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Hsp70 inhibitors have great potential as chemical probes and anticancer agents. Thus, it is important to elucidate their modes of action on cancer cell death. This protocol describes a step-by-step process for the synthesis of apoptozole as an inhibitor of Hsp70, analysis of internalization of apoptozole into lysosomes, and assessment of lysosomal membrane permeabilization induced by apoptozole. The current protocol can be used for detailed mechanistic studies of Hsp70 inhibitors and further substances targeting lysosomal proteins on cancer cell death.
Protocol

Synthesis of an Hsp70 inhibitor and its assessment of lysosomal membrane permeabilization

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SUMMARY

Hsp70 inhibitors have great potential as chemical probes and anticancer agents. Thus, it is important to elucidate their modes of action on cancer cell death. This protocol describes a step-by-step process for the synthesis of apoptozole as an inhibitor of Hsp70, analysis of internalization of apoptozole into lysosomes, and assessment of lysosomal membrane permeabilization induced by apoptozole. The current protocol can be used for detailed mechanistic studies of Hsp70 inhibitors and further substances targeting lysosomal proteins on cancer cell death. For complete information on the use and execution of this protocol, please refer to Park et al. (2018).

BEFORE YOU BEGIN

Note: All reagents were purchased from commercial suppliers and used without further purification.

Cell culture medium

© Timing: 10 min

1. Prepare the cell culture medium by mixing 450 mL of DMEM, 50 mL of fetal bovine serum, and 2.5 mL of a solution containing 10,000 units/mL of penicillin and 10 mg/mL of streptomycin in a sterile environment. Culture HeLa cancer cells in the above cell culture medium through this study. The cell culture medium can be stored at 4°C for several months without any problem. The cell culture medium should be warmed to 25°C prior to cell experiments.

Preparation of well plates with cover slips

© Timing: 0.1 h

2. Place sterile glass cover slips into a sterile 24-well culture plate.
3. Add 0.5 mL DPBS to each well.
4. Remove DPBS from each well by pipetting.

Cell culture and seeding

© Timing: 49 h
5. Culture HeLa cells in a 10 cm cell culture dish containing 8 mL cell culture medium in an incubator with 5% CO₂ for 24 h at 37°C.
6. Remove the cell culture medium from the dish and wash the cells with 3 mL DPBS once.
7. Add 1.5 mL of trypsin-EDTA solutions to the cells and place the dish in an incubator with 5% CO₂ for 5 min at 37°C.
8. Re-suspend the cells in 5 mL cell culture medium and transfer them to a 15 mL conical tube.
9. Centrifuge the cells at 260 × g for 3 min at 25°C.
10. Remove the cell culture medium from the conical tube and re-suspend the cells in 3 mL cell culture medium.
11. Count the number of cells under a microscope using a hemocytometer.
12. Seed 2 mL cell solutions (containing ca. 5 × 10⁵ cells) in a 6-well plate or 0.5 mL cell solutions (containing ca. 1 × 10⁵ cells) in a 24-well plate with a cover slip at the bottom of each well, prepared from steps 2–4, and place the plate in an incubator with 5% CO₂ for 24 h at 37°C.

**Note:** Cell seeding is chosen according to experiments. Isolation of lysosomes and western blotting can be conducted with a 6-well plate, and cell imaging experiments can be performed with a 24-well plate.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse monoclonal cathepsin B | Santa Cruz Biotechnology | Cat#sc-365558 |
| Mouse monoclonal β-actin | Santa Cruz Biotechnology | Cat#sc-47778 |
| Chemicals, peptides, and recombinant proteins | | |
| Acetic acid, glacial | Samchun chemicals | Cat#A0051 |
| Acetonitrile | Avantor J.T. Backer | Cat#901203 |
| Acridine orange | Immunochemistry Technologies | Cat#6130 |
| 4-(Aminomethyl)benzoic acid | Alfa Aesar | Cat#B23519 |
| Ammonium acetate | Sigma-Aldrich | Cat#A7262 |
| Apoptozole | This article | N/A |
| 3,5-Bis(trifluoromethyl)benzaldehyde | Tokyo Chemical Industry | Cat#B1751 |
| Bromophenol blue | Sigma-Aldrich | Cat#B5525 |
| tert-Butyl-2-[2-(2-aminoethoxy)ethoxy]ethylcarbamate | Sigma-Aldrich | Cat#89761 |
| Chloroform-D′ (CDCl₃) | Cambridge Isotope Laboratory | Cat#DLM-7 |
| Compound 1 | This article | N/A |
| Compound 2 | This article | N/A |
| Dichloromethane | Duksan Pure Chemicals | N/A |
| Digitonin | Sigma-Aldrich | Cat#D141 |
| 4,4′-Dimethoxybenzil | Tokyo Chemical Industry | Cat#A1028 |
| 4-Dimethylaminopyridine (DMAP) | Alfa Aesar | Cat#A13016 |
| N,N-Dimethylformamide (DMF) | Duksan Pure Chemicals | N/A |
| Dimethylsulfoxide (DMSO) | VWR Life Science | Cat#67-68-5 |
| Dulbecco’s modified Eagle’s medium (DMEM) | Thermo Fisher Scientific | Cat#11995-073 |
| Dulbecco’s phosphate buffered saline (DPBS) | Welgene | Cat#LB-001-02 |
| Ethyl acetate | Duksan Pure Chemicals | N/A |
| 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) | Glentham Life Sciences | Cat#GP0849 |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma-Aldrich | Cat#EDS |
| Ethyleneglycolbistetraacetic acid (EGTA) | Sigma-Aldrich | Cat#324626 |
| Fetal bovine serum (FBS) | Thermo Fisher Scientific | Cat#26140079 |
| Glycerol | Amresco | Cat#0942C411 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HEPES               | Sigma-Aldrich | Cat#H3375 |
| Hexane              | Duksan Pure Chemicals | N/A |
| Magnesium chloride (MgCl₂) | Sigma-Aldrich | Cat#M1028 |
| β-Mercaptoethanol   | Sigma-Aldrich | Cat#M3148 |
| Mounting solution   | Sigma-Aldrich | Cat#F4680 |
| Nitrogen gas        | Dong-A specialty gases | N/A |
| PBS                 | Intron | Cat#BP007A |
| Pefabloc            | Sigma-Aldrich | Cat#11429868001 |
| Penicillin/streptomycin solution | Thermo Fisher Scientific | Cat#15140122 |
| Polyvinylidene fluoride (PVDF) membrane | Merck Millipore | Cat#IPVH00010 |
| Potassium chloride (KCl) | Sigma-Aldrich | Cat#K9541 |
| Protease inhibitor cocktail | Roche | Cat#11836153001 |
| Silica gel 60 (0.040–0.063 mm) | Merck Millipore | Cat#109385 |
| Sodium acetate      | Sigma-Aldrich | Cat#S2839 |
| Sodium bicarbonate (NaHCO₃) | Samchun Chemicals | Cat#S0343 |
| Sodium chloride (NaCl) | Samchun Chemicals | Cat#S0343 |
| Sodium deoxycholate | Sigma-Aldrich | Cat#D6750 |
| Sodium dodecyl sulfate (SDS) | Intron | Cat#BS503 |
| Sucrose             | Sigma-Aldrich | Cat#S0389 |
| Syringe filter      | Cole-Parmer | Cat#EW-81054-42 |
| TLC silica gel 60 F₂₅₄ | Merck Millipore | Cat#105715 |
| Trichloroacetic acid | Sigma-Aldrich | Cat#T6399 |
| Trifluoroacetic acid (TFA) | Daejung Chemicals | Cat#S0558-4400 |
| Tris                | Biopure | Cat#TRS001 |
| Trypsin-EDTA        | Sigma-Aldrich | Cat#T4174 |
| Tween 20            | Amresco | Cat#2283C019 |
| West-ZOL plus       | Intron | Cat#16021 |
| **Other**           |        |            |
| Buchner funnel      | N/A    | N/A        |
| Column              | Daihan Scientific | N/A |
| Gas tight syringe 10 mL | Hamilton | Cat#4015-S4010 |
| Heating mantle      | Daihan Scientific | Cat#DH.WHM12113 |
| Magnetic stirrer     | Daihan Scientific | Cat#DH.WHM03120 |
| Magnetic stirring bar | Daihan Scientific | Cat#CW.001.620 |
| Reflux condenser     | N/A    | N/A        |
| Rotary evaporator   | EYELA  | Cat#N-1200A |
| 25 mL round-bottom flask | Daihan Scientific | Cat#FL.Fla2176 |
| 50 mL round-bottom flask | Daihan Scientific | Cat#FL.Fla2178 |
| Rubber balloon       | Neotex | N/A        |
| Rubber septum        | Sigma-Aldrich | Cat#Z553964 |
| Separatory funnel 250 mL | Daihan Scientific | Cat#GL.149.209.04 |
| Separatory funnel 500 mL | Daihan Scientific | Cat#GL.149.209.05 |
| 100 mL two-neck round-bottom flask | Daihan Scientific | Cat#FL.Fla2213 |
| **Critical commercial assays** | | |
| Lysosome isolation kit | Abcam | Cat#ab234047 |
| **Experimental models: cell lines** | | |
| HeLa cervical cancer cells | Korea Cell Line Bank | Cat#10002 |
| **Software and algorithms** | | |
| ZEN 2011            | Zeiss microscopy | https://www.zeiss.com/microscopy/int/downloads/zen.html |
**MATERIALS AND EQUIPMENT**

- Waters 600 HPLC system equipped with a Waters 2489 dual channel UV detector and a Vydac De-nali 238DE54 reversed-phase C18 column for analysis of internalization of an inhibitor of Hsp70 into lysosomes. Any other HPLC system equipped with a UV detector and an analytical reversed-phase column can be used as an alternative.
- Syngene G:Box Chemiluminescent Imaging system for Western blotting. Any other chemiluminescence imaging system can be used as an alternative.
- Carl Zeiss LSM800 confocal microscope with a 40× water-immersion objective for fluorescence cell imaging. Any other confocal microscope with a 40× water-immersion objective and excitation lasers at 488 and 561 nm can be used as an alternative.
- Waters 3100 ESI mass spectrometer for analysis of the molecular mass of synthesized compounds. Any other soft ionization mass spectrometer (e.g., MALDI-TOF) can be used as an alternative.
- Bruker 400 MHz FT-NMR spectrometer for confirming the structure of synthesized compounds. Any other FT-NMR spectrometer at least 250 MHz can be used as an alternative.
- Ultrasonic homogenizer for lysis of lysosomes.

### Digitonin extraction buffer

| Reagent                   | Final concentration | Amount  |
|---------------------------|---------------------|---------|
| Sucrose (1 M)             | 250 mM              | 12.5 mL |
| HEPES pH 7.4 (1 M)        | 20 mM               | 1 mL    |
| KCl (1 M)                 | 10 mM               | 0.5 mL  |
| MgCl₂ (0.15 M)            | 1.5 mM              | 0.5 mL  |
| EDTA (0.5 M)              | 1 mM                | 0.1 mL  |
| EGTA (0.5 M)              | 1 mM                | 0.1 mL  |
| Pefabloc (0.1 M)          | 0.5 mM              | 0.25 mL |
| ddH₂O                     | n/a                 | 35.05 mL|
| **Total**                 | n/a                 | 50 mL   |

Store at 4°C for a month. Right before use, mix 995 µL of digitonin extraction buffer with 5 µL digitonin (5 µg/mL). Do not store or reuse digitonin-added buffer.

### 2× SDS loading buffer

| Reagent                   | Final concentration | Amount  |
|---------------------------|---------------------|---------|
| SDS (20%)                 | 4%                  | 20 mL   |
| Glycerol                  | 20%                 | 20 mL   |
| Tris pH 6.8 (1 M)         | 0.1 M               | 10 mL   |
| Bromophenol blue          | 0.2%                | 0.2 g   |
| ddH₂O                     | n/a                 | 42 mL   |
| **Total**                 | n/a                 | 100 mL  |

Store at 4°C for a month. Right before use, mix 980 µL of 2× SDS loading buffer with 20 µL β-mercaptoethanol. Do not store or reuse β-mercaptoethanol-added buffer.

### TBST buffer

| Reagent | Final concentration | Amount  |
|---------|---------------------|---------|
| Tris    | 0.5 M               | 24.22 g |
| NaCl    | 1.4 M               | 81.82 g |
| Tween 20| 0.05%              | 0.5 mL  |
| ddH₂O   | n/a                 | 999.5 mL|
| **Total**| n/a               | 1 L    |

Store at 25°C for three months. Adjust pH to 7.6 with concentrated HCl.
STEP-BY-STEP METHOD DETAILS

**Synthesis of 4-((2-(3,5-bis(trifluoromethyl)phenyl)-4,5-bis(4-methoxyphenyl)-1H-imidazol-1-yl)methyl)benzoic acid (1)**

© Timing: 14 h

This step describes how to prepare compound 1. See Figure 1 and Table 1.

1. Weigh the above amount of 4-(aminomethyl)benzoic acid, 3,5-bis(trifluoromethyl)benzaldehyde, 4,4'-dimethoxybenzil and ammonium acetate in a 100 mL two-neck round-bottom flask containing a magnetic stirring bar.
2. Equip the main neck of the two-neck round-bottom flask with reflux condenser and place rubber septa both on the top of the condenser and the side neck of the two-neck round-bottom flask. Equip the top of the condenser with a nitrogen-filled balloon.
3. Connect water flow to the condenser and add 60 mL of glacial acetic acid through the septum on the side neck using a syringe.
4. Put the reaction flask on heating mantle and heat to 100°C. Stir the solution for 12 h.
5. Cool the reaction mixture to 25°C and transfer the solution into 500 mL separatory funnel. Add 200 mL of ethyl acetate to the solution.
6. Wash the mixture with 100 mL of distilled water and saturated aqueous sodium bicarbonate solution three times each. Dry the organic layer with 10 g of anhydrous sodium sulfate for 15 min and then filter to remove sodium sulfate. Condense the filtrate using a rotary evaporator.
7. Add 50 mL of cold dichloromethane to precipitate the product and filter the solid using Buchner funnel. Wash the solid with 50 mL of cold dichloromethane. Dry the collected solid under reduced pressure.

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**RIPA buffer**

| Reagent                             | Final concentration | Amount |
|-------------------------------------|---------------------|--------|
| Tris pH 8.0 (1 M)                   | 10 mM               | 1 mL   |
| EDTA (0.5 M)                        | 5 mM                | 1 mL   |
| Trition X-100                       | 1%                  | 1 mL   |
| Sodium deoxycholate (10%)           | 0.5%                | 5 mL   |
| SDS (10%)                           | 0.1%                | 1 mL   |
| NaCl (5 M)                          | 150 mM              | 3 mL   |
| ddH₂O                               | n/a                 | 88 mL  |
| **Total**                           | **n/a**             | **100 mL** |

Store at 4°C for a month.

**Blocking solution**

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| FBS     | 3%                  | 1.5 mL |
| PBS     | n/a                 | 48.5 mL |
| **Total** | **n/a**               | **50 mL** |

Store at 4°C for a month.

---

Figure 1. Synthetic scheme of compound 1
See also Figure S1.
pressure to afford a pure compound 1 (4.35 g; yield = 53% from 4-(aminomethyl)benzoic acid). (See Troubleshooting 1 for additional information).

8. Characterize the product by NMR spectroscopy (¹H NMR and ¹³C NMR) and mass spectrometry (MS) (see the EXPECTED OUTCOMES section).

Pause point: The product can be stored at −20°C for at least 2 years.

**Synthesis of tert-butyl (2-(2-(2-(4-((2-(3,5-bis(trifluoromethyl)phenyl)-4,5-bis(4-methoxyphenyl)-1H-imidazol-1-yl)methyl)benzamido)ethoxy)ethoxy)ethyl)carbamate (2)**

Timing: 10 h

This step describes how to prepare compound 2. See Figure 2 and Table 2.

9. Weigh the above amount of compound 1, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP) in 50 mL round-bottom flask containing a magnetic stirring bar.

10. Add 16 mL of anhydrous N,N-dimethylformamide (DMF) to the reaction flask and stir the solution for 30 min at 25°C.

11. Dissolve the above amount of tert-butyl 2-(2-aminoethoxy)ethoxyethylcarbamate in 4 mL of anhydrous DMF and add this solution to the above reaction mixture. Stir the reaction mixture for 8 h at 25°C.

12. Dilute the reaction mixture with 200 mL of ethyl acetate and transfer this solution to a 500 mL separatory funnel. Wash the organic layer with 150 mL of distilled water five times and 150 mL of brine twice.

13. Dry the organic layer with 10 g of anhydrous sodium sulfate for 15 min and filter to remove sodium sulfate. Condense the filtrate using a rotary evaporator.

14. Purify the residue by silica gel column chromatography with gradient from 1:1 to 1:3 hexane/ethyl acetate to obtain pure compound 2 (1.54 g; yield = 56% from compound 1) (See Troubleshooting 2 for additional information)

15. Characterize the product by NMR spectroscopy (¹H NMR and ¹³C NMR) and MS (see the Expected outcomes section).

Pause point: The product can be stored at −20°C for at least 2 years.

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Table 1. Reagents for synthesis of compound 1

| Reagent                         | Formula weight | Equivalent | Amount     |
|---------------------------------|----------------|------------|------------|
| 4-(Aminomethyl)benzoic acid     | 151.2 g/mol    | 1          | 2.00 g (13.2 mmol) |
| 3,5-Bis(trifluoromethyl)benzaldehyde | 242.1 g/mol    | 1.3        | 4.16 g (17.2 mmol) |
| 4,4’-Dimethoxybenzil            | 270.3 g/mol    | 1.3        | 4.65 g (17.2 mmol) |
| Ammonium acetate                | 77.08 g/mol    | 6          | 6.17 g (79.4 mmol) |

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**Figure 2. Synthetic scheme of compound 2**

See also Figure S2.
Synthesis of apoptozole

Timing: 3 h

This step describes how to prepare apoptozole. See Figure 3 and Table 3.

16. Weigh the above amount of compound 2 in 25 mL round-bottom flask containing a magnetic stirring bar.
17. Add 5.0 mL of dichloromethane into the round-bottom flask and stir the mixture at 25°C.
18. Add 5.0 mL of trifluoroacetic acid (TFA) dropwise to the reaction mixture while stirring. Stir the reaction mixture for 2 h at 25°C.
19. Remove volatile materials under reduced pressure using a rotary evaporator and dissolve the residue with 100 mL of ethyl acetate.
20. Transfer the solution in 250 mL separatory funnel and wash the organic solution with 100 mL of saturated aqueous sodium bicarbonate solution three times and 100 mL of brine. Dry the organic layer with 10 g of anhydrous sodium sulfate for 15 min and filter to remove sodium sulfate.
21. Concentrate the organic solution using rotary evaporator to obtain apoptozole (1.30 g; yield = 98% from compound 2)
22. Characterize the product by NMR spectroscopy (1H NMR and 13C NMR) and MS (see the EXPECTED OUTCOMES section).

Pause point: The product can be stored at −20°C for at least 2 years.

Assessment of internalization of apoptozole into lysosomes

Timing: 4 h

This step describes a method to determine internalization of apoptozole into lysosomes.

Note: This step would be appropriate for any similar Hsp70 inhibitors.

23. Remove the cell culture medium from a 6-well plate (Cell Culture and Seeding section, step 12) and wash the cells with 3 mL DPBS once.
24. Isolate lysosomes from cells using a lysosome isolation kit, according to the manufacturer’s instruction. Cells are collected, homogenized and centrifuged at 500 × g for 10 min at 4°C to obtain the supernatant. The supernatant is loaded onto a density gradient solution (17%–30%) and is centrifuged at 18,000 × g for 30 min at 4°C to isolate lysosomes.

25. Incubate isolated lysosomes with 100 μM apoptozole in PBS containing 1% DMSO (nontoxic effect) for 5 min at 4°C. (See Troubleshooting 3 for additional information).

26. Centrifuge the sample at 15,000 × g for 5 min at 4°C.

27. Discard the supernatant by pipetting and wash the pellet with 0.5 mL PBS twice.

28. Re-suspend the pellet in 0.5 mL PBS and lyse lysosomes with an ultrasonic homogenizer for 10 s.

29. Centrifuge the lysate at 15,000 × g for 5 min at 4°C.

30. Transfer 0.1 mL supernatant to a microcentrifuge tube and add 0.1 mL acetonitrile containing 1% TFA to the tube.

31. Filter the sample using the 0.2 μm pore size syringe filter.

32. Analyze the sample (50 μL) by analytic reversed-phase HPLC (C18 column, 250 × 4.6 mm; pore size, 5 μm) with a gradient of 5%–100% CH3CN in water (0.1% TFA) over 30 min (a flow rate; 1 mL/min, UV detection at 254 nm).

Assessment of lysosomal membrane permeabilization
Induction of lysosomal membrane permeabilization is generally assessed by determining both (A) an increase in the lysosomal pH and (B) the release of lysosomal cathepsin B to the cytosol.

Determination of an increase in lysosomal pH using acridine orange

© Timing: 4 h

Lysosomal membrane permeabilization induces proton leakage from acidic lysosomes to the cytosol, the event which increases the lysosomal pH. This step describes a method to determine an increase in the lysosomal pH using acridine orange.

33. Add apoptozole (final concentration: 5 μM) to HeLa cells (ca. 1 × 10^5 cells) in the 24-well plate (Cell Culture and Seeding section, step 9) containing 0.5 mL cell culture medium.

34. Place the plate in an incubator with 5% CO₂ for 3 h at 37°C.

35. Remove the cell culture medium from the plate and add 0.5 mL cell culture medium containing 100 nM acridine orange to the plate.

36. Place the plate into an incubator with 5% CO₂ for 30 min at 37°C.

37. Remove the cell culture medium from the plate and wash the cells with 3 mL DPBS three times.

38. Transfer cover slips from the plate to a glass bottom dish topped with a mounting solution for adhesion of cover slips onto the glass bottom.

39. Take fluorescence images of acridine orange in cells using a confocal fluorescence microscope (λex = 488 nm, 561 nm λem = 530–610 nm). (See Troubleshooting 4 for additional information)

Detection of cytosolic cathepsin B

© Timing: 9 h

Lysosomal membrane permeabilization causes the release of lysosomal enzymes, such as cathepsin B, to the cytosol across disrupted lysosomal membranes. This step describes a method to determine the level of cathepsin B released from lysosomes to the cytosol.

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**Table 3. Reagents for synthesis of apoptozole**

| Reagent | Formula weight | Equivalent | Amount |
|---------|----------------|------------|--------|
| Compound 2 | 856.9 g/mol | 1 | 1.50 g (1.75 mmol) |
| TFA | 114.0 g/mol | excess | 5.0 mL |
40. Add apoptozole (final concentration: 5 μM and 10 μM) to HeLa cells (ca. 5 x 10^5 cells) in the 6-well plate (Cell Culture and Seeding section, step 12) containing 2 mL cell culture medium.

*Note:* Untreated cells can be used as a negative control.

41. Place the plate in an incubator with 5% CO2 for 6 h at 37°C.

42. Remove the cell culture medium from the plate and add 800 μL of digitonin extraction buffer to the cells. (See Troubleshooting 5 for additional information)

*Note:* If necessary, cells treated with 200 μg/mL of digitonin alone, where lysosomes of cells are completely permeabilized, can be used as a positive control.

43. Shake the plate on ice for 12 min using a rocking table.

44. Centrifuge the sample at 10,000 x g for 5 min and collect 700 μL extract as a lysosome-free cytosolic fraction in a microcentrifuge tube.

45. Add trichloroacetic acid (final concentration: 5%) to the lysosome-free cytosolic fraction for precipitation and incubate for 10 min on ice.

46. Centrifuge the sample at 10,000 x g for 15 min at 4°C and discard the supernatant.

47. Add 50 μL of 2x SDS loading buffer to the pellet.

48. Perform a standard western blot analysis and carry out chemiluminescent detection of cathepsin B (1:1000 dilution) with WEST-ZOL plus by using a Chemiluminescent Imaging System.

**EXPECTED OUTCOMES**

**Preparation of apoptozole**

Apoptozole is obtained by applying a three-step synthesis with an overall yield of 29%. Characterization data are summarized in Table 4. Synthetic schemes are shown in Figures 1, 2, and 3 and NMR spectra in Figures S1–S3.

**Induction of LMP by apoptozole**

Hsp70 is present in the cytosol and subcellular organelles. Importantly, this protein is expressed in lysosomes of cancer cells but is rarely present in lysosomes of normal cells (Nylandsted et al., 2004). Lysosomal Hsp70 in cancer cells is known to act as a lysosome stabilizer through binding to lysosomal membranes in order to suppress lysosome-mediated cancer cell death (Kirkegaard et al., 2010). The previous studies suggest that inhibition of lysosomal Hsp70 induces lysosomal membrane permeabilization (LMP) by destabilizing lysosomal membranes, thereby leading to apoptotic cancer cell death (Kirkegaard et al., 2010). In this regard, inhibitors of lysosomal Hsp70 can be utilized as chemical probes to understand lysosome-mediated apoptosis in cancer cells as well as have great potential to treat cancer. For detailed understanding of modes of action of Hsp70 inhibitors on cancer cell death, their subcellular location should be determined and their functions in subcellular compartments should be elucidated. Nonetheless, mechanistic studies of most of Hsp70 inhibitors developed so far have been done without determining their subcellular location. The current protocol provides a method to prepare apoptozole as an Hsp70 inhibitor (Ko et al. 2015), to assess its lysosomal location, and to elucidate their functions in cancer cells.

Apoptozole is internalized into lysosomes on the basis of reversed-phase HPLC analysis of samples obtained from isolated lysosomes treated with the inhibitor (Figure 4). When apoptozole enters lysosomes to block lysosomal Hsp70, the inhibitor induces LMP and leads to proton leakage from acidic lysosomes (pH 4.5–5.0) into the cytosol (pH 7.2). An increase in the lysosomal pH is readily assessed using acridine orange which stains acidic lysosomes with red fluorescence but neutral lysosomes with greatly diminished red fluorescence (Boya and Kroemer, 2008). As shown in Figure 5, cancer cells exposed to apoptozole followed by treatment with acridine orange display remarkably attenuated red fluorescence of acridine orange, indicating that apoptozole increases the lysosomal
In addition to this event, LMP also results in the release of lysosomal enzymes, such as cathepsin B, to the cytosol across disrupted lysosomal membranes (Oberle et al., 2010). The release of cathepsin B to the cytosol is determined by measuring the level of cathepsin B in the cytosol by western blotting. As shown in Figure 6, apoptozole promotes the release of cathepsin B from lysosomes to the cytosol. As a consequence, apoptozole induces LMP by inhibiting lysosomal Hsp70 in order to eventually enhance apoptosis in cancer cells. The current protocol can be employed to understand detailed mechanisms of Hsp70 inhibitors on cancer cell death. Furthermore, this protocol can be utilized for elucidation of functions of inhibitors of other lysosomal proteins on cancer cell death.

**LIMITATIONS**

This protocol mainly deals with assessment of Hsp70 inhibitors for induction of LMP in HeLa cancer cells. However, this is not limited to HeLa cells but is applicable for assessment of LMP promoted by Hsp70 inhibitors in other cancer cells. Because LMP in cancer cells treated with Hsp70 inhibitors is not phenotypically observed, an increase in a lysosomal pH and the release of lysosomal proteins to the cytosol should be examined according to the current protocol. However, in the case of

**Table 4. Analytical data**

| Compound          | Yield and Rf value | $^1$H NMR (400 MHz, CDCl$_3$) δ [ppm] | $^{13}$C NMR (100 MHz, CDCl$_3$) δ [ppm] | MS (ESI)                  |
|-------------------|--------------------|--------------------------------------|----------------------------------------|--------------------------|
| 1 (C$_{33}$H$_{24}$F$_6$N$_2$O$_4$) | 53% from 4-(aminomethyl)benzoic acid, Rf = 0.5 (ethyl acetate/hexane = 3 : 1) | 8.06 (2H, s), 8.00 (2H, d, J = 8.2 Hz), 7.83 (1H, s), 7.51 (2H, d, J = 8.2 Hz), 7.19 (2H, d, J = 8.2 Hz), 6.99 (2H, d, J = 7.9 Hz), 6.90 (2H, d, J = 8.2 Hz), 5.16 (2H, s), 3.83 (3H, s), 3.78 (3H, s) | 166.8, 160.3, 158.7, 144.3, 139.1, 134.3, 133.0, 132.4, 132.3, 130.6, 128.7, 128.1, 128.0, 126.7, 125.9, 124.5, 122.2, 114.8, 113.8, 70.6, 70.5, 70.4, 70.2, 70.0, 55.4, 55.3, 48.3, 48.5 | Calcd. for C$_{33}$H$_{25}$F$_6$N$_2$O$_4$ [M+H]+ 627.2, found 627.1 |
| 2 (C$_{44}$H$_{46}$F$_6$N$_4$O$_7$) | 56% from compound 1, Rf = 0.4 (ethyl acetate/hexane = 3 : 1) | 8.07 (2H, s), 7.83 (1H, s), 7.73 (2H, d, J = 7.3 Hz), 7.51 (2H, d, J = 9.1 Hz), 7.18 (2H, d, J = 8.2 Hz), 6.97 (2H, d, J = 7.9 Hz), 6.90 (2H, d, J = 8.8 Hz), 6.80 (2H, d, J = 8.8 Hz), 6.70 (1H, br. s), 5.14 (2H, s), 3.83 (3H, s), 3.79 (3H, s), 3.60 - 3.70 (8H, m), 3.54 - 3.60 (8H, m), 3.30 - 3.40 (2H, t, J = 4.5 Hz), 3.00 - 3.10 (8H, m) | 166.8, 160.3, 158.7, 144.3, 139.1, 134.3, 133.0, 132.4, 132.3, 130.6, 128.7, 128.1, 128.0, 126.7, 125.9, 124.5, 122.2, 114.8, 113.8, 70.6, 70.5, 70.4, 70.2, 70.0, 55.4, 55.3, 48.3, 48.5 | Calcd. for C$_{44}$H$_{47}$F$_6$N$_4$O$_7$ [M+H]+ 857.3, found 857.5 |
| Apoptozole (C$_{39}$H$_{38}$F$_6$N$_4$O$_5$) | 98% from compound 1, Rf = 0.2 (dichloromethane/methanol = 10 : 1) | 8.07 (2H, s), 7.82 (1H, s), 7.75 (2H, d, J = 8.2 Hz), 7.51 (2H, d, J = 8.8 Hz), 7.18 (2H, d, J = 8.8 Hz), 7.09 (1H, t, J = 5.1 Hz), 6.96 (2H, d, J = 8.2 Hz), 6.89 (2H, d, J = 8.8 Hz), 6.80 (2H, d, J = 9.1 Hz), 5.13 (2H, s), 3.82 (3H, s), 3.78 (3H, s), 3.58 - 3.70 (8H, m), 2.86 (2H, t, J = 5.1 Hz), 2.86 (2H, t, J = 5.1 Hz) | 166.8, 160.3, 158.6, 144.2, 140.3, 139.0, 134.1, 132.9, 132.2, 132.1, 131.8, 130.4, 128.5, 128.0, 127.9, 126.6, 125.7, 122.1, 114.7, 113.7, 72.2, 70.3, 70.1, 69.9, 55.3, 55.2, 48.2, 41.2, 39.8 | Calcd. for C$_{39}$H$_{39}$F$_6$N$_4$O$_5$ [M+H]+ 757.3, found 757.3 |

Analytical data for 4-(2-(3,5-bis(trifluoromethyl)phenyl)-4,5-bis(4-methoxyphenyl)-1H-imidazol-1-yl)methylic benzoic acid (1), tert-butyl (2)-(2-(2-(4-(2-(3,5-bis(trifluoromethyl)phenyl)-4,5-bis(4-methoxyphenyl)-1H-imidazol-1-yl)methyl)benzamido)ethoxy)ethoxy)ethylcarbamate (2) and apoptozole.
inhibitors of other subcellular Hsp70, other methods should be used to understand the effect of the inhibitors on cancer cell death.

**TROUBLESHOOTING**

**Problem 1**
Insufficient yield of compound 1.

**Potential solution**
A considerable amount of compound 1 can exist in the filtrate. Concentrate the filtrate and filter it again or purify it by flash column chromatography (1:1 hexane/ethyl acetate) to obtain more compound 1.

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**Figure 4. Assessment of internalization of apoptozole into lysosomes**
Upper panel: Apoptozole only.
Middle panel: Isolated lysosomes only.
Lower panel: Isolated lysosomes incubated with apoptozole were analyzed by analytic reversed-phase HPLC.

**Figure 5. Determination of an increase in the lysosomal pH using acridine orange**
Left panel: HeLa cells untreated with apoptozole were incubated with acridine orange (scale bar, 10 μm).
Right panel: HeLa cells treated with apoptozole were incubated with acridine orange (scale bar, 10 μm).
The green signal is fluorescence arising from acridine orange in the neutral environment and red one is fluorescence from acridine orange in the acidic environment.
Problem 2
Insufficient yield of compound 2.

Potential solution
The residual acetic acid by insufficient washing of compound 1 can cause a low reaction yield of compound 2. Wash the compound 1 again with saturated aqueous sodium bicarbonate before amide coupling reaction.

Problem 3
False positive results of internalization of a substance into isolated lysosomes

Potential solution
Isolated lysosomes are not stable during relatively long-time incubation with a substance. If isolated lysosomes are disrupted during incubation, false positive results of internalization of a substance into isolated lysosomes will be given. To avoid this, incubate isolated lysosomes with a relatively high concentration of a substance (ca. 100 μM) for 5 min.

Problem 4
Inability of acridine orange to determine an accurate lysosomal pH

Potential solution
Acridine orange is appropriate for determination of a change in lysosomal pH because it stains acidic lysosomes with red fluorescence but neutral lysosomes with greatly diminished red fluorescence.

To accurately measure a lysosomal pH, use a ratiometric fluorescent probe, fluorescein-tetramethylrhodamine (TMR)-tagged dextran (Busschaert et al., 2017).

Problem 5
False positive and negative detection of cathepsin B in the cytosol.

Potential solution
An appropriate concentration of digitonin should be determined prior to experiments. If a low concentration of digitonin is added to cells, insufficient permeabilization of the cell membrane will take place (false negative detection). On the other hand, the use of a high concentration of digitonin will lead to permeabilization of lysosomal membranes and thus cause the release of lysosomal cathepsin B to the cytosol of cells (false positive detection). Thus, find a suitable concentration of digitonin by conducting both the lactate dehydrogenase (LDH) release assay and western blot analysis of cytosolic cathepsin B in cells in a digitonin concentration-dependent manner. Set the digitonin concentration at which the LDH is liberated but the cytosolic cathepsin B released from lysosomes is not detected. A suitable concentration of digitonin may vary depending on the cell type (Jäättelä and Nylandsted, 2015).
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Injae Shin (injae@yonsei.ac.kr).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2021.100349.

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AUTHOR CONTRIBUTIONS
Supervising, I.S.; Investigation, S.H.P. and S.P.; Writing – Original Draft, S.H.P. and S.P.; Writing – Review & Editing, S.H.P and I.S.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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