPEA, 3 molar equivalents) at room temperature. The mixture was stirred for 2 hours and then additioned of another 2 molar equivalents of the amine and of DIPEA and refluxed until complete conversion (TLC, gc-mass). The solvent was eliminated at reduced pressure and the residue, dissolved in $CHCl_3$, was hydrolyzed by a saturated solution of NH_4Cl. Organic products were repeatedly extracted ($CHCl_3$), the organic phase washed and dried. The solvent was eliminated at reduced pressure and the purification of the crude material by flash chromatography ($CHCl_3$/MeOH 85/15 v/v) afforded pure **6** (84%), showing: m.p.: 227°C; ^{1}H NMR ($CDCl_3$, 50°C): 1.31 (s, 9H, $C(CH_3)_3$); 3.74-3.81 (m, 16H, -C<u>H</u>$_2$-C<u>H</u>$_2$-OH); 4.40 (s, 4H, O<u>H</u>); ^{13}C NMR: 27.7, 30.3, 51.6, 51.9, 60.4, 62.1, 77.8, 98.2, 158.0, 164.6; $[M+H]^+$ = 368; $[M+Na]^+$ = 390. Found: C, 55.54; H, 7.93; N, 19.12. $C_{17}H_{29}N_5O_4$ requires C, 55.57; H, 7.96 ; N, 19.06 %.



6

2,4,6-tris(N-morpholino)-1,3,5-triazine (13): A cooled (0°C) solution of 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) in CH_3CN containing 3,5 molar equivalents of K_2CO_3 and a catalytic amount of 18-crown-6 was treated with a CH_3CN solution of morpholine (3,5 molar equivalents). The temperature was gradually raised and the mixture refluxed (80°C) until the complete disappearance of the cyanuric chloride (TLC, 6h). The solvent was eliminated at reduced pressure and the residue, dissolved in $CHCl_3$, was treated with a 0.6 M solution of HCl. The organic products were repeatedly extracted in $CHCl_3$, the organic phase washed and dried over anhydrous Na_2SO_4. The elimination of solvent at reduced pressure and the purification of crude material by flash chromatography (*n*.hexane/AcOEt/MeOH 70/8/22 v/v/v) afforded pure **13** (75%), showing: m.p.: 266°C; ^{1}H NMR (C_6D_6, 25°C): 3.66 (dd, 12H, J=5.1, J'=4.4 Hz, -OC<u>H</u>$_2$ CH_2); 3.48 (dd, 12H, J=5.0, J'=4.5 Hz, -NC<u>H</u>$_2$ CH_2); ^{13} C NMR: 43.7, 66.9, 165.4; M/e (I%) = 336 ($M^{+\cdot}$, 83.0), 306 (89.6), 279 (96.0), 261 (71.2), 249 (66.7), 221 (52.7), 153 (53.9), 138 (78.8), 94 (85.7), 81 (84.0), 68 (74.7), 55 (45.1), 42 (100). Found: C, 53.54; H, 7.16; N, 25.00. $C_{15}H_{24}N_6O_3$ requires C, 53.56; H, 7.19 ; N, 24.98 %.



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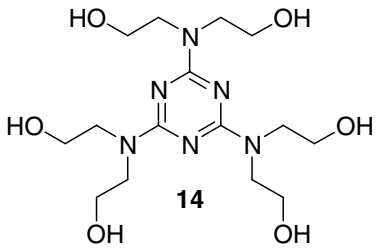
2,4,6-tris[di(2'-hydroxyethyl)amino]-1,3,5-triazine (14): A cooled (0°C) mixture of 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride), $NaHCO_3$ ([cyanuric chloride]/[$NaHCO_3$] = 1/3 molar

ratio) in water was treated with bis-(2-hydroxyethyl)amine ([cyanuric chloride]/[amine] = 1/3 molar ratio) and stirred at 0°C for 1h and at room temperature for 15h; the mixture was diluted with *i*.PrOH (water/*i*.PrOH = 1/1 v/v) and then stirred at 45°C for another 15h. After the complete conversion of the cyanuric chloride into **14** (HPLC: 250x4 mm Lichrocart Chiradex (5µm) column, CH_3CN/H_2O 50/50, 0.8 ml/min, 230nm and mass spectrometry), solvents were eliminated at reduced pressure and the purification of the crude residue by crystallization (*i*.PrOH/$CHCl_3$ 1/9 v/v) afforded pure **14** (30%), showing: m.p.: 175-177°C; ^{13}C NMR (DMSO d_6): 50.7, 59.8, 164.7; $[M+H]^+$ = 391; $[M+Na]^+$ = 413. Found: C, 46.16; H, 7.71; N, 21.50. $C_{15}H_{30}N_6O_6$ requires C, 46.14; H, 7.74 ; N, 21.52 %.



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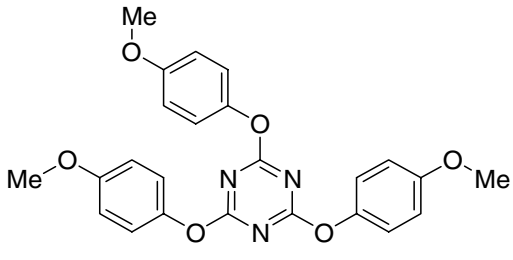
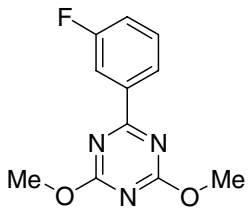
2,4,6-Tris-(4'-methoxyphenoxy)-1,3,5-triazine (16): A solution of 2-chloro-4,6-bis-(4'-methoxyphenoxy)-1,3,5-triazine (see reff. 18, 20) in toluene, containing 1,2 molar equivalents of K_2CO_3 and a catalytic amount of 18-crown-6 was treated with a toluene solution of 4-methoxyphenol (1,2 molar equivalents). The mixture was stirred at room temperature (12h), then refluxed (110°C) until the complete disappearance of the precursor (TLC, 96h) and finally filtered over a short package of celite. The elimination of solvent at reduced pressure afforded pure **16** (98%), showing: m.p.: 190-193°C; ^1H NMR ($CDCl_3$, 25°C): 7.08-7.01, 6.92-6.84 (2m, 12H, H_{Ar}); 3.79 (s, 9H, OCH_3); ^{13}C NMR: 55.6, 114.5, 122.2, 145.2, 157.4, 174.0; M/e (I%) = 324 ($M^{+\cdot}$, 76.2), 152 (21.5), 123 (100), 95 (76.6), 92 (73.4), 77 (65.3), 70 (33.4), 64 (32.6). Found: C, 64.39; H, 4.71; N, 9.42. $C_{24}H_{21}N_3O_6$ requires C, 64.42; H, 4.73; N, 9.39 %.



16

2-(3'-Fluorophenyl)-4,6-dimethoxy-1,3,5-triazine (21): A solution of 3-fluorophenyltributyltin (1.1 molar equivalents) in THF was added, under nitrogen atmosphere, to a solution of 2-chloro-4,6-dimethoxy-1,3,5-triazine (1 molar equivalent) and $PdCl_2(PPh_3)_2$ (0.03 molar equivalents) in THF. The mixture was stirred at room temperature until a complete conversion of the substrate (GC, TLC) was achieved (12 h). After addition of an aqueous saturated solution of NH_4Cl the mixture was extracted with $CHCl_3$ and treated with an aqueous solution of KF (60%) until no more $SnBu_3Cl$ was observed (GC, TLC). The organic layer was then dried over Na_2SO_4 and, after removal of the solvent at reduced pressure (20 mmHg) the crude product was finally purified by flash chromatography (n-hexane/acetone 85/15 v/v) affording pure **21** in 66% yield as a white solid with m.p. 93-95 °C, showing: ^1H NMR ($CDCl_3$, 25°C): 4.13 (s, 6H, OCH_3), 7.25 (tdd, J=8.3 Hz, J'=2.7 Hz, J''=1.0 Hz, 1H, H_{Ar}), 7.45 (td, J=8.1 Hz, J'=5.7 Hz, 1H, H_{Ar}), 8.17 (ddd, J=10 Hz, J'=2.5

Hz, J''=1.6 Hz, 1H, H_{Ar}), 8.28 (dt, J=7.9 Hz, J'=1.1 Hz, 1H, H_{Ar}); ^{13}C NMR: 55.2, 115.7 (d, J_{C-F}=20.0 Hz), 119.6 (d, J_{C-F}=25.0 Hz), 124.6 (d, J_{C-F}=5.0 Hz), 129.9 (d, J_{C-F}=10.0 Hz), 162.9 (d, J_{C-F}=244.0 Hz), 172.9, 173.8; M/e (I%) = 235 ($M^{+\cdot}$, 100), 205 (36), 190 (43), 147 (15), 122 (44), 95 (26), 69 (35). Found: C, 56.21; H, 4.25; F, 8.11; N, 17.84. $C_{11}H_{10}FN_6O_3$ requires C, 56.17; H, 4.29; F, 8.08; N, 17.86 %.



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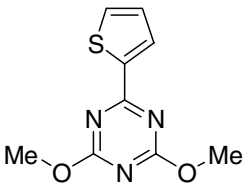
2-(2'-thienyl)-4,6-dimethoxy-1,3,5-triazine (22): A solution of 2-thienyltributyltin (1.1 molar equivalents) in THF was added, under nitrogen atmosphere, to a solution of 2-chloro-4,6-dimethoxy-1,3,5-triazine (1 molar equivalent) and $PdCl_2(PPh_3)_2$ (0.03 molar equivalents) in THF. The mixture was stirred at reflux of the solvent until a complete conversion of the substrate (GC, TLC) was achieved (12 h). After addition of an aqueous saturated solution of NH_4Cl the mixture was extracted with $CHCl_3$ and treated with an aqueous solution of KF (60%) until a complete disappearance of $SnBu_3Cl$ was achieved (GC, TLC). The organic layer was then dried over Na_2SO_4 and, after removal of the solvent at reduced pressure (20 mmHg) the crude product was finally purified by flash chromatography (n-hexane/acetone 75/25 v/v) affording pure **22** in 71% yield as a white solid with m.p. 85-87 °C, showing: ^{1}H NMR ($CDCl_3$, 25°C): 4.07 (s, 6H, OMe), 7.13 (t, J=4.9 Hz, 1H, H_{Ar}), 7.57 (dd, J=5.1 Hz, J'=0.8 Hz, 1H, H_{Ar}), 8.12 (dd, J=3.7 Hz, J'=0.8 Hz, 1H, H_{Ar}); ^{13}C NMR: 54.8, 128.0, 131.6, 132.2, 140.4, 170.3, 172.3; M/e (I%) = 223 ($M^{+\cdot}$, 100), 193 (30), 178 (14), 152 (31), 135 (13), 110 (48), 69 (23). Found: C, 48.44; H, 4.09; N, 18.78; S, 14.33. $C_9H_9N_3O_2S$ requires C, 48.42; H, 4.06; N, 18.82; S, 14.36 %.



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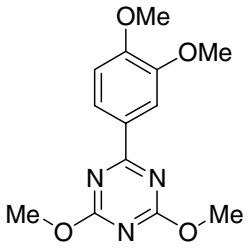
2-(3',4'-Dimethoxyphenyl)-4,6-dimethoxy-1,3,5-triazine (23): A solution of 3,4-dimethoxyphenyltributyltin (1.1 molar equivalents) in THF was added, under nitrogen atmosphere, to a solution of 2-chloro-4,6-dimethoxy-1,3,5-triazine (1 molar equivalent) and $PdCl_2(PPh_3)_2$ (0.03 molar equivalents) in THF. The mixture was stirred at reflux of the solvent until a complete conversion of the substrate (GC, TLC) was achieved (15 h). After addition of an aqueous saturated solution of NH_4Cl the mixture was extracted with $CHCl_3$ and treated with an aqueous solution of KF (60%) until a complete disappearance of $SnBu_3Cl$ was achieved (GC, TLC). The organic layer was then dried over Na_2SO_4 and, after removal of the solvent at reduced pressure (20 mmHg) the crude product was finally purified by flash chromatography ($CHCl_3$/petroleum spirit 75/25 v/v) affording pure **23** in 86% yield as a white solid with m.p. 124-125 °C, showing: ^{1}H NMR ($CDCl_3$,

25°C): 4.04 (s, 3H, Ph-OCH_3), 4.07 (s, 3H, Ph-OCH_3), 4.2 (s, 6H, OMe), 7.03 (d, J=8.4 Hz, 1H, H_{Ar}), 8.1 (d, J=1.83 Hz, 1H, H_{Ar}), 8.26 (dd, J=8.4 Hz, J'=1.83 Hz, 1H, H_{Ar}); ^{13}C NMR: 55.3, 56.3, 110.9, 111.7, 123.4, 128.0, 149.2, 153.5, 173.0, 174.7; M/e (I%) = 278 (15), 277 ($M^{+\cdot}$,100), 276 (53), 262 (20), 248 (7), 247 (11), 246 (21), 234 (12), 232 (13), 231 (14), 219 (5), 218 (6), 217 (8), 216 (5), 191 (11), 176 (6), 164 (7), 162 (6), 92 (5), 77 (6), 72 (10), 69 (7). Found: C, 56.33; H, 5.42; N, 15.12. $C_{13}H_{15}N_3O_4$ requires C, 56.31; H, 5.45 ; N, 15.15 %.



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Preparation of organotin compounds

In a typical procedure, in a round-bottomed flask equipped with a reflux condenser, a mechanical stirrer and a dropping funnel was placed a THF diluted solution of the appropriate Grignard reagent, under nitrogen atmosphere. Chlorotributylstannane (0.8 molar equivalent) in THF was then slowly added and the reaction mixture was refluxed until complete conversion of the chlorotributylstannane was achieved (GC-MS). The mixture was hydrolysed (saturated NH_4Cl_{aq}) extracted with diethyl ether and, after removal of the solvent at reduced pressure (20 mmHg) the crude product was purified by distillation: the recovered organotin compounds showed the following boiling points:

Tributyl(3-fluorophenyl)stannane: 176-178°C/15 mmHg
Tributyl(3,4-dimethoxyphenyl)stannane: 155°C/0.025mmHg
Tributyl(thiophen-2-yl)stannane: 125-126°C/0.35mmHg

Experimental procedures for the biological tests on HL60, L1210 and C6 cell lines

All cell lines were grown up in continuous culture; the L1210 murine leukemia cell line was grown up in RPMI 1640 medium with 10% horse serum, 2 mM glutamine, and antibiotics; the C6 rat glioma cell line was grown up in 50% minimal essential medium (MEM) with the addition of 10% fetal bovine serum, 2 mM glutamine, 0.45% glucose, and antibiotics; the HL60 human leukemia cell line was grown up in RPMI 1640 medium with 10% fetal bovine serum, 2 mM glutamine, and antibiotics.

All cell lines, except for the C6 cell line, grow as suspension cultures. Prior to seeding onto 96 well tissue culture plates (Nunc, Gibco Life Technologies, Paisley, Scotland), suspension cultures were reduced to a single cell suspension. The C6 cell lines were harvested by trypsinization (0.02% EDTA/0.05% trypsin; Sigma-Aldrich®) and then reduced to a single cell suspension.

Cells were plated at $2x10^4$ cells per well in a 100 µl aliquot. Weighed amounts of 1,3,5-triazine derivatives **5**-**16** (Figure 2) were dissolved in dimethylsulphoxide and stored as frozen solutions that were thawed immediately before use and diluted in tissue culture medium. Cells were drug-treated in continual exposure.

The 96 well plates were left for the required time (48 and 72h) at 37 °C and 5% CO_2 in a humidifying incubator. After the incubation period, 10 µl of a MTT (Sigma-Aldrich®) solution (5 mg/ml in phosphate buffered saline) was added to each well and plates were further on (4 h) incubated at 37 °C.

After the formation of the formazan crystals, the suspension cultures were centrifuged (5 min at 300 r/min) and the supernatant liquid was removed by aspiration. The crystalline formazan recovered was dissolved in 100 µl of 0.04 N HCl in *i*.propanol and gently stirred for 10 min.

Absorbances, recorded on a multititers plate reader (BioRad® laboratories) at 570 nm (reference wavelength 630 nm) were expressed as a fraction of those obtained for the control untreated wells. In all experiments three replicate wells were used for each drug concentration. Each assay was carried out at least three times.