Supplementary information

Rubrolide analogues and their derived lactams as potential anticancer agents

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1. Materials and instrumentation

Reagents and solvents were purified, when necessary, according to procedures described by Perrin and Armarego.\textsuperscript{1} The 3,4-dichlorofuran-2(5\textit{H})-ona \textsuperscript{9} was obtained by sodium borohydride reduction of mucocloric acid (\textsuperscript{8}) commercially available (Aldrich e Milwaukee, USA) according to a procedure described in the literature.\textsuperscript{2} It is important to mention that compound \textsuperscript{8} can alternatively be prepared from furfural as previously described.\textsuperscript{3} All reactions were carried out under a protective atmosphere of dry nitrogen. The \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Varian Mercury 300 instrument (300 MHz and 75 MHz,
respectively), using deuterated chloroform and acetone as a solvent and tetramethylsilane (TMS) as internal standard (δ = 0). Infrared spectra were recorded on a Varian 660-IR, equipped with GladiATR scanning from 4000 to 500 cm⁻¹. Mass spectra were recorded on a Shimadzu GCMS-QP5050A instrument under electron impact (70 eV) conditions. Melting points are uncorrected and were obtained from MQAPF-301 melting point apparatus (Microquimica, Brazil). High resolution mass spectra were recorded on a Bruker MicroToF (resolution = 10,000 FWHM) under electrospray ionization (ESI) and are given to four decimal places. Analytical thin layer chromatography analysis was conducted on aluminum packed precoated silica gel plates. Column chromatography was performed over silica gel (60-230 mesh).

2. Synthesis

2.1. General procedure for the synthesis of 10a-10h

2.1.1. 5(Z)-3,4-dichloro-5-(4-bromobenzylidene)furan-2(5H)-one (10a). To a two-neck round-bottom flask under nitrogen atmosphere were added 3,4-diclorofuran-2(5H)-one 9 (660 mg, 4.32 mmol), dichloromethane (18 mL), TBDMSOTf (1.39 mL, 6.04 mmol), DIPEA (1.51 mL, 8.63 mmol), and 4-bromobenzaldehyde (1.12 g, 6.04 mmol). The resulting mixture was stirred at room temperature over 1 h. After DBU (1.29 mL, 8.63 mmol) was added, the reaction mixture was refluxed for an additional 3 h before the addition of dichloromethane (120 mL). The resulting organic layer was washed with aqueous HCl 3 mol L⁻¹ solution (2 x 50 mL) and brine (2 x 40 mL). After separation, the organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. The resulting material was purified by column chromatography on silica gel eluted with hexane/dichloromethane (2:1 v/v) to afford compound 10a as yellow solid in 16% yield (225 mg, 0.70 mmol). Mp: 124.2-125.8 ºC. Rf = 0.45 (hexane:dichloromethane, 2:1, v/v). IR (ATR) νmax 3046, 1774, 1648, 1586, 1573, 1487, 1407, 1312, 1224, 1183, 1073, 998, 981, 877, 838, 812, 740, 526 cm⁻¹. ¹H NMR (300 MHz, CDCl₃) δ 6.33 (s, 1H, H-6); 7.54 (dcomp, 2H, J₃',₂' = J₅',₆' = 8.7; H-3'/H-5'); 7.64 (dcomp, 2H, J₂',₃' = J₆',₅' = 8.7; H-2'/H-6'). ¹³C NMR (75 MHz, CDCl₃) δ 111.3 (C-6); 119.8 (C-3); 124.7 (C-4'); 130.4 (C-1'); 132.3 (C-2'/C-3'/C-5'/C-6'); 142.6 (C-5); 143.6 (C-4); 161.9 (C-2). MS, m/z (%) 324 ([M+6]+, 6); 322 ([M+4]+, 42); 320 ([M+2]+, 91); 318 (C₁₁H₉Cl₂O₂Br, [M]+, 57); 183 (27); 89 (100); 63 (39); 39 (28).
2.1.2. 5(Z)-3,4-dichloro-5-(4-fluorobenzylidene)furan-2(5H)-one (10b). Compound 10b was synthesized using a method similar to that of 10a and was isolated as white solid in 15% yield; purified by column chromatography, eluent hexane/dichloromethane (3:1 v/v). Mp: 157.5-158.8 °C. R_f = 0.32 (hexane:dichloromethane, 3:1, v/v). IR (ATR) ν_max 3062, 1760, 1652, 1571, 1509, 1184, 1162, 1009, 982, 896, 821, 741, 530 cm⁻¹. ¹H NMR (300 MHz, CDCl₃) δ 6.37 (s, 1H, H-6); 7.12 (t, J_3',2' = J_5',6' = 8.7; H-3'/H-5'); 7.75-7.83 (m, 2H, H-2'/H-6'). ¹³C NMR (75 MHz, CDCl₃) δ 111.2 (C-6); 116.1 (d, J_C-F = 21.8; C-3'/C-5'); 119.3 (C-3); 128.7 (d, J_C-F = 3.0; C-1'); 132.9 (d, J_C-F = 9.0; C-2'/C-6'); 142.5 (C-5); 142.8 (C-4); 161.7 (C-2); 163.5 (d, J_C-F = 233.3; C-4'). MS, m/z (%) 262 ([M+4]^+, 8); 260 ([M+2]^+, 49); 258 (C₁₁H₅Cl₂O₂F, [M]^+, 76); 169 (17); 167 (54); 136 (68); 108 (100); 107 (57); 87 (17); 57 (22).

2.1.3. 5(Z)-3,4-dichloro-5-(4-trifluoromethylbenzylidene)furan-2(5H)-one (10c). Compound 10c was synthesized using a method similar to that of 10a and was isolated as green solid in 10% yield; purified by column chromatography, eluent hexane/dichloromethane (2:1 v/v). Mp: 101.9-103.2 °C. R_f = 0.40 (hexane:dichloromethane, 2:1, v/v). IR (ATR) ν_max 3077, 1791, 1576, 1417, 1322, 1223, 1159, 1098, 1063, 872, 821, 741, 592 cm⁻¹. ¹H NMR (300 MHz, CDCl₃) δ 6.42 (s, 1H, H-6); 7.67 (d, J_3',2' = J_5',6' = 8.2; H-3'/H-5'); 7.89 (d, J_2',3' = J_6',5' = 8.2; H-2'/H-6'). ¹³C NMR (75 MHz, CDCl₃) δ 110.7 (C-6); 120.9 (C-3); 124.0 (q, J_CF₃ = 271; CF₃); 126.1 (q, J_C-F = 3.8; C-3'/C-5'); 131.2 (C-2'/C-6'); 132.1 (q, J_C-F = 32.3; C-4'); 135.0 (C-1'); 142.8 (C-5); 144.9 (C-4); 161.9 (C-2). MS, m/z (%) 312 ([M+4]^+, 10); 310 ([M+2]^+, 67); 308 (C₁₂H₅Cl₂O₂F₃, [M]^+, 60); 273 (13); 245 (25); 219 (18); 217 (55); 186 (85); 158 (100); 108 (18); 89 (30); 87 (28); 63 (26); 39 (15).

2.1.4. 5(Z)-3,4-dichloro-5-(4-nitrobenzylidene)furan-2(5H)-one (10d). Compound 10d was synthesized using a method similar to that of 10a and was isolated as yellow solid in 10% yield; purified by column chromatography, eluent hexane/dichloromethane (3:1 v/v). Mp: 184.2-185.8 °C. R_f = 0.28 (hexane:dichloromethane, 3:1, v/v). IR (ATR) ν_max 3108, 3051, 1797, 1649, 1577, 1508, 1337, 1222, 1187, 1110, 980, 875, 826, 687 cm⁻¹. ¹H NMR (300 MHz, CDCl₃) δ 6.45 (s, 1H, H-6); 7.90 (d, J_comp., 2H, J₃',2' = J₅',6' = 9.0; H-3'/H-5'); 8.27 (d, J_comp., 2H, J₂',₃' = J₅',₆' = 9.0; H-2'/H-6'). ¹³C NMR (75 MHz, CDCl₃) δ 109.2 (C-6); 121.3 (C-3); 123.9 (C-2'/C-6'); 137.3 (C-1'); 142.4 (C-5); 145.3 (C-4/C-4'); 161.2 (C-2). MS, m/z (%) 289 ([M+4]^+, 11); 287 ([M+2]^+, 66); 285 (C₁₁H₅Cl₂NO₃, [M]^+,
2.1.5. 5(Z)-3,4-dichloro-5-(4-chlorobenzylidene)furan-2(5H)-one (10e). Compound 10e was synthesized using a method similar to that of 10a and was isolated as yellow solid in 10% yield; purified by column chromatography, eluent hexane/dichloromethane (3:1 v/v). Mp: 129.9-131.1 °C. R\textsubscript{f} = 0.33 (hexane:dichloromethane, 3:1, v/v). IR (ATR) \nu\textsubscript{max} 3068, 1775, 1649, 1577, 1484, 1408, 1225, 1088, 1013, 982, 873, 837, 740, 604, 529 cm\textsuperscript{-1}. \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \delta 6.39 (s, 1H, H-6); 7.39 (d\textsubscript{comp.}, 2H, J\textsubscript{3',2'} = J\textsubscript{5',6'} = 8.7; H-3'/H-5'); 7.71 (d\textsubscript{comp.}, 2H, J\textsubscript{2',3'} = J\textsubscript{6',5'} = 8.7; H-2'/H-6'). \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \delta 111.3 (C-6); 119.7 (C-3); 129.3 (C-3'/C-5'); 129.9 (C-1'); 132.1 (C-2'/C-6'); 136.2 (C-4'); 142.6 (C-5); 143.5 (C-4); 161.9 (C-2). MS, m/z (%) 278 ([M+4]\textsuperscript{+}, 15); 276 ([M+2]\textsuperscript{+}, 58); 274 (C\textsubscript{11}H\textsubscript{5}Cl\textsubscript{3}O\textsubscript{2}, [M]\textsuperscript{+}, 45); 239 (11); 185 (18); 183 (32); 152 (52); 124 (38); 89 (100); 87 (19); 63 (44); 39 (23).

2.1.6. 5(Z)-3,4-dichloro-5-(3-chlorobenzylidene)furan-2(5H)-one (10f). Compound 10f was synthesized using a method similar to that of 10a and was isolated as yellow solid in 35% yield; purified by column chromatography, eluent hexane/dichloromethane (3:1 v/v). Mp: 136.2-137.4 °C. R\textsubscript{f} = 0.84 (hexane:dichloromethane, 3:1, v/v). IR (ATR) \nu\textsubscript{max} 3066, 1767, 1649, 1567, 1473, 1431, 1230, 1189, 982, 886, 778, 727, 678 cm\textsuperscript{-1}. \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \delta 6.32 (s, 1H, H-6); 7.36-7.34 (m, 2H, H-4'/H-6'); 7.66-7.63 (m, 1H, H-5'); 7.76 (s, 1H, H-2'). \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \delta 111.0 (C-6); 120.3 (C-3); 130.3 (C-5'); 130.1 (C-4'); 130.6 (C-2'); 133.2 (C-1')*; 135.1 (C-3')*; 142.7 (C-5); 144.0 (C-4); 161.9 (C-2). *These assignments could be reversed. MS, m/z (%) 280 ([M+6]\textsuperscript{+}, 2); 278 ([M+4]\textsuperscript{+}, 19); 276 ([M+2]\textsuperscript{+}, 63); 274 (C\textsubscript{11}H\textsubscript{5}Cl\textsubscript{3}O\textsubscript{2}, [M]\textsuperscript{+}, 43); 239 (27); 185 (31); 183 (51); 152 (54); 124 (35); 89 (100); 87 (22); 63 (46); 39 (17).

2.1.7. 5(Z)-3,4-dichloro-5-(2-bromobenzylidene)furan-2(5H)-one (10g). Compound 10g was synthesized using a method similar to that of 10a and was isolated as yellow solid in 27% yield; purified by column chromatography, eluent hexane/dichloromethane (3:1 v/v). Mp: 126.5-128.1 °C. R\textsubscript{f} = 0.50 (hexane:ethyl acetate, 10:1, v/v). IR (ATR) \nu\textsubscript{max} 3057, 1779, 1644, 1574, 1461, 1432, 1222, 1189, 979, 750 cm\textsuperscript{-1}. \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \delta 6.88 (s, 1H, H-6); 7.23 (t, 1H, J\textsubscript{5',4'} = J\textsubscript{5',6'} = 7.8; H-5'); 7.38 (t, 1H, J\textsubscript{4',3'} = J\textsubscript{4',5'} = 7.8; H-4'); 7.64 (d, 1H, J\textsubscript{6',5'} = 7.8; H-6'); 8.12 (d, 1H, J\textsubscript{3',4'} = 7.8; H-3'). \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \delta 110.5 (C-6);
120.0 (C-3); 125.6 (C-2'); 127.8 (C-4'); 130.9 (C-5'); 131.0 (C-1'); 131.9 (C-3'); 133.1 (C-6'); 142.8 (C-5); 144.0 (C-4); 161.7 (C-2). MS, m/z (%) 324 ([M+6]⁺, 3); 322 ([M+4]⁺, 18); 320 ([M+2]⁺, 42); 318 (C₁₁H₂Cl₂BrO₂, [M]⁺, 24); 241 (37); 239 (57); 198 (16); 196 (15); 183 (21); 176 (19); 113 (17); 87 (17); 63 (48); 62 (23); 39 (21).

2.1.8. 5(Z)-3,4-dichloro-5-(3,4-metilenedioxibenzylidene)furan-2(5H)-one (10h). Compound 10h was synthesized using a method similar to that of 10a and was isolated as yellow solid in 58% yield; purified by column chromatography, eluent hexane/dichloromethane (1:1 v/v). Mp. 158.7-160.0 ºC. R<sub>f</sub> = 0.32 (hexane: dichloromethane, 10:1, v/v). IR (ATR) ν<sub>max</sub> 3074, 3012, 2906, 1762, 1649, 1567, 1493, 1451, 1251, 1103, 1013, 980, 889, 801, 739, 715, 611 cm<sup>-1</sup>. ¹H NMR (300 MHz, CDCl₃) δ 6.03 (s, 2H, -O-CH₂-O-); 6.30 (s, 1H, H-6); 6.84 (d, 1H, J<sub>5',6'</sub> = 8.1; H-5'); 7.19 (dd, 1H, J<sub>6',5'</sub> = 8.1; J<sub>6',2'</sub> = 1.6; H-6'); 7.42 (d, 1H; J<sub>2',6'</sub> = 1.6; H-2'). ¹³C NMR (75 MHz, CDCl₃) δ 101.7 (-O-CH₂-O-); 108.8 (C-5'); 110.1 (C-2'); 112.8 (C-6); 118.3, (C-3); 125.9 (C-1'); 127.2 (C-6'); 141.9 (C-5); 142.5 (C-4); 148.5 (C-4'); 149.5 (C-3'); 162.3 (C-2). MS, m/z (%) 288 ([M+4]⁺, 1); 286 ([M+2]⁺, 33); 284 (C₁₂H₆Cl₂O₄, [M]⁺, 65); 207 (19); 193 (52); 162 (30); 134 (53); 76 (100); 51 (15); 50 (92); 38 (23).

2.2. General procedure for the synthesis of 11a-11b

2.2.1. 5(Z)-3,4-dichloro-5-(4-bromobenzylidene)-5-hydroxy-1-isobutyl-pyrrol-2(5H)-one (11a). To a two-neck round bottom flask were added 5(Z)-3,4-dichloro-5-(4-bromobenzylidene)furan-2(5H)-one (10a) (312 mg, 0.98 mmol) dissolved in dichloromethane (5 mL) and the solution was cooled to 0 ºC. Then the isobutylamine (357 mg, 4.88 mmol) dissolved in dichloromethane was added in a dropwise fashion and the resulting mixture was stirred at 0 ºC for 3 h before the addition of dichloromethane (100 mL). After this time, the mixture was quenched with HCl aqueous solution (2 mol L<sup>-1</sup>, 2 x 30 mL), and washed with saturated NaHCO₃ solution (2 x 30 mL) and brine (2 x 30 mL). After separation, the organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. The resulting material was purified by column chromatography on silica gel eluted with hexane/ethyl acetate (3:1 v/v). The procedure described afforded compound 11a as white solid in 70% yield (267 mg, 0.68 mmol). Mp. 216.3-218.1 ºC. R<sub>f</sub> = 0.41 (hexane:ethyl acetate, 3:1, v/v). IR (ATR) ν<sub>max</sub> 3306, 2969, 2870, 1695, 1488, 1403, 1801, 1173, 1042, 721 cm<sup>-1</sup>. ¹H NMR (300 MHz, (CD₃)₂CO) δ 0.89 (d, 3H, J<sub>9,8</sub> = 6.6; H-9); 0.93 (d, 3H, J<sub>10,8</sub> = 6.6; H-10); 2.16-2.30 (m, 1H, H-8); 3.23 (d, 1H, J<sub>6a,6b</sub> = 14.1; H-6a); 6.61 (dd, 1H; J<sub>7a,7b</sub> = 14.1; J<sub>7a,k</sub> =
6.6; H-7a); 3.44 (d, 1H, $J_{6b,6a} = 14.1$; H-6b); 3.46 (dd, 1H, $J_{7b,7a} = 14.1$; $J_{7b,8} = 8.4$; H-7b); 6.18 (s, 1H, -OH); 7.10 (d$_{comp}$, 2H, $J_{3',2'} = J_{5',6'} = 8.5$; H-3'/H-5'); 7.44 (d$_{comp}$, 2H, $J_{2',3'} = J_{6',5'} = 8.5$; H-2'/H-6'). $^1$C NMR (75 MHz, (CD$_3$)$_2$CO)) $\delta$ 20.1 (C-9)*; 20.2 (C-10)*; 28.3 (C-8); 39.6 (C-7); 48.1 (C-6); 91.9 (C-5); 121.1 (C-1'); 126.1 (C-3); 131.5 (C-2'/C-6'); 131.8 (C-3'/C-5'); 133.4 (C-4'); 145.0 (C-4); 161.6 (C-2). *These assignments could be reversed.

MS, m/z (%) 224 (48); 222 (71); 172 (27); 170 (34); 168 (44); 166 (63); 90 (25); 89 (27); 57 (100); 41 (41).

2.2. 5(Z)-3,4-dichloro-5-(4-fluorobenzylidene)-5-hydroxy-1-propyl-pyrrol-2(5H)-one (11b). Compound 11b was synthesized using a method similar to that of 11a and was isolated as white solid in 84% yield, purified by column chromatography, eluent hexane/ethyl acetate (2:1 v/v). Mp. 217.5-219.1 °C. R$_f$ = 0.40 (hexane:ethyl acetate, 2:1, v/v). IR (ATR) $\nu_{max}$ 3290, 2940, 2878, 1685, 1631, 1511, 1415, 1220, 1041, 849, 658 cm$^{-1}$. $^1$H NMR (300 MHz, (CD$_3$)$_2$CO)) $\delta$ 0.92 (t, 3H, $J_{9,8} = 7.2$; H-9); 1.66-1.80 (m, 2H, H-8); 3.23 (d, 1H, $J_{6a,6b} = 14.1$; H-6a); 3.41 (d, 1H, $J_{6b,6a} = 14.1$; H-6b); 3.41 (ddd, 1H, $J_{7a,7b} = 13.9$, $J_{7a,8a} = 9.2$, $J_{7a,8b} = 6.6$; H-7a); 3.56 (ddd, 1H, $J_{7b,7a} = 13.9$, $J_{7b,8b} = 9.2$, $J_{7b,8a} = 6.6$; H-7b); 6.00 (s, 1H, -OH); 7.02 (t$_{comp}$, 2H, $J_{3',2'} = J_{5',6'} = J_{5',F} = 8.9$; H-3'/H-5'); 7.14-7.21 (m, 2H, H-2'/H-6'). $^1$C NMR (75 MHz, (CD$_3$)$_2$CO)) $\delta$ 11.2 (C-9); 22.5 (C-8); 39.3 (C-7); 42.4 (C-6); 92.0 (C-5); 115.1 (d, $J_{C,F} = 21.2$; C-3'/C-5'); 126.1 (C-3); 130.2 (d, $J_{C,F} = 3.5$; C-1'); 131.6 (d, $J_{C,F} = 8.1$; C-2'/C-6'); 145.1 (C-4); 160.6 (C-2); 162.6 (d, $J_{C,F} = 186.5$; C-4'). MS, m/z (%) 210 (61); 208 (91); 168 (68); 166 (96); 148 (21); 110 (93); 109 (100); 87 (20); 83 (37); 43 (44); 41 (36).

2.3. General procedure for the synthesis of 12a-12b and 13a-13b

2.3.1. Synthesis of 5(Z)-3,4-dichloro-5-(4-bromobenzylidene)-1-isobutyl-pyrrol-2(5H)-one (12a) and 5(E)-3,4-dichloro-5-(4-bromobenzylidene)-1-isobutyl-pyrrol-2(5H)-one (13a). To a round-bottom flask under nitrogen atmosphere were added 5(Z)-3,4-dichloro-5-(4-bromobenzylidene)-5-hydroxy-1-isobutyl-pyrrol-2(5H)-one (11a) (100 mg, 0.26 mmol) dissolved in dry CHCl$_3$ (7 mL) and p-toluenesulfonic acid (24 mg, 0.13 mmol). After reflux for 2 h, the reaction was diluted with additional chloroform (50 mL) and quenched with saturated aqueous NaHCO$_3$ solution (3 x 15 mL) and washed with brine (3 x 15 mL). The organic layer was dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The resulting material was purified by column chromatography on silica gel eluted with
hexane/ethyl acetate (6:1 v/v). The procedure described afforded compound 12a in 56% yield (54 mg, 0.14 mmol) and 13a in 36% yield (34 mg, 0.09 mmol).

Data for 12a: amorphous yellow solid. Mp. 95.5-97.1 °C. R<sub>f</sub> = 0.65 (hexane:ethyl acetate, 6:1, v/v). IR (ATR) ν<sub>max</sub> 2957, 2921, 2851, 1714, 1638, 1592, 1457, 1250, 1064, 736 cm<sup>-1</sup>. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 0.50 (d, 6H, <i>J</i><sub>9,8</sub> = <i>J</i><sub>10,8</sub> = 6.9; H-9/H-10); 1.28-1.41 (m, 1H, H-8); 3.41 (d, 2H, <i>J</i><sub>7,8</sub> = 7.5; H-7); 6.59 (s, 1H, H-6); 7.17 (d<sub>comp.</sub>, 2H, <i>J</i><sub>3',2'</sub> = <i>J</i><sub>5',6'</sub> = 8.4; H-3'/H-5'); 7.55 (d<sub>comp.</sub>, 2H, <i>J</i><sub>2',3'</sub> = 8.4; H-2'/H-6'). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 19.4 (C-9/C-10); 27.6 (C-8); 49.1 (C-7); 122.7 (C-1'); 131.0 (C-3'/C-5'); 131.5 (C-2'/C-6'); 132.3 (C-4'); 134.8 (C-5); 164.4 (C-2). MS, m/z (%) 379 (M+6)*, 3); 377 (M+4)*, 20); 375 (M+2)*, 44); 373 (C<sub>15</sub>H<sub>14</sub>Cl<sub>2</sub>BrNO, M+*, 27); 253 (41); 252 (71); 251 (62); 250 (100); 240 (43); 238 (74); 203 (21); 126 (20); 41 (53); 39 (29).

Data for 13a: amorphous yellow solid. Mp. 94.8-96.5 °C. R<sub>f</sub> = 0.48 (hexane:ethyl acetate, 6:1, v/v). IR (ATR) ν<sub>max</sub> 2959, 2926, 1701, 1575, 1485, 1457, 1318, 1071, 1010, 739 cm<sup>-1</sup>. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 0.97 (d, 6H, <i>J</i><sub>9,8</sub> = <i>J</i><sub>10,8</sub> = 6.6; H-9/H-10); 2.01-2.15 (m, 1H, H-8); 3.57 (d, 2H, <i>J</i><sub>7,8</sub> = 7.5; H-7); 6.53 (s, 1H, H-6); 7.20 (d<sub>comp.</sub>, 2H, <i>J</i><sub>3',2'</sub> = <i>J</i><sub>5',6'</sub> = 8.4; H-3'/H-5'); 7.49 (d<sub>comp.</sub>, 2H, <i>J</i><sub>2',3'</sub> = 8.4; H-2'/H-6'). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 20.3 (C-9/C-10); 28.3 (C-8); 47.8 (C-7); 112.8 (C-3); 131.2 (C-1'); 131.7 (C-4'); 135.3 (C-4); 162.1 (C-2). MS, m/z (%) 379 ([M+6]*, 3); 377 ([M+4]*, 20); 375 ([M+2]*, 44); 373 (C<sub>15</sub>H<sub>14</sub>Cl<sub>2</sub>BrNO, [M]*, 26); 319 (22); 253 (41); 252 (73); 251 (62); 250 (100); 240 (41); 238 (75); 203 (21); 126 (21); 41 (53); 39 (30).

2.3.2. 5(Z)-3,4-dichloro-5-(4-fluorobenzylidene)-1-propyl-pyrrol-2(5H)-one (12b) and 5(E)-3,4-dichloro-5-(4-fluorobenzylidene)-1-propyl-pyrrol-2(5H)-one (13b). Compounds 12b and 13b were synthesized using a method similar to that of 12a and 13a were isolated in 71% and 27% yield, respectively.

Data for 12b: amorphous yellow solid, purified by column chromatography, eluent hexane/ethyl acetate (6:1 v/v). Mp. 96.6-98.1 °C. R<sub>f</sub> = 0.53 (hexane:ethyl acetate, 6:1, v/v). IR (ATR) ν<sub>max</sub> 2957, 2872, 1695, 1591, 1504, 1343, 1217, 1135, 1055, 823, 526 cm<sup>-1</sup>. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 0.49 (t, 3H, <i>J</i><sub>9,8</sub> = 7.5; H-9); 1.17 (sext, 2H, <i>J</i><sub>8,7</sub> = <i>J</i><sub>8,9</sub> = 7.5; H-8); 6.64 (s, 1H, H-6); 7.10 (t<sub>comp.</sub>, 2H, <i>J</i><sub>3',2'</sub> = <i>J</i><sub>5',6'</sub> = <i>J</i><sub>3',F</sub> = <i>J</i><sub>5',F</sub> = 8.6; H-3'/H-5'); 7.27-7.32 (m, 2H, H-2'/H-6'). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 10.8 (C-9);
21.6 (C-8); 43.9 (C-7); 112.9 (C-6); 115.7 (d, J_C-F = 21.8; C-3’/C-5’); 123.9 (C-3); 129.6 (d, J_C-F = 3.5; C-1’); 131.3 (d, J_C-F = 8.0; C-2’/C-6’); 134.8 (C-5); 137.2 (C-4); 162.8 (d, J_C-F = 248.3; C-4’); 164.6 (C-2). MS, m/z (%): 303 ([M+4]+ ▪, 8); 301 ([M+2]+ ▪, 50); 299 (C_{14}H_{12}Cl_{2}FNO, [M]+ ▪, 78); 270 (33); 235 (50); 234 (35); 224 (31); 222 (100); 206 (26); 158 (22); 144 (30); 107 (21); 41 (39).

Data for 13b: amorphous yellow solid, purified by column chromatography, eluent hexane/ethyl acetate (6:1 v/v). Mp. 97.3-99.1 °C. R_f = 0.38 (hexane:ethyl acetate, 6:1, v/v). IR (ATR) ν_{max} 2971, 2917, 2849, 1697, 1574, 1504, 1323, 1221, 1064, 747, 532 cm^{-1}. ^{1}H NMR (300 MHz, CDCl_3) δ 0.97 (t, 3H, J_{9,8} = 7.5; H-9); 1.69 (sext, 2H, J_{8,7} = J_{8,9} = 7.5; H-8); 3.70-3.75 (m, 2H; H-7); 6.60 (s, 1H, H-6); 7.05 (t_{comp}, 2H, J_{3’,2’} = J_{3’,F} = J_{5’,6’} = J_{5’,F} = 8.6; H-3’/H-5’); 7.29-7.34 (m, 2H, H-2’/H-6’). ^{13}C NMR (75 MHz, CDCl_3) δ 11.5 (C-9); 22.2 (C-8); 42.0 (C-7); 115.0 (C-6); 115.4 (d, J_C-F = 21.7; C-3’/C-5’); 128.0 (C-3); 128.8 (d, J_C-F = 3.5; C-1’); 132.1 (d, J_C-F = 8.1; C-2’/C-6’); 132.6 (C-5); 134.7 (C-4); 161.8 (C-2); 163.0 (d, J_C-F = 247.4; C-4’). MS, m/z (%): 303 ([M+4]+ ▪, 8); 301 ([M+2]+ ▪, 47); 299 (C_{14}H_{12}Cl_{2}FNO, [M]+ ▪, 78); 270 (31); 235 (49); 234 (37); 224 (33); 222 (100); 206 (25); 158 (23); 144 (33); 107 (22); 41 (44).

3. Biological assays

3.1. Cytotoxic assay in human cancer cell lines and normal cells

The cytotoxicity was tested against HL-60 (human leukemia), HCT-116 (human colon), SF-295 (human central nervous system) and OVCAR-8 (human ovarian) cancer cell lines and L929 normal cells (mouse fibroblast), obtained from the National Cancer Institute, Bethesda, MD, USA. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% antibiotics and incubated at 37 °C under a 5% CO_{2} atmosphere. The cytotoxicity of the samples was assessed by the MTT method. For experiments, cells were seeded in 96-well plates (0.7 x 10^5 for adherent cells or 0.3 x 10^6 for suspended cells in 100 mL medium). The compounds (0.05 – 25 μg/mL) dissolved in DMSO 5% were added to each well (using the HTS - high-throughput screening - biomek 3000 - Beckman Coulter, Inc. Fullerton, California, USA) and incubated for 3 days (72 h). After 69 h of incubation, the supernatant was replaced by fresh medium containing MTT (0.5 mg/mL). Three hours later, the MTT formazan product was dissolved in 150 μL of DMSO, and absorbance was measured.
at 595 nm (DTX 880 Multimode Detector, Beckman Coulter, Inc. Fullerton, California, USA). Doxorubicin (0.009 to 5 µg/mL) was used as positive control. Control groups received the same amount of DMSO. Doxorubicin was used as positive control.

3.2. Study the type of cell death in human HL-60 cells

3.3.1. Analysis of morphological changes

Untreated and treated HL-60 cells were examined for morphological changes by light microscopy (Olympus, Tokyo, Japan) after 48 h of incubation. To evaluate nuclear and cell morphology, cells were harvested, transferred to cytopsin slides, fixed with methanol for 1 min and stained with May-Grunwald-Giemsa (Bioclin, Brazil).

3.3.2. Morphological analysis with fluorescence microscopy

Cell death pattern was determined by differential staining with acridine orange/ethidium bromide (AO/EB) (Sigma-Aldrich). Briefly, cells were pelleted and resuspended in 25 µL in PBS. Afterwards, 1 µL of aqueous solution of acridine orange/ethidium bromide (AO/EB, 100 µg/mL) was added and the cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan). Three hundred cells were analyzed using a fluorescence microscope with filter for 470/40 nm. The cells were then classified as follows: live cells, apoptotic cells and necrotic cells. The percentage of apoptotic and necrotic cells was then calculated as described.5

3.3.3. Flow Cytometry Analysis

For tested compounds, five thousand events were evaluated per experiment and cellular debris was omitted from the analysis. HL-60 cell fluorescence was then determined by flow cytometry in a Guava EasyCyte Mine using Guava Express Plus software. Five thousand events were analyzed for each replicate in three independent experiments. Internucleosomal DNA fragmentation and cell cycle were analyzed by ModFit LT for Win32 version 3.1.

DNA fragmentation and cell cycle were analyzed by flow cytometry after DNA staining with propidium iodide. Briefly, 100 µL of treated and untreated cells were incubated
for 30 min, in the dark, with hypotonic solution containing 50 µg/mL propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Fluorescence was measured and DNA fragmentation and cell cycle were analyzed.\textsuperscript{6}

PS externalization was analyzed after PS staining with Annexin V according to the method described by Vermes and coworkers.\textsuperscript{7} Guava Nexin Assay Kit was used to determine early apoptosis. Cells were washed twice with cold PBS and then resuspended in 135 µL of PBS with 5 µL of 7-amino-actinomycin D (7AAD) and 10 µL of Annexin V-PE. The cells were gently vortexed and incubated for 20 min at room temperature (20–25 °C) in the dark. Afterwards, the cells were analyzed by flow cytometry (EasyCyte from Guava Technologies). Annexin V is a phospholipid-binding protein that has a high affinity for PS. 7-AAD, a cell impermeant dye, is used as an indicator of membrane structural integrity. Fluorescence of annexin V-PE was measured: yellow fluorescence-583 nm and 7-AAD in red fluorescence-680 nm. The percentage of early and late apoptotic cells and necrotic cells was then calculated.

Mitochondrial depolarization was evaluated by incorporation of Rhodamine 123 (Sigma Aldrich Co. - St. Louis, MO/USA). Rhodamine 123 is a cell-permeable, cationic, fluorescent dye that is readily sequestered by active mitochondria without inducing cytotoxic effects. Briefly, cells were centrifuged at 2000 rpm for 5 min and the pellet was resuspended in 500 µL of 1 µg/mL of rhodamine 123 for 15 min in the dark. After incubation, cells were centrifuged at 2000 rpm for 5 min and the pellet was resuspended in 500 µL in phosphate-buffered saline (PBS) and incubated for 30 min in the dark. Fluorescence was measured and percentage mitochondrial depolarization was analyzed.\textsuperscript{5}

4. Statistical analysis

For cytotoxicity assays, the drug effect was quantified as percentage of the absorbance reduced dye at 595 nm in relation to control wells. Additionally, the \( \text{RC}_{50} \) values ± SEM were obtained by nonlinear regression using the Graphpad program (Intuitive Software for Science, San Diego, CA). Data obtained from the studies of cell death are presented as means ± SEM and evaluated by ANOVA followed by Dunnet’s test.
5. References

6. Spectra of some selected compounds

**Fig. S1** $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 10a.

**Fig. S2** $^{13}$C NMR spectrum (75 MHz, CDCl$_3$) of compound 10a.
**Fig. S3** $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 10b.

**Fig. S4** $^{13}$C NMR spectrum (75 MHz, CDCl$_3$) of compound 10b.
Fig. S5 $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 10c.

Fig. S6 $^{13}$C NMR spectrum (75 MHz, CDCl$_3$) of compound 10c.
**Fig. S7** $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 10d.

**Fig. S8** $^{13}$C NMR spectrum (75 MHz, CDCl$_3$) of compound 10d.
Fig. S9 $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 10e.

Fig. S10 $^{13}$C NMR spectrum (75 MHz, CDCl$_3$) of compound 10e.
**Fig. S11** $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 10f.

**Fig. S12** $^{13}$C NMR spectrum (75 MHz, CDCl$_3$) of compound 10f.
Fig. S13 $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 10g.

Fig. S14 $^{13}$C NMR spectrum (75 MHz, CDCl$_3$) of compound 10g.
**Fig. S15** $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 10h.

**Fig. S16** $^{13}$C NMR spectrum (75 MHz, CDCl$_3$) of compound 10h.
Fig. S17 $^1$H NMR spectrum (300 MHz, (CD$_3$)$_2$CO) of compound 11a.

Fig. S18 $^{13}$C NMR spectrum (75 MHz, (CD$_3$)$_2$CO) of compound 11a.
Fig. S19 $^1$H NMR spectrum (300 MHz, (CD$_3$)$_2$CO) of compound 11b.

Fig. S20 $^{13}$C NMR spectrum (75 MHz, (CD$_3$)$_2$CO) of compound 11b.
Fig. S21 $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 12a.

Fig. S22 $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 13a.
Fig. S23 $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 12b.

Fig. S24 $^1$H NMR spectrum (300 MHz, CDCl$_3$) of compound 13b.