Novel indazole-based small compounds enhance TRAIL-induced apoptosis by inhibiting the M KK7-TIPRL interaction in hepatocellular carcinoma

SUPPLEMENTARY MATERIALS

MATERIALS AND METHODS

Preparation of 1-(5-acetyl-2,4-dichlorophenyl)-5-amino-1H-indazole (1)

A mixture of 5-amino-1H-indazole (6.40 g, 48.1 mmol), 1-acetyl-2,4-dichloro-5-fluorobenene (10.0 g, 48.3 mmol), and potassium carbonate (66.8 g, 483 mmol) in N,N-dimethylformamide (200 mL) was heated to 110 °C for 20 h. After cooling to room temperature, the resulting mixture was partitioned between dichloromethane and water and the organic layer was washed with water several times and dried over magnesium sulfate. The solvent was evaporated in vacuo to afford the indazole 1 (1.00 g, 6 %). 1H NMR (500 MHz, CDCl3): δ 8.11 (s, 1 H), 8.05 (s, 1 H), 7.94 (s, 1 H), 7.09 (d, J = 8.2 Hz, 1 H), 7.30 (t, J = 8.7 Hz, 2 H), 7.23 (t, J = 9.3 Hz, 1 H), 7.08 (m, 4 H); MS (ESI): m/z for C15H11Cl2N2O3, found 258 [M+H+]

Preparation of 1-(4-fluorophenyl)-5-nitro-1H-indazole (3, TRT-0029)

Benzenesulfonyl chloride (14 µL, 0.11 mmol) was added to a solution of compound 1 (30 mg, 0.094 mmol) and pyridine (11 µL, 0.14 mmol) in dichloromethane (2 mL) at room temperature and the resulting mixture was stirred at the same temperature for 15 h. The reaction mixture was partitioned between dichloromethane and water, and the organic layer was dried over magnesium sulfate. The solvent was evaporated in vacuo, and the residue was separated by chromatography on a silica gel column (n-hexane : ethyl acetate = 1 : 1) to give the final product 3 (25 mg, 58 %). 1H NMR (500 MHz, CDCl3): δ 8.18 (s, 1 H), 7.76 (t, J = 7.5 Hz, 3 H), 7.72 (s, 1 H), 7.57-7.54 (m, 2 H), 7.46 (t, J = 8.00 Hz, 2 H), 7.13 (d, J = 1.5 Hz, 2 H), 6.69 (s, 1 H), 2.70 (s, 3 H); MS (ESI): m/z for C15H11Cl2N2O3, found 460 [M+H+]

Preparation of 1-(2-fluoro-5-nitrobenzylidene)-2-(4-fluorophenyl)hydrazine (7)

4-fluorophenylhydrazine (37 mg, 0.29 mmol) and toluenesulfonic acid monohydrate (3.0 mg, 0.02 mmol) were added to a solution of 2-fluoro-5-nitrobenzaldehyde (50 mg, 0.27 mmol) in ethanol, and the mixture was stirred at reflux for 1 h. After cooling to room temperature, the precipitated solid was collected by filtration to give the hydrazone 7 (65 mg, 87 %). 1H NMR (300 MHz, CDCl3); δ 8.84 (dd, J = 6.2, 2.9 Hz, 1 H), 8.12 (m, 1 H), 7.86 (s, 1 H), 7.23 (t, J = 9.3 Hz, 1 H), 7.08 (m, 4 H); MS (ESI): m/z for C15H14F2N4O2, found 278 [M+H+]

Preparation of 1-(4-fluorophenyl)-5-nitro-1H-indazole (8)

A mixture of compound 7 (61 mg, 0.22 mmol) and potassium carbonate (134 mg, 0.97 mmol) in N,N-dimethylformamide (2 mL) was heated to 100 °C for 15 h. After cooling to room temperature, the reaction mixture was partitioned between ethyl acetate and water, and the organic layer was washed with water several times and dried over magnesium sulfate. The solvent was evaporated in vacuo, and the residue was separated by chromatography on a silica gel column (n-hexane : ethyl acetate = 1 : 1) to give the final product 8 (17 mg, 30 %). 1H NMR (300 MHz, CDCl3): δ 8.80 (d, J = 2.0 Hz, 1 H), 8.40 (s, 1 H), 8.32 (dd, J = 9.2, 2.1 Hz, 1 H), 7.68 (m, 3 H), 7.30 (t, J = 6.1 Hz, 2 H); MS (ESI): m/z for C15H14F2N4O2, found 258 [M+H+]

Preparation of 5-amino-1-(4-fluorophenyl)-1H-indazole (9)

To a solution of compound 8 (17 mg, 0.066 mmol) in dichloromethane (5 mL), 10 % palladium on carbon (10 mg) was added at room temperature, and the mixture was shaken under a hydrogen atmosphere (60–70 psi) at the same temperature for 5 h. The reaction mixture was filtered through Celite and the filtrate was evaporated in vacuo to afford the indazole 9 (14 mg, 93 %). 1H NMR (300 MHz, CDCl3): δ 8.03 (s, 1 H), 7.74 (dd, J = 8.1, 5.2 Hz, 2 H), 7.55 (d, J = 8.9 Hz, 1 H), 7.38 (t, J = 8.7 Hz, 2 H), 6.88 (t, J = 8.2 Hz, 2 H), 5.00 (s, 1 H); MS (ESI): m/z for C15H18FN3, found 228 [M+H+]

Preparation of 1-(4-fluorophenyl)-5-(4-methoxybenzenesulfonylamo)-1H-indazole (4, TRT-0173)

4-methoxybenzenesulfonyl chloride (8 mg, 0.04 mmol) was added to a solution of compound 9 (7 mg, 0.03
mmol) and pyridine (4 µL, 0.05 mmol) in dichloromethane (2 mL) at room temperature, and the mixture was stirred at the same temperature for 15 h. The reaction mixture was partitioned between dichloromethane and water, and the organic layer was dried over magnesium sulfate. The solvent was evaporated in vacuo and the residue was chromatographed on a silica gel column (n-hexane : ethyl acetate = 4 : 1) to give the final product 4 (8 mg, 70 %).

1H NMR (500 MHz, CDCl3): δ 8.13 (s, 1 H), 7.69-7.64 (m, 4 H), 7.61 (d, J = 9.0 Hz, 1 H), 7.50 (d, J = 1.8 Hz, 1 H), 7.25 (t, J = 8.6 Hz, 2 H), 7.17 (dd, J = 9.0, 2.0 Hz, 1 H), 6.91 (dd, J = 7.2, 1.7 Hz, 2 H), 6.55 (s, 1 H), 3.85 (s, 1 H); MS (ESI): m/z for C20H16FN3O3S, found 398 [M+H+].

Supplementary Figure 1: The hit chemical compound, TRT-0002, enhances TRAIL-induced apoptosis by inhibiting MKK7-TIPRL interaction. (A) ELISA analysis results of 6,000 compounds. (B) Inhibition of the MKK7-TIPRL interaction by TRT-0002 using an ELISA system. (C) MTT assay for the viability of Huh7 cells treated with TRAIL, TRT-0002, or a combination of TRAIL and TRT-0002 for 48 h. Differences in the viabilities of treated cells were assessed using Student’s t-test (***p < 0.001). (D) Huh7 cells were treated with TRT-0002 (40 µM) and TRAIL (100 ng/ml) for 48 h. The levels of PARP and caspase-3 expression were examined by Western blotting. GAPDH was used as a loading control. (E) Huh7 cells were treated with 40 µM TRT-0002 in the presence or absence of 100 ng/ml TRAIL for the indicated time periods. The phosphorylation of JNK was examined by Western blotting.
Supplementary Figure 3: Lead compounds enhance TRAIL-induced cell death in HepG2 cells, but not in normal cells. (A) MTT assay of HepG2 cells following treatment with lead compounds, TRAIL, or a combination of lead compounds (10 μM for TRT-0029 and 20 μM for TRT-0173) and TRAIL (100 ng/ml) for 24 h. Differences in cell viabilities were assessed using Student’s t-test (**p < 0.01, ***p < 0.001). (B) WI-38 cells were treated with lead compounds and/or TRAIL for 24 h. The MTT assay was performed to measure cell viability.
Supplementary Table 1: Average Z’factor of over 6,000 chemical compounds in an in-house chemical library

| Plate number | Z’ factor<sup>a</sup> | Plate number | Z’ factor<sup>a</sup> |
|--------------|-----------------------|--------------|-----------------------|
| 1            | 0.52                  | 11           | 0.57                  |
| 2            | 0.79                  | 12           | 0.58                  |
| 3            | 0.66                  | 13           | 0.51                  |
| 4            | 0.67                  | 14           | 0.53                  |
| 5            | 0.47                  | 15           | 0.60                  |
| 6            | 0.59                  | 16           | 0.70                  |
| 7            | 0.50                  | 17           | 0.76                  |
| 8            | 0.50                  | 18           | 0.50                  |
| 9            | 0.65                  | 19           | 0.71                  |
| 10           | 0.66                  | 20           | 0.63                  |

<sup>a</sup>Z’factor’ = 1-((3 × SD of sample + 3x SD of control)/(mean of sample – mean of control))

Supplementary Figure 4: Treatments with lead compounds and TRAIL do not show general cytotoxicity in the Huh7 tumor xenograft model. (A) The body weights of mice at 0 and 11 days. (B) Representative image of H&E stained liver tissues after 11 days. Pictures were taken at a magnification of × 400.
## Supplementary Table 2: Screening of hit compounds using ELISA and MTT assay

| Compounds | ELISA IC\(_{50}\) (µM) | Cell viability assay (%) |
|-----------|--------------------------|--------------------------|
|           |                          | Compound only            | Compound + TRAIL         |
| 1         | 2.7                      | 88                       | 87                       |
| 2         | 5.9                      | 89                       | 42                       |
| 3         | 1.7                      | 42                       | 22                       |
| 4         | 0.089                    | 86                       | 91                       |
| 5         | 1.4                      | 81                       | 79                       |
| 6         | 2.0                      | 93                       | 99                       |
| 7         | 8.9                      | 89                       | 88                       |
| 8         | 2.8                      | 69                       | 84                       |
| 9         | 1.0                      | 90                       | 96                       |
| 10        | 3.5                      | 111                      | 113                      |
| 11        | 3.6                      | 86                       | 97                       |
| 12        | 0.37                     | 30                       | 24                       |
| 13        | 5.1                      | 87                       | 89                       |
| 14        | 19.8                     | N.D                      | N.D                      |
| 15        | 0.77                     | 91                       | 95                       |
| 16        | 1.0                      | 80                       | 51                       |
| 17        | 0.89                     | 96                       | 95                       |
| 18        | 12.7                     | 89                       | 86                       |
| 19        | 0.036                    | 6                        | 6                        |
| 20        | 3.5                      | 102                      | 113                      |
| 21        | 0.15                     | 84                       | 80                       |
| 22        | 4.7                      | 59                       | 10                       |
| 23        | 7.5                      | 87                       | 82                       |
| 24        | 17.2                     | N.D                      | N.D                      |
| 25        | 0.34                     | 93                       | 60                       |
| 26        | 2.7                      | 97                       | 91                       |
| 27        | 1.3                      | 40                       | 40                       |

\(IC_{50}\): half maximal inhibitory concentration

N.D: Not Determined
Supplementary Table 3: Selection of the lead compounds (TRT-0029 and TRT-0173) from 280 analogs of TRT-0002

| Compound  | Cell viability assay | Apoptotic protein detection | Caspase 3 activity | JNK phosphorylation | Inhibition assay of MKK7-TIPRL |
|-----------|----------------------|------------------------------|------------------|-------------------|-------------------------------|
|           | Woking concentration (µM) | Treatment time (hrs) | Compound only (%) | Compound + TRAIL (%) | Compound + TRAIL/Compound (ratio) |                               |
| TRT-0002  | 40                   | 48                           | 81               | 29                | +++                          | 1.80                         | ++                            | ++                            |
| TRT-0004  | 40                   | 24                           | 84               | 34                | +++                          | 2.58                         | ND                            |                               |
| TRT-0006  | 40                   | 24                           | 62               | 23                | +++                          | 1.01                         |                               |                                |
| TRT-0029  | 10                   | 24                           | 69               | 11                | +++                          | 2.20                         | +++                          | +++                            |
| TRT-0030  | 20                   | 24                           | 51               | 23                | ND                           |                               |                               |                                |
| TRT-0049  | 40                   | 24                           | 61               | 25                | +++                          | 3.81                         | ND                            |                               |
| TRT-0051  | 40                   | 24                           | 54               | 28                | +++                          | 1.67                         |                               |                                |
| TRT-0060  | 40                   | 24                           | 50               | 36                | ND                           |                               |                               |                                |
| TRT-0061  | 40                   | 24                           | 85               | 69                | +++                          | 2.94                         | ND                            |                               |
| TRT-0062  | 40                   | 24                           | 86               | 64                | +++                          | 1.69                         |                               |                                |
| TRT-0065  | 40                   | 24                           | 88               | 72                | ND                           |                               |                               |                                |
| TRT-0083  | 40                   | 24                           | 52               | 36                | +++                          | 1.67                         |                               |                                |
| TRT-0086  | 40                   | 24                           | 59               | 43                | +++                          | 5.71                         | ND                            |                               |
| TRT-0088  | 40                   | 24                           | 50               | 34                | +++                          | 4.77                         | ND                            |                               |
| TRT-0105  | 10                   | 24                           | 88               | 48                | +                            | 5.01                         | ND                            |                               |
| TRT-0106  | 40                   | 24                           | 42               | 24                | +++                          | 5.48                         | ND                            |                               |
| TRT-0173  | 10                   | 24                           | 53               | 7                 | +++                          | 2.10                         | +++                          | +++                            |
| TRT-0208  | 20                   | 24                           | 92               | 6                 | +++                          | 1.21                         |                               |                                |
| TRT-0211  | 20                   | 24                           | 87               | 4                 | +++                          | 1.47                         |                               |                                |

a : Expression levels of cleaved caspase-3 and PARP on western blotting (Compound + TRAIL/TRAIl)
b : Phosphorylation levels of JNK on western blotting (Compound + TRAIL/Compound)
c : Inhibition activities of MKK7-TIPRL on GST pull-down assay (Compound + TRAIL/TRAIl)