Supporting Information

Indolylmaleimide derivative IM-17 shows cardioprotective effects in ischemia-reperfusion injury

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Chemistry

General

Melting points were determined with a Yanaco MP-J8 micro melting point apparatus. $^1$H and $^{13}$C NMR spectra were recorded on a JEOL JNM-LA400, JEOL JNM-AL400 or JNM-AL300 spectrometer. Chemical shifts were reported in the scale relative to CDCl$_3$ as an internal reference. FAB-MS was taken on a Hitachi M-80B or JEOL JMS-700. ESI-MS was taken on a Bruker Daltonics micrOTOF-QII-RSL. IR spectra were measured on Thermo Nicolet AVATAR 370 FT-IR. Column chromatography was performed with silica gel 60 (40-100 µm) purchased from Kanto Chemical Co. Dehydrated stabilizer-free tetrahydrofuran (THF), dehydrated dichloromethane, dehydrated dimethylformamide (DMF) were purchased from Kanto Chemical Co., and used as received. The purity of IM-17, 18, 19 was checked by HPLC. The purity of other IM derivatives was assessed by elemental analysis.

Synthesis of indolylmaleimide derivatives

The synthetic schemes of IM-20, 12, 13, 54, and 25 were shown in the previous report.$^1$ IM-17, 18, 19, 27, 90, and 91 were similarly synthesized from IM-3 or IM-4 (Scheme S1). Typical synthetic procedures for IM-20, IM-12, and IM-17 are described below. The purity of indolylmaleimide derivatives was assessed by elemental analysis, except for IM-17, 18, 19.

Scheme S1

![Scheme S1](image_url)
1-Methyl-3-(1-methyl-1H-indol-3-yl)-4-(methylamino)-1H-pyrrole-2,5-dione (IM-20)

To a solution of IM-3 (54.2 mg, 0.197 mmol) in THF (1 mL) was added a 40% aqueous solution of methylamine (0.20 mL). The mixture was stirred for 24 h at room temperature, and then water was added. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were washed with brine. The organic layer was dried over Na₂SO₄, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/ethyl acetate = 4/1 ~ 2/1) to give the title compound (50.3 mg, 95%) as a yellow solid: mp 195-198 °C (sub); IR (neat, cm⁻¹) 3312, 2962, 2931, 2848, 1753, 1701, 1652, 1608, 1547, 1508, 1442, 1416, 1385, 1333, 1298, 1245, 1219, 1184, 1153, 1122, 1101, 1048, 1004, 982, 734; ¹H NMR (400 MHz, CDCl₃) δ 2.83 (d, J = 5.5 Hz, 3H), 3.06 (s, 3H), 3.82 (s, 3H), 5.17 (br, 1H), 7.11 (s, 1H), 7.11 (dd, J = 7.1 and 7.8 Hz, 1H), 7.23 (dd, J = 7.1 and 8.3 Hz, 1H), 7.32 (d, J = 8.3 Hz, 1H), 7.52 (d, J = 7.8 Hz, 1H); ¹³C NMR (68 MHz, CDCl₃) δ 10.72, 23.18, 23.66, 32.74, 45.10, 93.43, 103.16, 109.19, 119.46, 119.96, 121.65, 128.76, 129.23, 136.37, 142.65, 168.39, 172.85; HRMS (ESI⁺) calcd. for C₁₅H₁₅N₃NaO₂ ([M+Na⁺]⁺) m/z 292.1056; found 292.1064; Anal calcd. for (C₁₅H₁₅N₃O₂): C, 66.90; H, 5.61; N, 15.60; found: C, 66.91; H, 5.72; N, 15.48.

1-Methyl-3-(1-methyl-1H-indol-3-yl)-4-(propylamino)-1H-pyrrole-2,5-dione (IM-12)

To a solution of IM-3 (200 mg, 0.73 mmol) in CH₂Cl₂ (8 mL) was added n-propylamine (600 µL, 7.3 mmol), and the mixture was stirred at room temperature for 4 days. After evaporation of the solvent, the residue was purified by silica gel column chromatography (hexane/ethyl acetate = 4/1) to give IM-12 (178 mg, 82%) as an orange solid: mp 137-140 °C; IR (neat, cm⁻¹) 3330, 2960, 1691, 1653, 1533, 1440, 744; ¹H NMR (270 MHz, CDCl₃) δ 0.68 (t, J = 7.4 Hz, 3H), 1.35 (tq, J = 7.4 and 7.4 Hz, 2H), 3.04 (s, 3H), 3.09 (dt, J = 7.4 and 7.4 Hz, 2H), 3.79 (s, 3H), 5.17 (t, J = 7.4 Hz, 1H), 7.06-7.16 (m, 2H), 7.23 (dd, J = 8.0 and 8.0 Hz, 1H), 7.32 (d, J = 8.0 Hz, 1H), 7.48 (d, J = 8.0 Hz, 1H); ¹³C NMR (68 MHz, CDCl₃) δ 10.72, 23.18, 23.66, 32.74, 45.10, 93.43, 103.16, 109.19, 119.46, 119.96, 121.65, 128.76, 129.23, 136.37, 142.65, 168.39, 172.85; HRMS (ESI⁺) calcd. for C₁₇H₁₉N₃NaO₂ ([M+Na⁺]⁺) m/z 320.1369; found 320.1375; Anal calcd. for (C₁₇H₁₉N₃O₂•0.1H₂O): C, 68.25; H, 6.47; N, 14.05; found: C, 68.24; H, 6.59; N, 13.85.
4-(Butylamino)-1-methyl-3-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (IM-13)

Synthesized from IM-3 (76%) as an orange solid; mp 144-147 °C; IR (neat, cm⁻¹) 3211, 2928, 1693, 1650, 1541, 1441, 742; ¹H NMR (270 MHz, CDCl₃) δ 0.69 (t, J = 6.8 Hz, 3H), 1.11 (tq, J = 6.8 and 6.8 Hz, 2H), 1.34 (tt, J = 6.8 and 6.8 Hz, 2H), 3.06 (s, 3H), 3.14 (dt, J = 6.8 and 6.8 Hz, 2H), 3.92 (s, 3H), 5.17 (t, J = 6.8 Hz, 1H), 7.08-7.16 (m, 2H), 7.23 (dd, J = 8.0 and 8.0 Hz, 1H), 7.32 (d, J = 8.0 Hz, 1H), 7.48 (d, J = 8.0 Hz, 1H); ¹³C NMR (68 MHz, CDCl₃) δ 13.33, 19.44, 23.76, 32.05, 32.82, 43.25, 93.42, 103.23, 109.24, 119.53, 120.01, 121.74, 128.89, 129.28, 136.44, 142.73, 168.47, 172.93; HRMS (ESI⁺) calcd. for C₁₈H₂₁N₃O₂ ([M+Na]⁺) m/z 334.1526; found 334.1532; Anal calcd. for (C₁₈H₂₁N₃O₂): C, 69.43; H, 6.80; N, 13.49; found: C, 69.20; H, 6.87; N, 13.36.

1-Methyl-3-(1-methyl-1H-indol-3-yl)-4-(pentylamino)-1H-pyrrole-2,5-dione (IM-54)

Synthesized from IM-4 (59%) as an orange solid; mp 110-111 °C; IR (neat, cm⁻¹) 3330, 2962, 2931, 2853, 1753, 1692, 1648, 1613, 1543, 1512, 1442, 1385, 1328, 1293, 1232, 742; ¹H NMR (400 MHz, CDCl₃) δ 0.75 (t, J = 6.8 Hz, 3H), 0.96-1.17 (m, 4H), 1.35 (quintet, J = 7.0 Hz, 2H), 3.06 (s, 3H), 3.13 (dt, J = 6.8 and 7.0 Hz, 2H), 3.82 (s, 3H), 5.17 (br s, 1H), 7.11 (s, 1H), 7.12 (dd, J = 7.3 and 8.0 Hz, 1H), 7.23 (dd, J = 7.3 and 8.3 Hz, 1H), 7.33 (d, J = 8.3 Hz, 1H), 7.48 (d, J = 8.0 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 13.80, 22.08, 23.87, 28.50, 29.83, 32.96, 43.66, 93.56, 103.32, 109.32, 119.65, 120.11, 121.85, 128.98, 129.35, 136.55, 142.77, 168.58, 173.03; HRMS (ESI⁺) calcd. for C₁₉H₂₃N₃NaO₂ ([M+Na]⁺) m/z 348.1682; found 348.1672; Anal calcd. for (C₁₉H₂₃N₃O₂): C, 70.13; H, 7.12; N, 12.91; found: C, 70.07; H, 7.20; N, 12.82.
3-(Hexylamino)-1-methyl-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (IM-25)

Synthesized from IM-4 (83%) as a yellow solid; mp 60-63 °C; IR (neat, cm⁻¹) 3339, 3045, 2958, 2927, 2857, 1753, 1692, 1653, 1534, 1504, 1434, 1381, 1328, 1293, 1241, 1158, 1127, 1092, 1044, 1009, 978, 816, 781, 746, 650; ¹H NMR (270 MHz, CDCl₃): δ 0.79 (t, J = 7.3 Hz, 3H), 0.98-1.39 (m, 8H), 3.06 (s, 3H), 3.12 (dt, J = 7.3 and 7.3 Hz, 2H), 3.82 (s, 3H), 5.18 (t, J = 7.3 Hz, 1H), 7.11 (dd, J = 8.0 and 8.0 Hz, 1H), 7.12 (s, 1H), 7.23 (dd, J = 8.0 and 8.0 Hz, 1H), 7.31 (d, J = 8.0 Hz, 1H), 7.48 (d, J = 8.0 Hz, 1H); ¹³C NMR (68 MHz, CDCl₃): δ 13.92, 22.37, 23.86, 26.03, 30.10, 31.14, 32.94, 43.66, 93.52, 103.32, 109.32, 119.65, 120.10, 121.85, 128.99, 129.34, 136.53, 142.78, 168.57, 173.03; HRMS (ESI⁺) calcd. for C₂₀H₂₅N₃O₂ ([M+Na]⁺) m/z 362.1839; found 362.1839; Anal calcd. for (C₂₀H₂₅N₃O₂): C, 70.77; H, 7.42; N, 12.38, found: C, 70.94; H, 7.64; N, 12.07.

3-(2-Aminoethylamino)-1-methyl-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (IM-17)

To a solution of IM-4 (389 mg, 1.21 mmol) in THF (12 mL) was added 1,2-diaminoethane (809 µL, 12.1 mmol). The mixture was stirred for 9 days at room temperature, and then saturated aqueous NaHCO₃ solution was added to it. The aqueous layer was extracted with ether and ethyl acetate, and the combined organic layer was washed with brine. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/ethyl acetate = 4/1) to give IM-17 (273 mg, 76%) as an orange solid; mp 137-140 °C; IR (neat, cm⁻¹) 3361, 3308, 1696, 1648, 1600, 1543, 1530, 1433, 1330, 1056, 732; ¹H NMR (400 MHz, CDCl₃) δ 1.35 (br s, 2H), 2.66 (t, J = 5.8 Hz, 2H), 3.06 (s, 3H), 3.19 (dt, J = 5.8 and 5.8 Hz, 2H), 3.82 (s, 3H), 5.64 (br s, 1H), 7.12 (dd, J = 7.9 and 8.1 Hz, 1H), 7.14 (s, 1H), 7.23 (dd, J = 7.9 and 8.3 Hz, 1H), 7.32 (d, J = 8.3 Hz, 1H), 7.50 (d, J = 8.1 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 23.85, 32.99, 41.53, 45.91, 94.20, 103.20, 109.42, 119.70, 120.06, 121.90, 128.82, 129.44, 136.55, 142.74, 168.50, 172.95; MS (FAB, mNBA) m/z 299 ([M+H]⁺); HRMS calcd. for C₁₆H₁₀N₃O₂ ([M+H]⁺) 299.1508; found 299.1506; purity >98% (HPLC).
3-(3-Aminopropylamino)-1-methyl-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (IM-18).

Red amorphous solid; IR (neat, cm\(^{-1}\)) 3363, 3310, 1705, 1656, 1543, 1530, 1442, 1381, 1056, 724; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.31 (br s, 2H), 1.47 (tt, \(J = 6.4\) and 6.4 Hz, 2H), 2.58 (t, \(J = 6.4\) Hz, 2H), 3.05 (s, 3H), 3.23 (dt, \(J = 6.4\) and 6.4 Hz, 2H), 3.81 (s, 3H), 6.04 (br s, 1H), 7.09-7.15 (m, 1H), 7.12 (s, 1H), 7.22 (dd, \(J = 7.8\) and 7.8 Hz, 1H), 7.31 (d, \(J = 7.8\) Hz, 1H), 7.49 (d, \(J = 7.8\) Hz, 1H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 23.79, 32.72, 32.92, 39.79, 42.34, 93.26, 103.34, 109.34, 119.59, 120.06, 121.83, 128.96, 129.39, 136.47, 142.94, 168.49, 173.02; MS (FAB, mNBA) m/z 313 ([M+H]+); HRMS calcd. for C\(_{17}\)H\(_{21}\)N\(_4\)O\(_2\) ([M+H]+) 313.1665; found 313.1664; purity >98% (HPLC).

3-(4-Aminobutylamino)-1-methyl-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (IM-19).

Red amorphous solid; IR (neat, cm\(^{-1}\)) 3317, 2927, 1700, 1656, 1609, 1578, 1547, 1438, 1385, 1367, 1236, 1091, 732; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.20 (tt, \(J = 6.8\) and 6.8 Hz, 2H), 1.27 (br s, 2H), 1.38 (tt, \(J = 6.8\) and 6.8 Hz, 2H), 2.45 (t, \(J = 6.8\) Hz, 2H), 3.08 (s, 3H), 3.15 (dt, \(J = 6.8\) and 6.8 Hz, 2H), 3.81 (s, 3H), 5.57 (br s, 1H), 7.09-7.15 (m, 1H), 7.11 (s, 1H), 7.22 (dd, \(J = 8.1\) and 8.1 Hz, 1H), 7.31 (d, \(J = 8.1\) Hz, 1H), 7.48 (d, \(J = 8.1\) Hz, 1H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 23.79, 27.48, 30.30, 32.91, 41.31, 43.43, 93.45, 103.28, 109.32, 119.60, 121.01, 121.83, 128.89, 129.35, 136.47, 142.81, 168.50, 172.97; MS (FAB, mNBA) m/z 327 ([M+H]+); HRMS calcd. for C\(_{18}\)H\(_{23}\)N\(_4\)O\(_2\) ([M+H]+) 327.1821; found 327.1827; purity >98% (HPLC).

3-(2-Hydroxyethylamino)-1-methyl-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (IM-27).
Red solid; mp 144-147 °C; IR (neat, cm⁻¹) 3509, 3479, 3457, 3439, 3413, 3365, 3339, 3063, 3001, 2927, 1749, 1705, 1653, 1609, 1543, 1442, 1385, 1355, 1328, 1298, 1237, 1162, 1127, 1101, 1053, 1013, 982, 750, 637; ¹H NMR (400 MHz, CDCl₃) δ 1.25 (s, 1H), 3.06 (s, 3H), 3.35 (m, 2H), 3.55 (d, J = 7.0 Hz, 2H), 3.83 (s, 3H), 5.50 (t, J = 7.0 Hz, 1H), 7.15 (dd, J = 7.8 and 7.8 Hz, 1H), 7.16 (s, 1H), 7.22-7.26 (m, 1H), 7.33 (d, J = 7.8 Hz, 1H), 7.50 (d, J = 7.8 Hz, 1H); HRMS(ESI⁺) calcd. for C₁₆H₁₇N₃NaO₃ ([M+Na⁺]⁺) m/z 322.1162; found 322.1156; Anal calcd. for (C₁₆H₁₇N₃O₃): C, 64.20; H, 5.72; N, 14.04; found C, 64.07; H, 5.81; N, 13.89.

![Image of IM-90](image-url)

3-(3-Hydroxypropylamino)-1-methyl-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (IM-90).

Red solid; mp 121-124 °C; IR (neat, cm⁻¹) 3514, 3457, 3356, 3330, 2923, 2874, 2848, 1753, 1696, 1648, 1613, 1543, 1512, 1447, 1385, 1328, 1289, 1232, 1153, 1123, 1101, 1048, 982, 816, 746, 676, 645; ¹H NMR (400 MHz, CDCl₃) δ 1.58 (s, 1H), 1.61 (tt, J = 6.3 and 6.3 Hz, 2H), 3.06 (s, 3H), 3.32 (dt, J = 6.3 and 6.3 Hz, 2H), 3.55 (dt, J = 6.3 and 6.3 Hz, 2H), 3.83 (s, 3H), 5.52 (t, J = 6.3 Hz, 1H), 7.13 (dd, J = 8.0 and 8.0 Hz, 1H), 7.14 (s, 1H), 7.24 (dd, J = 8.0 and 8.0 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.50 (d, J = 8.0 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 23.87, 32.42, 33.01, 41.31, 60.47, 94.01, 103.20, 109.50, 119.70, 120.03, 121.96, 128.80, 129.48, 136.56, 142.73, 168.54, 172.92; HRMS (ESI⁺) calcd. for C₁₇H₁₉N₃O₃ ([M+Na⁺⁺]⁺) m/z 336.1319; found 336.1320; Anal calcd. for (C₁₇H₁₉N₃O₃): C, 65.16; H, 6.11; N, 13.41; found C, 65.03; H, 6.18; N, 13.25.

![Image of IM-91](image-url)

3-(4-Hydroxybutylamino)-1-methyl-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (IM-91).

Red solid; mp 137-140 °C; IR (neat, cm⁻¹) 3549, 3514, 3444, 3326, 2997, 2927, 2866, 1753, 1692, 1657, 1613, 1539, 1508, 1438, 1381, 1328, 1289, 1241, 1149, 1131, 1096, 1048, 1026, 1009, 982, 812, 742, 658; ¹H NMR (400 MHz, CDCl₃) δ 1.33 (tt, J = 5.8 and 5.8 Hz, 2H), 1.46 (tt, J = 5.8 and 5.8 Hz, 2H), 1.58 (s, 1H), 3.06 (s, 3H), 3.19 (dt, J = 5.8 and 5.8 Hz, 2H), 3.39 (dt, J = 5.8 and 5.8 Hz, 2H), 3.83 (s, 3H), 5.33 (t, J = 5.8 Hz, 1H), 7.13 (dd, J = 7.8 and 7.8 Hz, 1H), 7.14 (s, 1H), 7.24 (dd, J = 7.8 and 7.8 Hz, 2H), 7.33 (d, J = 7.8 Hz, 1H), 7.50 (d, J = 7.8 Hz, 1H), 7.50 (d, J = 7.8 Hz, 1H); HRMS (ESI⁺) calcd. for C₁₉H₂₁N₃O₅ ([M+Na⁺⁺]⁺) m/z 351.1531; found 351.1341; Anal calcd. for (C₁₉H₂₁N₃O₅): C, 66.32; H, 6.45; N, 13.04; found C, 66.31; H, 6.43; N, 13.03.
Hz, 1H), 7.33 (d, J = 7.8 Hz, 1H), 7.49 (d, J = 7.8 Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 24.02, 26.82, 29.47, 33.14, 43.54, 62.19, 93.92, 103.42, 109.58, 119.83, 120.22, 122.09, 129.06, 129.60, 136.68, 142.86, 168.70, 173.13; HRMS (ESI$^+$) calcd. for C$_{18}$H$_{22}$N$_3$O$_3$ ([M+H]$^+$) m/z 328.1656; found 328.1656; Anal calcd. for (C$_{18}$H$_{21}$N$_3$O$_3$): C, 66.04; H, 6.47; N, 12.84; found C, 65.82; H, 6.56; N, 12.68.

Scheme S2

3-(2-Aminoethylamino)-1-methyl-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione hydrochloride (IM-17•HCl)

To a solution of IM-17 (90 mg, 0.3 mmol) in CH$_2$Cl$_2$ (1.5 mL) was slowly added a saturated HCl diethylether solution (1 mL) at 0℃. The mixture was stirred for 15 min, and the resulting precipitate was collected by filtration, washed with diethylether, and dried to afford IM-17•HCl (95 mg, 95%) as an orange solid; $^1$H NMR (400 MHz, DMSO-d$_6$) δ 2.67-2.72 (m, 2H), 2.91 (s, 3H), 3.33-3.39 (m, 2H), 3.73-3.80 (m, 2H), 3.81 (s, 3H), 7.03 (dd, J = 7.2 and 7.2 Hz, 1H), 7.15-7.19 (m, 2H), 7.36-7.46 (m, 3H), 7.80 (br, 1H); HRMS (ESI$^+$) calcd. for C$_{16}$H$_{18}$N$_4$O$_2$ ([M+H]$^+$) m/z 299.1503; found 299.1506.
HPLC analysis of purity

The purity of indolylmaleimide derivatives **IM-17, 18, 19** was assessed by HPLC using a Waters HPLC system under two different conditions.

**Condition 1**: Type: TOSOH TSK-GEL ODS-100S; size: 4.6 mm (ID)–150 mm (L); flow rate 0.50 ml / min; detector: UV 254 nm; eluent: CH$_3$CN / H$_2$O with 0.2 % AcOH = 1 / 4; temperature 35 °C; run time 30 min.

**Condition 2**: Type: Nakarai tesque COSMOSIL 5C$_{18}$-PAQ; size: 4.6 mm (ID)–250 mm (L); flow rate 1.0 ml / min; detector: UV 254 nm; eluent: CH$_3$CN / H$_2$O with 0.2 % AcOH = 1 / 4; temperature 35 °C; run time 30 min.

**IM-17**: condition 1: 98.78 % ($t_R = 3.8$ min); condition 2: 99.14 % ($t_R = 3.5$ min)

**IM-18**: condition 1: 98.33 % ($t_R = 4.0$ min); condition 2: 98.62 % ($t_R = 3.2$ min)

**IM-19**: condition 1: 99.89 % ($t_R = 4.0$ min); condition 2: 98.66 % ($t_R = 3.6$ min)
IM-18

Condition 1

Condition 2

IM-19

Condition 1

Condition 2
Biology

Cell culture

HL-60 cells were maintained in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 5% heat-inactivated fetal bovine serum (FBS). Jurkat cells were maintained in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). H9c2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). Cells were grown in a humidified incubator at 37 °C under 5% CO2/95% air.

AlamarBlue assay

HL-60 cells (3 x 10^4 cells/well for Fas ligand (FasL) and 4 x 10^4 cells/well for others) or Jurkat cells (2 x 10^4 cells/well for FasL/CHX/Z-VAD treatment or 3 x 10^4 cells/well for FasL treatment) were suspended in fresh medium in a 96-well plate. After 2 h incubation, the cells were treated with test compounds (DMSO solution, 0.5 µL/well) for 1 h and then cell death inducer (in medium, 4 µL/well) was added (final volume 100 µL/well). In all experiments, the final DMSO concentration was the same (0.5%). At 3 h later, 10 µL of AlamarBlue (Biosource International) was added to each well. The cell viability was determined based on the increase of fluorescence (excitation 560 nm/emission 590 nm) during 3-4 h incubation. Data are presented as mean ± S.D. (n = 4).

LDH assay

Lactate dehydrogenase (LDH) that leaked into the culture medium was measured with a Cytotoxicity detection kitPLUS (Roche Applied Science) according to the manufacturer’s protocol. HL-60 cells were treated with test compounds and H2O2 (100 µM) in a 96-well plate according to the same method as described for AlamarBlue assay (triplicate). H9c2 cells (1 x 10^4 cells/well) were treated with test compounds and TBHP (300 µM) for 4 h (triplicate). The change in absorbance at 490 nm was then measured to calculate the percentage of LDH release. IC50 values were calculated by Origin 8.0 software, and data are presented as mean ± S.D. (n = 3, three independent experiments).

Cell imaging

Phase-contrast images were taken with an Olympus IX71 microscope equipped with an Olympus DP70 cooled CCD camera.
Cell death inhibitors and inducers

Fas ligand was purchased from Enzo Life Sciences Inc. (SuperFasligand, cat. No. ALX-522-020-C005), Z-VAD was purchased from Peptide Institute Inc., and other reagents (actinomycin D, camptotecin, cycloheximide, CsA, DPQ, etoposide, H$_2$O$_2$, 3-MA, TBHP, Nec-1) were purchased from Sigma.

Analysis of stability to liver metabolism

To compare the drug-like properties of IM derivatives, stabilities of IM-12, IM-54, and IM-17 to liver metabolism were examined. Each compound in DMSO was mixed with 1 mL of rat liver S9 fraction containing cofactors (Ieda Trading Corporation, cat. No. S-9MIX) for a final concentration of 5 µM with 0.1% DMSO. The mixture was incubated at 37 °C for 0 min, 5 min, 15 min and 30 min. After the incubation, the mixture was moved to ice quickly. Then ethyl acetate (250 µL) was added to the mixture and the compound was extracted. The extraction was repeated 5 times. The organic layer was combined and concentrated in vacuo. The residue was diluted with MeOH (30 µL) and analyzed by HPLC. Residual compound was quantified based on the peak area detected by UV (n = 3, three independent experiments). As shown in figure S1, IM-12 and IM-54 were decreased rapidly and were not detected after 5 min incubation with S9 fraction. In contrast, IM-17 was detected even after 15 min incubation with 40% residual rate. These results demonstrated the higher stability of IM-17 to liver metabolism than IM-12 and IM-54.

Figure S1. Stabilities of IM-12, IM-17, IM-54 to liver metabolism.

IM-12, IM-17, and IM-54 (5 µM) were respectively incubated with rat S9 liver solution for 0, 5, 15, or 30 min at 37 °C. Then residual compound was extracted with ethyl acetate and quantified by HPLC. Data are presented as mean ± S.D. (n = 3, three independent experiments).

HPLC system: Thermo Fisher UltiMate 3000

Type: GL Sciences InertSustain C18; size: 1.0 mm (ID)–150 mm (L) 3 µm (particle size); flow rate 50 µl / min; detector: UV 230 nm; eluent: mobile phase A, H$_2$O with 0.1% trifluoroacetic acid; mobile phase B, CH$_3$CN with 0.1% trifluoroacetic acid; temperature 35 °C; injection volume 2 µL.
Analysis of IM-12: Gradient curve: 40% B (0–2 min), 40% to 60% B (2–6 min), 60% B (6–12.5 min), 60% to 40% B (12.5–13.5 min), 40% B (13.5–15 min); run time 15 min; $t_R = 11.3$ min.
Standard curve: $y = 0.7609x + 3.4515$, $R^2 = 0.9933$ (eight concentrations from 200 nM to 200 µM).

Analysis of IM-17: Gradient curve: 15% B (0–2 min), 15% to 35% B (2–12 min), 35% B (12–14 min), 35% to 15% B (14–16 min), 15% B (16–20 min); run time 20 min; $t_R = 12.6$ min.
Standard curve: $y = 0.3794x - 0.285$, $R^2 = 0.9994$ (seven concentrations from 200 nM to 100 µM).

Analysis of IM-54: Gradient curve: 40% B (0–2 min), 40% to 70% B (2–6 min), 70% B (6–12.5 min), 70% to 40% B (12.5–13.5 min), 40% B (13.5–15 min); run time 15 min; $t_R = 11.9$ min.
Standard curve: $y = 0.6997x + 2.2325$, $R^2 = 0.9949$ (eight concentrations from 200 nM to 200 µM).

Langendorff isolated rat heart model
The isolated rat heart preparations used in this study have been described previously\(^2\). The experiments were performed according to the Guide for the Care and Use of Laboratory Animals promulgated by the National Research Council. Briefly, male Sprague–Dawley rats (270–320 g) were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and heparinized (1000 IU/kg, i.v.). The hearts were rapidly removed and mounted on a Langendorff perfusion system (Technical Supply Co. Ltd., Tokyo). The environmental temperature was maintained at 37 °C with a heated glass water-bath throughout the experiments. Through the cannulated aorta, the hearts were perfused with warmed (37 °C) and gassed (95% O\(_2\) and 5% CO\(_2\), pO\(_2\)>600 mmHg) Krebs–Henseleit solution containing (in mM) NaCl 120, KCl 4.7, CaCl\(_2\) 1.25, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, glucose 11 at a constant perfusion pressure (70 ± 5 mm Hg). To measure the coronary flow (CF) continuously, a cannulating-type flow probe (FF-030T, Nihon Kohden, Tokyo) connected to an electro-magnetic blood flowmeter (MFV-3700, Nihon Kohden, Tokyo) was inserted into a perfusion line connected to the heart. The left ventricular pressure was measured through a water-filled latex balloon (LB-2, Technical Service Corporation, Miyagi, Japan) inserted into the left ventricle via the left atrium, with a pressure transducer (DX-360, Ohmeda, Tokyo) connected to an amplifier (AP-601G, Nihon Kohden, Tokyo). Left ventricular end-diastolic pressure (LVEDP) was adjusted to about 5 mm Hg by adjusting the volume of the balloon, and the left ventricular developed pressure (LVDP) was obtained by deducting LVEDP from left ventricular systolic pressure. The heart rate was measured by using a cardio-tachometer (AT-600G, Nihon Kohden, Tokyo) triggered by the pulse of left ventricular pressure. All hemodynamic parameters and CF were continuously recorded on a multi-channel recorder (WR3701, Graphtec, Tokyo). The hearts were paced at 300 beats/min using an electric stimulator (SEN-7103, Nihon Kohden, Tokyo), and an isolator unit (SS-104J, Nihon Kohden, Tokyo) with the bipolar electrode placed on the left atrium. After a 15-min period of
equilibration, vehicle, IM-12 (0.3 µM) or IM-17 (3 µM) was infused for 10 min with an infusion pump (Harvard Apparatus, U.S.A.) through a drug infusion line connected to the main perfusion line of the Langendorff system at a flow rate of 1/100 of the CF rate. Subsequent to drug treatment for 5 min, hearts were subjected to 30 min of global ischemia and 60 min of reperfusion. Global ischemia was induced by completely stopping the flow. LVDP, LVEDP and CF were measured before and 10 min after infusion of the drug, 10, 20 and 30 min after the induction of global ischemia, and 10, 20 and 30 min after reperfusion. Data are presented as mean ± S.D. (n = 3).

Measurement of CK activity: The coronary effluent was collected before ischemia and during reperfusion for measurement of the creatine kinase (CK) activity with a commercial assay reagent and a clinical chemistry analyzer (CL-8000, Shimadzu, Kyoto, Japan). Data are presented as mean ± S.D. (n = 3).

Figure S2. Cardioprotective effect of IM-12 in a Langendorff rat heart Ischemia-reperfusion injury model. IM-12 (0.3 µM) was added to the perfusion buffer 10 min before ischemia. No-flow ischemia was maintained for 30 min, and reperfusion was accomplished by restoring flow for 60 min. The cardioprotective effect of IM-12 was examined based on recovery of LVDP (left ventricular developed pressure) (a) and release of CK (creatine kinase) (b).

Ischemia-reperfusion-induced arrhythmia model

Male Sprague-Dawley rats were anesthetized with sodium pentobarbital. The experiments were performed according to the Guide for the Care and Use of Laboratory Animals promulgated by the National Research Council. The femoral vein was cannulated for intravenous test drug administration. Heart rate was measured by a cardiotachometer (AT-601G, Nihon Kohden). A left thoracotomy at the fifth intercostal space and pericardiectomy were performed and an ELP 5-0 nylon ligature (L14-50N, Akiyama Seisakusho, Tokyo) was placed around the left coronary artery about 2-3 mm from its origin.
Thereafter, both ends of the nylon ligature were passed through a small polyethylene tube to make a coronary snare. The standard limb lead II electrocardiogram (ECG) was monitored by a cardiograph (ECG-6303, Nihon Kohden). ECG, blood pressure and heart rate data were collected by an ECG processor (Softron, Tokyo) and stored on the MO disk for further data processing. Myocardial ischemia was initiated by tightening the coronary snare and successful ischemia was confirmed by typical elevation of the ST segment in the ECG. At 5 min after the start of ischemia, reperfusion was initiated by releasing the snare. Ventricular fibrillation (VF) after reperfusion was evaluated by ECG analysis based on the reported guideline. Total duration of VF was calculated as the sum of the duration of episodes that occurred within 10 min after reperfusion. 0.9% saline was used as a control. IM-17 was intravenously injected (1 ml/kg, over 1 min) at 5 min before ischemia (pre-ischemia treatment, 1 to 3 mg/kg) or at 1 min before reperfusion (post-ischemia treatment, 3 mg/kg).
**KinaseProfiler assay of IM-54**

As IM-54 was originally developed from ATP-competitive PKC inhibitor bisindolylmaleimide I (BM I), we previously analyzed its kinase-inhibitory activities against all PKC subtypes and confirmed that no PKC was inhibited by IM-54. We also evaluated its inhibitory activities against various kinases related to cell-death signaling pathways. Protein kinase inhibition assays were performed using the KinaseProfiler service (Upstate USA, Inc.). Briefly, protein kinases were assayed for their ability to phosphorylate the appropriate peptide/protein substrates in the presence of 50 µM IM-54 and 100 µM or 10 µM ATP. Activities are given as mean percentages of those in control incubations (averages of duplicate determinations). IM-54 did not inhibit more than half of any kinase activity, indicating that IC$_{50}$ values against all tested kinases were over 50 µM.

**Table S1. KinaseProfiler assay of IM-54.** The activity of various kinases was measured in the presence and absence of IM-54 (50 µM). ATP was present at 100 µM (left) or 10 µM (right).

| kinase         | kinase activity (% of control) with IM-54 (50 µM) |
|----------------|--------------------------------------------------|
| PKA            | 75                                               |
| JNK1α1         | 91                                               |
| JNK2α2         | 110                                              |
| JNK3           | 87                                               |
| MAPK1/ERK1     | 74                                               |
| MAPK2/ERK2     | 91                                               |
| MAPKAP-K2      | 89                                               |
| MKK4           | 106                                              |
| MKK6           | 99                                               |
| MKK7β          | 100                                              |
| SAPK2a/p38α    | 95                                               |
| SAPK2b/p38β2   | 102                                              |
| SAPK3/p38γ     | 100                                              |
| SAPK4/p38δ     | 96                                               |
| RIPK2          | 96                                               |

| kinase         | kinase activity (% of control) with IM-54 (50 µM) |
|----------------|--------------------------------------------------|
| ASK1           | 104                                              |
| c-Raf          | 87                                               |
| MEK1           | 102                                              |
| PKBα           | 85                                               |
| PKBβ           | 110                                              |
| PKBγ           | 92                                               |
| RSK1           | 57                                               |
| RSK2           | 105                                              |
| RSK3           | 108                                              |
| RSK4           | 107                                              |
| Src            | 69                                               |

**KINOMEscan screening of IM-17 and IM-54**

To examine the broad pattern of kinase inhibitory activities, KINOMEscan screening (DiscoverX Corp.) was applied for IM-54 and IM-17 at 10 µM. KINOMEscan evaluated the binding affinity to kinases, which was determined based on the ATP site-dependent competition for beads-immobilized kinase inhibitors. Activities are given as percentages of control values of bound kinases to beads-immobilized kinase inhibitors (averages of duplicate determinations). Less than 35 % of control at 10 µM indicates
significant inhibition of kinase. As shown in Table S2, no significant inhibition of 467 kinases by IM-17 or IM-54 was observed, supporting the view that these compounds do not have kinase-inhibitory activity.

Table S2. KINOMEscan screening of IM-17 and IM-54. Compounds were tested against 467 kinases at 10 µM (averages of duplicate determinations).

| DiscoveRx Gene Symbol | Entrez Gene Symbol | IM-17 | IM-54 |
|-----------------------|--------------------|-------|-------|
| AAK1                  | AAK1               | 100   | 69    |
| ABL1(E255K)-phosphorylated | ABL1             | 90    | 69    |
| ABL1(F317I)-nonphosphorylated | ABL1          | 100   | 95    |
| ABL1(F317I)-phosphorylated  | ABL1             | 97    | 93    |
| ABL1(F317L)-nonphosphorylated  | ABL1            | 100   | 91    |
| ABL1(F317L)-phosphorylated  | ABL1             | 91    | 98    |
| ABL1(H396P)-nonphosphorylated  | ABL1            | 100   | 85    |
| ABL1(H396P)-phosphorylated  | ABL1             | 89    | 77    |
| ABL1(M351T)-phosphorylated  | ABL1             | 95    | 75    |
| ABL1(Q252H)-nonphosphorylated  | ABL1            | 95    | 66    |
| ABL1(Q252H)-phosphorylated  | ABL1             | 96    | 97    |
| ABL1(T315I)-nonphosphorylated  | ABL1            | 100   | 69    |
| ABL1(T315I)-phosphorylated  | ABL1             | 69    | 66    |
| ABL1(Y253F)-phosphorylated  | ABL1             | 93    | 79    |
| ABL1-nonphosphorylated  | ABL1             | 93    | 85    |
| ABL1-phosphorylated  | ABL1             | 96    | 82    |
| ABL2                  | ABL2               | 99    | 100   |
| ACVR1                 | ACVR1             | 96    | 100   |
| ACVR1B                | ACVR1B            | 91    | 100   |
| ACVR2A                | ACVR2A            | 97    | 100   |
| ACVR2B                | ACVR2B            | 100   | 100   |
| ACVR1L                | ACVR1L            | 90    | 100   |
| ADCK3                 | CABC1             | 97    | 97    |
| ADCK4                 | ADCK4             | 100   | 100   |
| AKT1                  | AKT1              | 91    | 84    |
| AKT2                  | AKT2              | 86    | 100   |
| AKT3                  | AKT3              | 85    | 100   |
| ALK                   | ALK               | 96    | 98    |
| ALK(C1156Y)           | ALK               | 93    | 54    |
| ALK(L1196M)           | ALK               | 97    | 59    |
| AMPK-alpha1           | PRKAA1            | 100   | 100   |
| AMPK-alpha2           | PRKAA2            | 97    | 99    |
| ANKK1                 | ANKK1             | 91    | 98    |
| ARK5                  | NUAK1             | 78    | 100   |
| ASK1                  | MAP3K5            | 100   | 84    |
| ASK2                  | MAP3K6            | 100   | 90    |
| AURKA                 | AURKA             | 79    | 87    |
| Protein     | Protein     | 96 | 92 |
|------------|------------|----|----|
| AURKB      | AURKB      | 96 | 92 |
| AURKC      | AURKC      | 100| 95 |
| Axl        | AxL        | 95 | 91 |
| BIKE       | BMP2K      | 99 | 91 |
| Blk        | BlK        | 97 | 74 |
| BMPR1A     | BMPR1A     | 100| 100|
| BMPR1B     | BMPR1B     | 94 | 100|
| BMPR2      | BMPR2      | 69 | 72 |
| Bmx        | Bmx        | 89 | 100|
| Braf       | Braf       | 93 | 89 |
| Braf(V600E)| Braf       | 100| 86 |
| Brk        | PTK6       | 100| 97 |
| BRSK1      | BRSK1      | 96 | 97 |
| BRSK2      | BRSK2      | 99 | 88 |
| Btk        | Btk        | 96 | 79 |
| Bub1       | Bub1       | 83 | 72 |
| Camk1      | Camk1      | 96 | 100|
| Camk1B     | PNCK       | 86 | 92 |
| Camk1D     | Camk1D     | 100| 100|
| Camk1G     | Camk1G     | 100| 100|
| Camk2A     | Camk2A     | 100| 96 |
| Camk2B     | Camk2B     | 100| 100|
| Camk2D     | Camk2D     | 89 | 100|
| Camk2G     | Camk2G     | 100| 100|
| Camk4      | Camk4      | 95 | 100|
| Camkk1     | Camkk1     | 84 | 85 |
| Camkk2     | Camkk2     | 100| 98 |
| Cask       | CasK       | 100| 91 |
| Cdc2L1     | CDK11B     | 98 | 100|
| Cdc2L2     | CDC2L2     | 92 | 99 |
| Cdc2L5     | CDK13      | 99 | 90 |
| Cdk11      | CDK19      | 93 | 100|
| Cdk2       | Cdk2       | 97 | 91 |
| Cdk3       | Cdk3       | 100| 94 |
| Cdk4-cyclinD1 | Cdk4     | 82 | 89 |
| Cdk4-cyclinD3 | Cdk4     | 98 | 100|
| Cdk5       | Cdk5       | 100| 100|
| Cdk7       | Cdk7       | 92 | 84 |
| Cdk8       | Cdk8       | 92 | 100|
| Cdk9       | Cdk9       | 97 | 100|
| Cdk1L1     | CDK1L1     | 100| 78 |
| Cdk1L2     | CDK1L2     | 99 | 84 |
| Cdk1L3     | CDK1L3     | 95 | 100|
| Cdk1L5     | CDK1L5     | 92 | 93 |
| Protein         | Protein         | Percentage | Percentage |
|-----------------|-----------------|------------|------------|
| CHEK1           | CHEK1           | 90         | 93         |
| CHEK2           | CHEK2           | 82         | 95         |
| CIT             | CIT             | 100        | 100        |
| CLK1            | CLK1            | 100        | 77         |
| CLK2            | CLK2            | 100        | 100        |
| CLK3            | CLK3            | 100        | 89         |
| CLK4            | CLK4            | 95         | 100        |
| CSF1R           | CSF1R           | 100        | 100        |
| CSF1R-autoinhibited | CSF1R       | 98         | 100        |
| CSK             | CSK             | 95         | 95         |
| CSNK1A1         | CSNK1A1         | 90         | 70         |
| CSNK1A1L        | CSNK1A1L        | 100        | 100        |
| CSNK1D          | CSNK1D          | 96         | 100        |
| CSNK1E          | CSNK1E          | 100        | 92         |
| CSNK1G1         | CSNK1G1         | 100        | 99         |
| CSNK1G2         | CSNK1G2         | 85         | 82         |
| CSNK1G3         | CSNK1G3         | 100        | 81         |
| CSNK2A1         | CSNK2A1         | 91         | 90         |
| CSNK2A2         | CSNK2A2         | 82         | 74         |
| CTK             | MATK            | 85         | 82         |
| DAPK1           | DAPK1           | 100        | 77         |
| DAPK2           | DAPK2           | 100        | 98         |
| DAPK3           | DAPK3           | 100        | 94         |
| DCAMKL1         | DCLK1           | 90         | 81         |
| DCAMKL2         | DCLK2           | 94         | 100        |
| DCAMKL3         | DCLK3           | 78         | 100        |
| DDR1            | DDR1            | 100        | 91         |
| DDR2            | DDR2            | 95         | 62         |
| DLK             | MAP3K12         | 87         | 94         |
| DMPK            | DMPK            | 100        | 98         |
| DMPK2           | CDC42BPG        | 96         | 99         |
| DRAK1           | STK17A          | 85         | 93         |
| DRAK2           | STK17B          | 80         | 73         |
| DYRK1A          | DYRK1A          | 90         | 100        |
| DYRK1B          | DYRK1B          | 82         | 100        |
| DYRK2           | DYRK2           | 89         | 100        |
| EGFR            | EGFR            | 94         | 68         |
| EGFR(E746-A750del) | EGFR          | 100        | 100        |
| EGFR(G719C)     | EGFR            | 99         | 86         |
| EGFR(G719S)     | EGFR            | 100        | 90         |
| EGFR(L747-E749del, A750P) | EGFR      | 81         | 90         |
| EGFR(L747-S752del, P753S) | EGFR      | 100        | 100        |
| EGFR(L747-T751del,Sins) | EGFR      | 100        | 90         |
| EGFR(L858R)     | EGFR            | 92         | 87         |
| Gene  | % Expression | % Potential |
|-------|--------------|-------------|
| EGFR(L858R,T790M) | EGFR | 100 | 86 |
| EGFR(L861Q) | EGFR | 100 | 91 |
| EGFR(S752-I759del) | EGFR | 97 | 99 |
| EGFR(T790M) | EGFR | 100 | 73 |
| EIF2AK1 | EIF2AK1 | 82 | 80 |
| EPHA1 | EPHA1 | 93 | 93 |
| EPHA2 | EPHA2 | 99 | 100 |
| EPHA3 | EPHA3 | 100 | 100 |
| EPHA4 | EPHA4 | 100 | 100 |
| EPHA5 | EPHA5 | 100 | 100 |
| EPHA6 | EPHA6 | 95 | 100 |
| EPHA7 | EPHA7 | 100 | 98 |
| EPHA8 | EPHA8 | 100 | 100 |
| EPHB1 | EPHB1 | 100 | 92 |
| EPHB2 | EPHB2 | 100 | 97 |
| EPHB3 | EPHB3 | 100 | 97 |
| EPHB4 | EPHB4 | 100 | 100 |
| EPHB6 | EPHB6 | 85 | 97 |
| ERBB2 | ERBB2 | 96 | 92 |
| ERBB3 | ERBB3 | 98 | 100 |
| ERBB4 | ERBB4 | 100 | 100 |
| ERK1 | MAPK3 | 88 | 90 |
| ERK2 | MAPK1 | 100 | 100 |
| ERK3 | MAPK6 | 99 | 100 |
| ERK4 | MAPK4 | 100 | 100 |
| ERK5 | MAPK7 | 100 | 100 |
| ERK8 | MAPK15 | 100 | 97 |
| ERN1 | ERN1 | 90 | 90 |
| FAK | PTK2 | 100 | 100 |
| FER | FER | 85 | 98 |
| FES | FES | 99 | 100 |
| FGFR1 | FGFR1 | 100 | 95 |
| FGFR2 | FGFR2 | 100 | 100 |
| FGFR3 | FGFR3 | 78 | 100 |
| FGFR3(G697C) | FGFR3 | 91 | 78 |
| FGFR4 | FGFR4 | 97 | 94 |
| FGR | FGR | 100 | 100 |
| FLT1 | FLT1 | 94 | 100 |
| FLT3 | FLT3 | 100 | 100 |
| FLT3(D835H) | FLT3 | 94 | 100 |
| FLT3(D835V) | FLT3 | 84 | 100 |
| FLT3(D835Y) | FLT3 | 95 | 100 |
| FLT3(ITD) | FLT3 | 85 | 94 |
| FLT3(ITD,D835V) | FLT3 | 76 | 95 |
| Protein                  | Gene   | Normal   | Abnormal |
|-------------------------|--------|----------|----------|
| FLT3(ITD,F691L)         | FLT3   | 72       | 100      |
| FLT3(K663Q)             | FLT3   | 91       | 100      |
| FLT3(N841I)             | FLT3   | 99       | 87       |
| FLT3(R834Q)             | FLT3   | 78       | 90       |
| FLT3-autoinhibited      | FLT3   | 79       | 71       |
| FLT4                    | FLT4   | 97       | 97       |
| FRK                     | FRK    | 99       | 100      |
| FYN                     | FYN    | 100      | 90       |
| GAK                     | GAK    | 96       | 100      |
| GCN2(Kin.Dom.2,S808G)   | EIF2AK4| 100      | 100      |
| GRK1                    | GRK1   | 100      | 92       |
| GRK2                    | ADRBK1 | 77       | 94       |
| GRK3                    | ADRBK2 | 74       | 92       |
| GRK4                    | GRK4   | 97       | 100      |
| GRK7                    | GRK7   | 100      | 98       |
| GSK3A                   | GSK3A  | 100      | 70       |
| GSK3B                   | GSK3B  | 86       | 87       |
| HASPIN                  | GSG2   | 92       | 100      |
| HCK                     | HCK    | 99       | 98       |
| HIPK1                   | HIPK1  | 92       | 92       |
| HIPK2                   | HIPK2  | 94       | 83       |
| HIPK3                   | HIPK3  | 85       | 77       |
| HIPK4                   | HIPK4  | 88       | 92       |
| HPK1                    | MAP4K1 | 97       | 100      |
| HUNK                    | HUNK   | 100      | 94       |
| ICK                     | ICK    | 92       | 67       |
| IGF1R                   | IGF1R  | 93       | 88       |
| IKK-alpha               | CHUK   | 81       | 78       |
| IKK-beta                | IKBKB  | 91       | 84       |
| IKK-epsilon             | IKBKE  | 86       | 87       |
| INSR                    | INSR   | 100      | 61       |
| INSRR                   | INSRR  | 100      | 92       |
| IRAK1                   | IRAK1  | 76       | 88       |
| IRAK3                   | IRAK3  | 86       | 100      |
| IRAK4                   | IRAK4  | 66       | 77       |
| ITK                     | ITK    | 100      | 100      |
| JAK1(JH1domain-catalytic)| JAK1  | 92       | 100      |
| JAK1(JH2domain-pseudokinase) | JAK1 | 86   | 86       |
| JAK2(JH1domain-catalytic) | JAK2  | 97       | 71       |
| JAK3(JH1domain-catalytic) | JAK3  | 82       | 100      |
| JNK1                    | MAPK8  | 67       | 90       |
| JNK2                    | MAPK9  | 86       | 85       |
| JNK3                    | MAPK10 | 97       | 67       |
| KIT                     | KIT    | 100      | 97       |
| Protein | Protein | Activity |
|---------|---------|----------|
| KIT(A829P) | KIT | 80 90 |
| KIT(D816H) | KIT | 70 100 |
| KIT(D816V) | KIT | 100 100 |
| KIT(L576P) | KIT | 95 83 |
| KIT(V559D) | KIT | 100 95 |
| KIT(V559D,T670I) | KIT | 99 100 |
| KIT(V559D,V654A) | KIT | 89 87 |
| KIT-autoinhibited | KIT | 96 88 |
| LATS1 | LATS1 | 93 100 |
| LATS2 | LATS2 | 100 94 |
| LCK | LCK | 96 100 |
| LIMK1 | LIMK1 | 96 100 |
| LIMK2 | LIMK2 | 97 97 |
| LKB1 | STK11 | 100 65 |
| LOK | STK10 | 100 97 |
| LRRK2 | LRRK2 | 77 99 |
| LRRK2(G2019S) | LRRK2 | 100 91 |
| LTK | LTK | 100 100 |
| LYN | LYN | 98 100 |
| LZK | MAP3K13 | 77 98 |
| MAK | MAK | 100 91 |
| MAP3K1 | MAP3K1 | 79 80 |
| MAP3K15 | MAP3K15 | 97 80 |
| MAP3K2 | MAP3K2 | 91 95 |
| MAP3K3 | MAP3K3 | 100 86 |
| MAP3K4 | MAP3K4 | 88 100 |
| MAP4K2 | MAP4K2 | 100 56 |
| MAP4K3 | MAP4K3 | 100 100 |
| MAP4K4 | MAP4K4 | 100 100 |
| MAP4K5 | MAP4K5 | 100 100 |
| MAPKAPK2 | MAPKAPK2 | 71 100 |
| MAPKAPK5 | MAPKAPK5 | 94 100 |
| MARK1 | MARK1 | 100 96 |
| MARK2 | MARK2 | 93 100 |
| MARK3 | MARK3 | 86 100 |
| MARK4 | MARK4 | 92 100 |
| MAST1 | MAST1 | 100 69 |
| MEK1 | MAP2K1 | 97 88 |
| MEK2 | MAP2K2 | 100 88 |
| MEK3 | MAP2K3 | 98 79 |
| MEK4 | MAP2K4 | 100 90 |
| MEK5 | MAP2K5 | 97 86 |
| MEK6 | MAP2K6 | 100 89 |
| MELK | MELK | 100 86 |
| Protein | Annotation | Percent | Activity |
|---------|------------|---------|----------|
| MERTK  | MERTK      | 100     | 68       |
| MET    | MET        | 100     | 91       |
| MET(M1250T) | MET     | 88      | 100      |
| MET(Y1235D) | MET    | 100     | 98       |
| MINK   | MINK1     | 94      | 100      |
| MKK7   | MAP2K7    | 97      | 98       |
| MKNK1  | MKNK1     | 94      | 80       |
| MKNK2  | MKNK2     | 91      | 64       |
| MLCK   | MYLK3     | 78      | 97       |
| MLK1   | MAP3K9    | 100     | 92       |
| MLK2   | MAP3K10   | 100     | 93       |
| MLK3   | MAP3K11   | 100     | 100      |
| MRCKA  | CDC42BPA  | 100     | 100      |
| MRCKB  | CDC42BPB  | 100     | 100      |
| MST1   | STK4      | 95      | 100      |
| MST1R  | MST1R     | 100     | 98       |
| MST2   | STK3      | 100     | 90       |
| MST3   | STK24     | 100     | 100      |
| MST4   | MST4      | 100     | 100      |
| MTOR   | MTOR      | 93      | 91       |
| MUSK   | MUSK      | 100     | 80       |
| MYLK   | MYLK      | 73      | 95       |
| MYLK2  | MYLK2     | 100     | 95       |
| MYLK4  | MYLK4     | 99      | 99       |
| MYO3A  | MYO3A     | 100     | 100      |
| MYO3B  | MYO3B     | 91      | 93       |
| NDR1   | STK38     | 100     | 100      |
| NDR2   | STK38L    | 98      | 100      |
| NEK1   | NEK1      | 95      | 99       |
| NEK10  | NEK10     | 100     | 95       |
| NEK11  | NEK11     | 55      | 99       |
| NEK2   | NEK2      | 96      | 100      |
| NEK3   | NEK3      | 87      | 77       |
| NEK4   | NEK4      | 83      | 97       |
| NEK5   | NEK5      | 96      | 94       |
| NEK6   | NEK6      | 100     | 100      |
| NEK7   | NEK7      | 99      | 100      |
| NEK9   | NEK9      | 98      | 100      |
| NIK    | MAP3K14   | 91      | 79       |
| NIM1   | MGC42105  | 100     | 74       |
| NLK    | NLK       | 99      | 100      |
| OSR1   | OXSR1     | 93      | 84       |
| p38-alpha | MAPK14 | 100     | 100      |
| p38-beta | MAPK11  | 88      | 100      |
| Protein                      | Gene       | p38-delta | p38-gamma | PAK1 | PAK2 | PAK3 | PAK4 | PAK6 | PAK7 | PCTK1 | PCTK2 | PCTK3 | PDGFRA | PDGFRB | PDPK1 | PFCDPK1(P.falciparum) | PFPK5(P.falciparum) | PFTAIRE2 | PFTK1 | PHKG1 | PHKG2 | PIK3C2B | PIK3C2G | PIK3CA | PIK3CA(C420R) | PIK3CA(E542K) | PIK3CA(E545A) | PIK3CA(E545K) | PIK3CA(H1047L) | PIK3CA(H1047Y) | PIK3CA(I800L) | PIK3CA(M1043I) | PIK3CA(Q546K) | PIK3CB | PIK3CD | PIK3CG | PIK4CB | PIKFYVE | PIM1 | PIM2 | PIM3 | PIP5K1A | PIP5K1C | PIP5K2B | PIP5K2C | PIP4K2C |
|-----------------------------|------------|-----------|-----------|------|------|------|------|------|------|------|-------|-------|-------|---------|---------|-------|---------------------|---------------------|----------|-------|-------|--------|--------|--------|-------|----------|----------|-----------|----------|------------|-----------|----------|-----------|----------|---------|-------|-------|-------|--------|--------|--------|--------|--------|
| gene                  | protein   | ratio (new) | ratio (old) |
|-----------------------|-----------|-------------|-------------|
| PKAC-alpha            | PRKACA    | 90          | 82          |
| PKAC-beta             | PRKACB    | 100         | 100         |
| PKMYT1                | PKMYT1    | 96          | 100         |
| PKN1                  | PKN1      | 95          | 88          |
| PKN2                  | PKN2      | 96          | 92          |
| PKNB(M. tuberculosis) | pknB      | 97          | 61          |
| PLK1                  | PLK1      | 100         | 91          |
| PLK2                  | PLK2      | 89          | 100         |
| PLK3                  | PLK3      | 91          | 85          |
| PLK4                  | PLK4      | 72          | 83          |
| PRKCD                 | PRKCD     | 100         | 87          |
| PRKCE                 | PRKCE     | 100         | 100         |
| PRKCH                 | PRKCH     | 96          | 100         |
| PRKCI                 | PRKCI     | 100         | 100         |
| PRKCQ                 | PRKCQ     | 70          | 100         |
| PRKD1                 | PRKD1     | 90          | 94          |
| PRKD2                 | PRKD2     | 100         | 100         |
| PRKD3                 | PRKD3     | 83          | 96          |
| PRKG1                 | PRKG1     | 98          | 100         |
| PRKG2                 | PRKG2     | 94          | 87          |
| PRKR                  | EIF2AK2   | 100         | 70          |
| PRKX                  | PRKX      | 100         | 100         |
| PRP4                  | PRPF4B    | 78          | 100         |
| PYK2                  | PTK2B     | 99          | 100         |
| QSK                   | KIAA0999  | 98          | 100         |
| RAF1                  | RAF1      | 99          | 99          |
| RET                   | RET       | 100         | 100         |
| RET(M918T)            | RET       | 100         | 93          |
| RET(V804L)            | RET       | 100         | 94          |
| RET(V804M)            | RET       | 96          | 100         |
| RIOK1                 | RIOK1     | 83          | 66          |
| RIOK2                 | RIOK2     | 94          | 64          |
| RIOK3                 | RIOK3     | 81          | 100         |
| RIPK1                 | RIPK1     | 100         | 100         |
| RIPK2                 | RIPK2     | 98          | 97          |
| RIPK4                 | RIPK4     | 93          | 91          |
| RIPK5                 | DSTYK     | 95          | 78          |
| ROCK1                 | ROCK1     | 80          | 100         |
| ROCK2                 | ROCK2     | 99          | 96          |
| ROS1                  | ROS1      | 98          | 97          |
| RPS6KA4(Kin.Dom.1-N-terminal) | RPS6KA4 | 100       | 100         |
| RPS6KA4(Kin.Dom.2-C-terminal) | RPS6KA4 | 99       | 100         |
| RPS6KA5(Kin.Dom.1-N-terminal) | RPS6KA5 | 96       | 100         |
| RPS6KA5(Kin.Dom.2-C-terminal) | RPS6KA5 | 98       | 100         |
| Kinase | Reference Gene | % Phosphorylation |
|--------|----------------|-------------------|
| RSK1 (Kin.Dom.1-N-terminal) | RPS6KA1 | 96 100 |
| RSK1 (Kin.Dom.2-C-terminal) | RPS6KA1 | 100 100 |
| RSK2 (Kin.Dom.1-N-terminal) | RPS6KA3 | 86 83 |
| RSK2 (Kin.Dom.2-C-terminal) | RPS6KA3 | 100 100 |
| RSK3 (Kin.Dom.1-N-terminal) | RPS6KA2 | 99 100 |
| RSK3 (Kin.Dom.2-C-terminal) | RPS6KA2 | 100 96 |
| RSK4 (Kin.Dom.1-N-terminal) | RPS6KA6 | 86 83 |
| RSK4 (Kin.Dom.2-C-terminal) | RPS6KA6 | 91 100 |
| S6K1 | RPS6KB1 | 95 100 |
| SBK1 | SBK1 | 95 73 |
| SGK | SGK1 | 94 92 |
| SgK110 | SgK110 | 89 100 |
| SGK2 | SGK2 | 91 100 |
| SGK3 | SGK3 | 91 100 |
| SIK | SIK1 | 98 100 |
| SIK2 | SIK2 | 99 100 |
| SLK | SLK | 95 100 |
| SNARK | NUAK2 | 97 94 |
| SNRK | SNRK | 87 97 |
| SRC | SRC | 91 100 |
| SRMS | SRMS | 100 97 |
| SRPK1 | SRPK1 | 100 100 |
| SRPK2 | SRPK2 | 63 100 |
| SRPK3 | SRPK3 | 94 100 |
| STK16 | STK16 | 92 100 |
| STK33 | STK33 | 100 95 |
| STK35 | STK35 | 96 100 |
| STK36 | STK36 | 100 100 |
| STK39 | STK39 | 97 86 |
| SYK | SYK | 100 77 |
| TAK1 | MAP3K7 | 98 93 |
| TAOK1 | TAOK1 | 100 78 |
| TAOK2 | TAOK2 | 88 89 |
| TAOK3 | TAOK3 | 98 94 |
| TBK1 | TBK1 | 85 78 |
| TEC | TEC | 96 100 |
| TESK1 | TESK1 | 96 100 |
| TGFBR1 | TGFBR1 | 92 100 |
| TGFBR2 | TGFBR2 | 85 97 |
| TIE1 | TIE1 | 100 74 |
| TIE2 | TEK | 97 100 |
| TLK1 | TLK1 | 100 100 |
| TLK2 | TLK2 | 100 97 |
| TNIK | TNIK | 100 100 |
| Gene       | Protein    | %100   | %90   |
|------------|------------|--------|-------|
| TNK1       | TNK1       | 95     | 100   |
| TNK2       | TNK2       | 98     | 100   |
| TNN13K     | TNN13K     | 90     | 99    |
| TRKA       | NTRK1      | 100    | 99    |
| TRKB       | NTRK2      | 100    | 100   |
| TRKC       | NTRK3      | 90     | 90    |
| TRPM6      | TRPM6      | 100    | 100   |
| TSSK1B     | TSSK1B     | 84     | 100   |
| TSSK3      | TSSK3      | 100    | 95    |
| TTK        | TTK        | 100    | 98    |
| TXK        | TXK        | 95     | 100   |
| TYK2(JH1domain-catalytic) | TYK2 | 98     | 88    |
| TYK2(JH2domain-pseudokinase) | TYK2 | 99     | 93    |
| TYRO3      | TYRO3      | 90     | 80    |
| ULK1       | ULK1       | 100    | 74    |
| ULK2       | ULK2       | 74     | 76    |
| ULK3       | ULK3       | 92     | 78    |
| VEGFR2     | KDR        | 89     | 93    |
| VPS34      | PIK3c3     | 92     | 97    |
| VRK2       | VRK2       | 89     | 89    |
| WEE1       | WEE1       | 100    | 100   |
| WEE2       | WEE2       | 91     | 100   |
| WNK1       | WNK1       | 94     | 97    |
| WNK2       | WNK2       | 94     | 100   |
| WNK3       | WNK3       | 97     | 50    |
| WNK4       | WNK4       | 98     | 100   |
| YANK1      | STK32A     | 99     | 100   |
| YANK2      | STK32B     | 97     | 96    |
| YANK3      | STK32C     | 96     | 100   |
| YES        | YES1       | 97     | 100   |
| YSK1       | STK25      | 90     | 99    |
| YSK4       | MAP3K19    | 84     | 82    |
| ZAK        | ZAK        | 95     | 100   |
| ZAP70      | ZAP70      | 100    | 100   |
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