Supplementary Materials

A novel nucleolin-binding peptide for cancer theranostics

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Figure S1

A

1. Combinatorial OBOC library

2. OBOC library

3. Incubation

4. Positive & Negative hits

5. Collect positive hits

6. Automatic Edman microsequencing

7. Sequence of peptide candidates

B

C

1. AGM-330
2. AGM-331
3. AGM-332
4. AGM-333
5. AGM-334
6. AGM-335
7. AGM-336
8. AGM-337

ID | Sequence | MDA-MB-231 | MCF-10A
---|----------|------------|------------
AGM-330 | R H G A M V Y L K | ++++ | -
AGM-331 | A D H R H R R S G | ++++ | ++
AGM-332 | A V A R A R R R R R | +++ | +++
AGM-333 | R F L K N K K A R | ++ | ++++
AGM-334 | R W L K N K K A R | ++ | +++
AGM-335 | F G R L K K P L K | + | +
AGM-336 | K R R R R E R A G | +++ | +++
AGM-337 | K R R K A P T D | + | +

Note: Semiquantitative relative binding activity: "++++" very strong binding (>40 cells per bead), "+++" strong binding (30-20 cells per bead), "++" moderate binding (20-10 cells per bead), "+" weak binding (5-10 cells per bead), "-" no binding.
Figure S1. A, Steps performed in peptide library synthesis and screening. 1, The split-mix synthesis method was performed to construct the combinatorial OBOC libraries. 2, Approximately ~2,600,000 libraries were synthesized. 3, OBOC libraries were incubated with cancer cells in a CO₂ incubator. 4, Beads carrying ligands with an affinity for cell surface molecules became covered with cells. 5, The positive beads were picked with a pipette under an inverted microscope. 6, The peptide sequences for positive beads were determined by Edman microsequencing. 7, Cancer-specific peptide ligand candidates were identified from screening OBOC combinatorial peptide libraries. B, The binding specificities of the peptides against cancer cell and normal cells were determined. C, Whole-cell binding assay results showing the cell binding specificity of 8 selected beads. Multiple breast and colorectal cancer cells and normal breast and colorectal cells were re-suspended at 10⁶ cells/ml and incubated with beads. All experiments were repeated 3 times. Scale bar, 200 μm.
Figure S2

A

Remaining peptide (%) vs. Time (h)

B

Peptide concentration (µg/mL) vs. Time (h)

C

| Parameters   | AGM-330m       | AGM-330d       | AGM-330       |
|--------------|----------------|----------------|---------------|
| T₁/₂ (h)     | 0.42 ± 0.33    | 1.82 ± 0.36    | 9.43 ± 1.21   |
| Tₘₐₓ (h)     | 0.167          | 0.167          | 0.167         |
| Cₘₐₓ (µg/ml) | 1.27 ± 0.12    | 1.71 ± 0.11    | 1.875 ± 0.67  |
| AUC (µg/h/ml)| 0.64 ± 0.09    | 1.77 ± 0.12    | 21.42 ± 0.81  |

T₁/₂: The distribution half-time; Tₘₐₓ: Time to reach maximum plasma concentration; Cₘₐₓ: Maximum plasma concentration; AUC: Area under the plasma concentration-time curve
Figure S2. A, AGM-330m, AGM-330d and AGM-330 was incubated with pre-warmed human serum and incubated at 37 °C for 0, 3, 6, 9, and 24 h. The portion of serum-treated peptide remaining was determined by calculating the height of the respective elution peak in analytical-HPLC and comparing it with control without incubation. B, C, Mean plasma concentration-time profile (B) and pharmacokinetic parameters (C) of AGM-330m, AGM-330d and AGM-330 in mice (n = 6) after single intravenous injection. Plasma peptide concentrations were quantified by ELISA, and pharmacokinetic profiles were analyzed using Phoenix WinNonlin 8.1 (Pharsight Corporation, Mountain View, CA, USA). The data are presented as the means ± standard deviation. AGM-330m: Monomeric AGM-330, AGM-330d: Dimeric AGM-330, AGM-330: Tetrameric AGM-330.
Figure S3

A. Media + 0% FBS

B. Media + 1% FBS

C. Media + 5% FBS

D. Media + 1% FBS

E. Media + 5% FBS

**Cells and Tissues**

- MCF-10A
- MCF-7
- MDA-MB-231
- CCD-18Co
- HT-29
- HCT-116

**Concentration [nM]**

- AGM-330
- AGM-330d
Figure S3. A-C, Cancer and normal cells (1 × 10^4 cells/well) were seeded in 96-well plates. After 24 h of incubation, the cells were treated with an increasing concentration of dimeric or tetrameric AGM-330 for 48 h. The viability of cells grown in 0 % FBS (A), 5 % FBS (B) or 10 % FBS (C) was assessed by a CellVia WST-1 assay (Young In Frontier, Seoul, Korea) according to the manufacturer’s instruction. The numbers of viable cells were measured at a wavelength of 450 nm using an VersaMax ELISA plate reader (Molecular Devices). D, E, Binding specificity of AGM-330. A whole-cell binding assay was performed to determine the cell binding of AGM-330 in 1 % FBS (D) or 5 % FBS (E). Scale bar, 200 μm.
Figure S4

$K_D = 57.7 \pm 12 \text{ nM}$
Figure S4. AGM-330 affinity for recombinant NCL was assessed by SPR using an increasing concentration (20 nM - 2.5 μM) of AGM-330. The SPR sensorgrams shown are from an experiment that is representative of a set of three separate experiments using different sensor chips with similar results. Recombinant NCL was immobilized onto a CM5 sensorchip. Different concentrations of AGM-330 were incubated with immobilized NCL and analyzed on a Biacore T-200 apparatus. The resulting $K_D$ values are also indicated.
| Membrane fraction         | In-put | Biotin-pull down |
|---------------------------|--------|------------------|
| α2-3,6,8,9 Neuraminidase A | -      | +                |
| O-glycosidase             | -      | +                |
| AGM-330                   | -      | +                |
| Bead                      | -      | +                |

| Size (kDa) | IB: NCL | IB: P-cadherin | IB: Lamin A/C | IB: β-actin |
|------------|---------|----------------|---------------|------------|
| 100        | -       | +              |               |            |
| 75         |         |                |               |            |
| 130        |         |                |               |            |
| 70         |         |                |               |            |
| 40         |         |                |               |            |
Figure S5. Analysis of the interaction between AGM-330 and O-glycan-cleaved membrane NCL by a biotin pull-down assay. Immunoblotting analysis of the eluted proteins from the biotin pull-down using an anti-NCL antibody. Lane 1: Input of membrane NCL; Lane 2: Input of O-glycan-cleaved membrane NCL; Lane 3: Elution after incubation with membrane NCL; Lane 4: Elution after incubation with O-glycan-cleaved membrane NCL.
Figure S6

|      | DAPI | AGM-330-FITC | Merge | Zoom |
|------|------|--------------|-------|------|
| 1 h  | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| 3 h  | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| 6 h  | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| 12 h | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |
**Figure S6.** Time-dependent *in vitro* binding assay of AGM-330-FITC toward cancer cells. MDA-MB-231 cells were incubated with 5 μmol/l AGM-330-FITC for 1, 3, 6 and 12 h at 37 °C. The cells were then fixed with 4 % paraformaldehyde, and the nuclei were stained with DAPI. MERGE represents a merged image of AGM-330-FITC and nuclear staining by DAPI. AGM-330-FITC: FITC-conjugated AGM-330. Scale bar, 20 μm.
Figure S7

A

| Protein                  | CCD-18Co | HT-29 | HCT-116 |
|--------------------------|----------|-------|---------|
| Total nucleolin          |          |       |         |
| Cell surface nucleolin   |          |       |         |
| P-cadherin               |          |       |         |
| Cytosolic nucleolin      |          |       |         |
| β-actin                  |          |       |         |
| Nuclear nucleolin        |          |       |         |
| Lamin A/C                |          |       |         |

| Protein | CCD-18Co | HT-29 | HCT-116 |
|---------|----------|-------|---------|
| 100kDa  |          |       |         |
| 130kDa  |          |       |         |
| 100kDa  |          |       |         |
| 40kDa   |          |       |         |
| 100kDa  |          |       |         |
| 70kDa   |          |       |         |

B

Relative mRNA expression level

Control: Scr: siNCL1: siNCL2: siNCL3

NS: ***
Figure S7

C

Alexa680, Non-tumor-bearing mice

| Time  | BMI | FMI | Merge |
|-------|-----|-----|-------|
| 30min | ![Image] | ![Image] | ![Image] |
| 1h    | ![Image] | ![Image] | ![Image] |
| 6h    | ![Image] | ![Image] | ![Image] |
| 24h   | ![Image] | ![Image] | ![Image] |

**Epi-Fluorescence**

Min = 2.00e^8
Max = 2.50e^8

**Radiant Efficiency**

\[
\frac{p/sec/cm^2/sr}{\mu W/cm^2}
\]

Color Scale

Min = 2.00e^8
Max = 2.50e^8
Figure S7. A, The NCL distribution in various subcellular fractions was analyzed in CCD-18Co, HT-29, and HCT-116 cells. Plasma membrane, cytosol and nuclear NCL were immunoblotted using an anti-NCL antibody. B, MDA-MB-231 cells were transfected with non-targeting scrambled siRNA (Scr) or NCL-targeting siNCL1, siNCL2, or siNCL3. After 24 h, the efficiencies of gene silencing were determined by real-time PCR. Based on the observed mRNA levels, one set of siRNAs (siNCL1) showed significant target gene knockdown efficiency and was used in further experiments. C, In vivo fluorescence images of mice 30 min, 1 h, 6 h, and 24 h after intravenous injections of 10 nmol free Alexa680 in non-tumor-bearing mice. Fluorescence images of the major organs, including the heart, spleen, lung, brain, liver, kidney, and tumors removed from mice with different treatments. The bar graphs show the means ± SD, and statistical analyses were performed using one-way ANOVA with Dunnett’s multiple comparison. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
Figure S8

A

|            | IgG | -50 | - | (μg/mL) |
|------------|-----|-----|---|---------|
| NCL Ab     | -   | -   | 50 | (μg/mL) |
| p-PI3K     |     |     |    |         |
| PI3K       |     |     |    |         |
| β-actin    |     |     |    |         |

B

|            | IgG | -50 | - | (μg/mL) |
|------------|-----|-----|---|---------|
| NCL Ab     | -   | -   | 50 | (μg/mL) |
| p-Akt      |     |     |    |         |
| Akt        |     |     |    |         |
| β-actin    |     |     |    |         |

C

|            | AGM-330 | -10 | 50 | 100 | (μM) |
|------------|---------|-----|----|-----|------|
| p-PI3K     |         |     |    |     |      |
| PI3K       |         |     |    |     |      |
| β-actin    |         |     |    |     |      |

D

|            | AGM-330 | -10 | 50 | 100 | (μM) |
|------------|---------|-----|----|-----|------|
| p-Akt      |         |     |    |     |      |
| Akt        |         |     |    |     |      |
| β-actin    |         |     |    |     |      |
Figure S8. A-D, Western blot analysis of PI3K/Akt signaling in MDA-MB-231 cells stimulated by increasing concentrations of an anti-NCL antibody and AGM-330. After treatment with the anti-NCL antibody (A,B) or AGM-330 (C,D), the protein levels of p-PI3K, PI3K, p-Akt and Akt were assessed by immunoblot analysis. The bar graphs show the means ± SD, and statistical analyses were performed using one-way ANOVA with Dunnett’s multiple comparison. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
Figure S9

A

PTX

Maleimide-linker

Maleimide-PTX

B

PTX

C

Maleimide-PTX

D

876.3

E

Maleimide-PTX

m/z

m/z
Figure S9

Maleimide-PTX-Rh
Solvent: CD3OD
Chemical Formula: C82H85N4O19+
Molecular Weight: 1430.6

AGM-330

AGM-330-PTX-Rh

AGM-330-PTX-Rh

AGM-330-PTX-Rh

AGM-330-PTX-Rh

AGM-330-PTX-Rh

AGM-330-PTX-Rh
Figure S9. A, Conjugation of paclitaxel (PTX) with a maleimide linker. B–E, HPLC chromatograms and MS spectra of PTX and maleimide-PTX. The HPLC chromatograms of PTX (B) and maleimide-PTX (C). MS spectra of PTX (D) by MALDI-TOF and maleimide-PTX (E) by ESI-MS. F, Conjugation of AGM-330 with maleimide-PTX. G–J, HPLC chromatograms and MS spectra of AGM-330 and AGM-330-PTX. The HPLC chromatograms of AGM-330 (G) and AGM-330-PTX (H). MS spectra of AGM-330 (I) and AGM-330-PTX (J) by MALDI-TOF. K, 1H-NMR spectra of maleimide-PTX- Rh. 1H-NMR: (400 MHz, CD3OD): δ 8.27 (d, J = 0.8Hz, 1H), 8.25–8.11 (m, 2H), 8.09–7.77 (m, 4H), 7.69–7.65 (m, 1H), 7.59–7.53 (m, 3H), 7.49–7.39 (m, 7H), 7.31 (t, J = 7.2Hz, 1H), 7.20 (d, J = 9.2Hz, 1H), 7.13–7.10 (m, 2H), 7.03 (d, J = 0.8Hz, 1H), 6.99–6.95 (m, 2H), 6.82–6.68 (m, 2H), 6.16 (s, 1H), 6.02 (t, J = 8Hz, 1H), 5.87 (d, J = 6Hz, 1H), 5.59–5.49 (m, 3H), 4.80 (d, J = 8.8Hz, 1H), 4.14 (d, J = 8.4Hz, 1H), 4.06 (d, J = 8.4Hz, 1H), 3.82–3.70 (m, 7H), 3.68–3.63 (m, 4H), 2.84–2.79 (m, 2H), 2.60 (t, J = 7.2Hz, 1H), 2.38 (s, 3H), 2.22–2.07 (m, 2H), 2.05 (s, 3H), 1.96–1.90 (m, 1H), 1.76 (s, 3H), 1.40 (s, 3H), 1.37–1.25 (m, 12H), 1.13–1.08 (m, 4H), 1.01 (s, 3H). L, Conjugation of AGM-330 with maleimide-PTX-Rh. M, N, HPLC chromatogram (M) and MS spectrum (N) of AGM-330-PTX-Rh. Maleimide-PTX: maleimide-linker conjugated PTX; AGM-330-PTX: maleimide-PTX conjugated AGM-330; maleimide-PTX-Rh: rhodamine B conjugated maleimide-PTX; AGM-330-PTX-Rh: maleimide-PTX-Rh conjugated AGM-330.
Figure S10

A

AGM-330-PTX

5 min

AGM-330

1 h

AGM-330

2 h

B

AGM-330-PTX

5 min

AGM-330

6 h

AGM-330

12 h

C

8146.3

10000

15000

m/z

D

7325.3

5000

10000

15000

m/z

E

DAPI

AGM-330

Alexa488

PTX

Rhodamine

Merge

Zoom

30 min

1 h

2 h
Figure S10. **A, B.** Changes in the HPLC chromatogram of AGM-330-PTX after incubation with human serum (**A**) or PBS (**B**). **C, D.** MS spectra of AGM-330-PTX (**C**) and AGM-330 (**D**). **E.** Time-dependent fluorescence imaging of AGM-330-PTX-Rh in MDA-MB-231 cells. Cells were incubated with 5 μmol/l AGM-330-PTX-Rh. AGM-330 was stained with a primary anti-AGM-330 antibody, and the complex was revealed using an anti-rabbit secondary antibody coupled to Alexa Fluor 488. Cells were then fixed with 4 % paraformaldehyde, and the nuclei were stained with DAPI. MERGE represents a merged image of AGM-330, PTX, and nuclear staining by DAPI. Scale bar, 20 μm.
Figure S11. A-E, NPG™ mice bearing MDA-MB-231-luc tumors were treated twice a week for 21 days with intravenous doses of vehicle (PBS), PTX (2 or 10 mg/kg), AGM-330 (16.68 mg/kg, molar equivalent to 2 mg/kg PTX) and AGM-330-PTX (19.05 mg/kg, molar equivalent to 2 mg/kg PTX) (n = 5). Blood was collected from xenografted mice under isoflurane-induced deep anesthesia by cardiac puncture. After allowing for blood coagulation at 4 °C, serum was collected by centrifugation at 3000 rpm for 10 min at 4 °C. Analysis of the levels of (A), ALB, (B), GOT, (C), GPT, (D), TP-PS, and (E), UA in the serum was performed using a veterinary hematology analyzer (Fuji DRI-Chem 3500 s, Fujifilm, Tokyo, Japan) according to the manufacturer’s provided protocols. ALB, albumin; GOT, glutamic oxaloacetic transaminase; GTP, glutamate pyruvate transaminase; TP-PS, total protein/protein scan; UA, uric acid. F, Body weight changes after 3 weeks of different treatments indicated in xenograft mice. AGM-330d-PTX: PTX-conjugated dimeric AGM-330. The bar graphs show the means ± SD, and statistical analyses were performed using one-way ANOVA with Dunnett’s multiple comparison. *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively.
Figure S12

A

B

C

D

E

F

G

H

S.C injection
MDA-MB-231-luc

Tail vein injection
(~100 mm$^3$)

Sacrificed &
necropsy

Control
AGM-330d 8.34 mg/kg
PTX 2 mg/kg
PTX 10 mg/kg
AGM-330d-PTX 10.71 mg/kg

Day 1
Day 5
Day 10
Day 15
Day 20

Control
AGM-330d 8.34 mg/kg
PTX 2 mg/kg
PTX 10 mg/kg
AGM-330d-PTX 10.71 mg/kg

Tumor formation
monitoring

Drug treatment

Radiance
(p/sec/cm²/ster)

Color Scale
Min = 4.38×10$^6$
Max = 1.40×10$^8$

RHGAMVYLK

Control
AGM-330d 8.34 mg/kg
PTX 2 mg/kg
PTX 10 mg/kg
AGM-330d-PTX 10.71 mg/kg

Total flux (X10$^10$ p/s)

Control
AGM-330d 8.34 mg/kg
PTX 2 mg/kg
PTX 10 mg/kg
AGM-330d-PTX 10.71 mg/kg

Tumor volume
(×10$^3$ mm$^3$)

Control
AGM-330d 8.34 mg/kg
PTX 2 mg/kg
PTX 10 mg/kg
AGM-330d-PTX 10.71 mg/kg

Body weight (g)

Control
AGM-330d 8.34 mg/kg
PTX 2 mg/kg
PTX 10 mg/kg
AGM-330d-PTX 10.71 mg/kg
Figure S12. A, Structure of AGM-330d-PTX. B, Schematic representation of the experimental protocol as described in the Materials and Methods section. Anesthetized 6 week-old male NPG mice were inoculated with a 1:1 mix of Matrigel and $1 \times 10^6$ MDA-MB-231-luc cells into the mammary fat pad. When the volume of the primary tumors reached approximately 100 mm$^3$, tumor-bearing mice were treated with vehicle (PBS), PTX (2 or 10 mg/kg), AGM-330d (8.34 mg/kg, molar equivalent to 2 mg/kg PTX), or AGM-330d-PTX (10.71 mg/kg, molar equivalent to 2 mg/kg PTX) ($n$ = 5/group). C, Monitoring of tumor growth through whole-body bioluminescence imaging. The mice were subjected to bioluminescence imaging every 5 days. D, Bioluminescence was quantified for each time point using the region of interest tool in the Living Image software program. E-G, The therapeutic effect of AGM-330d-PTX was evaluated in breast cancer xenograft models. MDA-MB-231-luc cells were subcutaneously inoculated into the mammary fat pads of NPG mice. (E), The primary tumor volume was measured twice per week until the day of sacrifice. The primary tumor volume was calculated using the following formula: volume (mm$^3$) = [length (mm)] $\times$ [width (mm)]$^2$ $\times$ 0.5. (F), On the day of sacrifice, all primary tumors were isolated, and (G) the primary tumor weights were evaluated. H, Body weight changes after 3 weeks of different treatments in the indicated xenograft mice. AGM-330d-PTX: PTX-conjugated dimeric AGM-330. Bar graphs represent the means $\pm$ SD, and statistical analyses were performed using one-way ANOVA with Dunnett’s multiple comparison. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.