Supporting Information

Grafting Multi-Maleimides on Antisense Oligonucleotide to Enhance Its Cellular Uptake and Gene Silencing Capability

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Materials and methods

Materials

3-maleimidopropionic acid, dimethylaminopyridine (DMAP), dichloromethane (DCM), dimethyl sulfoxide (DMSO), and dicyclohexylcarbodiimide (DCC) were purchased from Tansoole-reagent, China. (4-(bromomethyl)phenyl)methanol was purchased from Shanghai Bide pharmaceutical technology co. LTD, China. 40% acrylamide (19:1, acrylamide/bisacrylamide) solution was purchased from Sangon Biotech co. LTD, China. N-ethylmaleimide (NEM), iodoacetic acid sodium and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich, USA. Dulbecco's Modified Eagle Medium (DMEM) was obtained from Hyclone, USA. Fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma-Aldrich, USA. LysoTracker DND-26 (Green), Early Endosomes-GFP BacMam 2.0, Late Endosome-GFP BacMam 2.0 and Alexa fluor®488/Annexin V cell apoptosis assay kit were purchased from Thermo Fisher Scientific. Oligonucleotides used in this study were purchased from Shanghai Biogene Co. Ltd.

Ultrapure water was used in all experiments.

Table S1. DNA sequences used in this study.

Oligonucleotides	Oligonucleotide sequences (5'-3')
^{3}PS-ASO-Cy3	T*CTCCCAGC*GTGCGCCA*T-Cy3
^{5}PS-ASO-Cy3	T*CTCC*CAGC*GTGC*GCCA*T-Cy3
^{7}PS-ASO-Cy3	T*CTC*CCA*GC*GT*GCG*CCA*T-Cy3
^{9}PS-ASO-Cy3	T*CT*CC*CA*GC*GT*GC*GC*CA*T-Cy3
^{7}PS-ASO	T*CTC*CCA*GC*GT*GCG*CCA*T
Complementary ASO (C-ASO)	ATGGCGCACGCTGGGAGA

Notes: * represent for phosphorothioate (PS) modification sites. The ASO used in this study was the Bcl-2 protein targeting antisense.

Methods

Synthesis of benzyl-bromide modified maleimide (Mal-Bz-Br). 3-maleimidopropionic acid (1 eq.) and (4-(bromomethyl)phenyl)methanol (1 eq.) were dissolved in DCM. And then DMAP (0.2 eq.) was added into the mixture. After stirring for a few minutes, the DCC (1.2 eq, dissolved in dry DCM) was added dropwisely under ice-bath. The reaction was monitored by thin layer chromatography (TLC) using DCM/CH_3OH as the eluent reagent.

The crude Mal-Bz-Br was purified by silica-gel column chromatography using DCM/Methanol as the eluent reagent. The structure of Mal-Bz-Br was confirmed by nuclear magnetic resonance (NMR) and MS spectra as shown in Fig. S2-S4. The MS spectrum showed an m/z of 376, which was attributed to the $[M + Na]^+$ ion of Mal-Bz-Br (Fig. S4).

Synthesis of multi maleimide-grafted antisense oligonucleotides (Mal-*g*-ASO). The Mal-*g*-ASO conjugates were synthesized according to the previously reported[1] method with slight change. ASO strands with multi-PS modification (^{n}PS-ASO, **n** indicates the number of PS in ASO, listed in Table S1), was used to obtain multitude maleimides grafted ASO, expressed as ^{n}Mal-*g*-ASO. The PS-ASO aqueous solution was dehydrated by evaporation and then dissolved with Mal-Bz-Br in DMSO at 55 ºC at a DNA concentration of 5 OD/mL. The molecular ratio between PS group on phosphorothioate ASO and Mal-Bz-Br was 1:50. After reacting for 12 h, the DMSO was removed to obtain dried product, which was further washed with DCM for 3 times to remove excess compounds. The final product was dissolved in phosphate buffer solution (PBS, 10 mM) and then performing ultrafiltration by filter tube (Mw=3000 Da) to remove the undesired water-soluble substances. The final product was characterized by 15% denaturing polyacrylamide gel electrophoresis (PAGE) as shown in Fig. S5.

In vitro molecular recognition verification of Mal-*g*-ASO. The ^{7}Mal-*g*-ASO and its complementary sequence (C-ASO) were mixed equivalently in 1×TAE/Mg^{2+} buffer (40 mM Tris, 2 mM EDTA·2Na·2H_2O, 20 mM acetic acid, 12.5 mM $(CH_3COO)_2Mg·4H_2O$) and heated to 85 ºC for 5 min, and then naturally cooled to room temperature. The free PS-ASO was used as control group. The successful base paring was characterized by 15% native PAGE gel analysis as shown in Fig. S6.

RNase H mediated degradation of Mal-*g*-ASO/mRNA fragment hybrid. The hybrid duplexes (20 μM) of Mal-*g*-ASO/mRNA fragment (39 nt in length) were incubated with RNase H (0 U/mL, 10 U/mL, 50 U/mL, 100 U/mL and 200 U/mL) for 1 h at 37 °C. Then sample mixtures were analyzed by 10% denaturing PAGE gels, which were stained with GelRed. Sequence of mRNA fragments used in this experiment was listed as follows: rCrUrCrUrGrGrGrArArGrGrArUrGrGrCrGrCrArCrGrCrUrGrGrGrArGrArArCrGrGrGrGrUrArCrG, in which underlined segment is the target sequence of Mal-*g*-ASO.

Denaturing PAGE gel analysis. 15% denaturing polyacrylamide (19:1, acrylamide/bisacrylamide) solution containing 8.3 M urea was used to perform gel analysis. Samples were mixed with loading buffer (1×TBE buffer containing 8.3 M urea, 0.03% bromophenol blue and 0.03% xylene cyano) and loaded into the gel. The 1 × TBE buffer (89.0 mM Tris, 89.0 mM boric acid, and 2.0 mM EDTA·2Na·2H_2O) was used as the electrophoretic buffer. After 1-2 hours' electrophoresis, the gel was stained with ethidium bromide solution and then imaged by BioRad imaging system.

Native PAGE gel analysis. 15% native PAGE gel was prepared using the following formulations: 3.75 mL 40% acrylamide (19:1, acrylamide/bisacrylamide) solution, 1 mL 10 × TAE/Mg^{2+} buffer, 5.25 mL ultrapure water, 75 μL ammonium persulfate and 7.5 μL tetramethyl ethylenediamine (TEMED). Each sample was mixed with loading buffer and then loaded into the gel. The electrophoresis was performed in 1 × TAE/Mg^{2+} buffer. After electrophoresis, the gel was immersed into EB solution for staining and then imaged by BioRad imaging system.

Cytotoxicity of Mal-*g*-ASO. In vitro cytotoxicity of Mal-*g*-ASO was determined by MTT assay using normal cell line L929 (mouse fibroblast cells). L929 cells were seeded at 96-well plates with a density of 1 × 10^4 cell per well and incubated for overnight, followed by refreshing with 200 μL opti-MEM culture medium. Afterwards, ^{7}Mal-*g*-ASO was added with different concentrations (0.1, 0.5, 1, 5, 10 μM) and incubated for 24 h. Thereafter, 20 μL MTT solution (5 mg/mL 3-[4, 5-dimethylthiazol-2-yl]-3, 5-diphenyltetrazolium bromide in PBS buffer) was added to each well and incubated for another 4 h at 37 ºC. Then the medium was removed and 150 μL DMSO was added to dissolve formazan crystal. The absorbance at 490 nm was measured by Synergy H4 of BioTek®.

Cell uptake of Mal-*g*-ASO by flow cytometry. Cyanine 3 modified PS-ASO (PS-ASO-Cy3) was used to synthesize fluorescent Mal-*g*-ASO (Mal-*g*-ASO-Cy3) to perform the cell uptake assay. Both cancer cells including HeLa cells (human cervical carcinoma cell), MCF-7 cells (human breast cancer cell), A549 cells (non-small lung cancer cell), and BEL7402 cells (liver cancer cell), and normal cells including L929 (mouse fibroblast cells), MRC5 cells (human lung fibroblast cell), BRL3A (rat hepatocytes) and HUVEC cells (human umbilical vein endothelial cells) were chosen to investigate the cell uptake of Mal-*g*-ASO. 2 × 10^4 cells (per well) were seeded at 24-well plate and incubated for overnight. And then refreshed with 300 μL opti-MEM culture medium. Samples including free PS-ASO-Cy3 and ^{7}Mal-*g*-ASO-Cy3 were added and incubated at 37 ºC for 2 h. The concentration of Cy3 was 1 μM. Thereafter, the cells were harvested and washed with PBS for 2 times, and then the fluorescence of cells were determined by flow cytometry.

In order to investigate the influence of maleimide number on cell uptake, Mal-Bz-Br was conjugated to ^{n}PS-ASO-Cy3 (n=3, 5, 7, 9, Table S1) to obtain corresponding ^{n}Mal-*g*-ASO-Cy3. MCF-7 cells and BEL7402 cells were seeded at 24-well plate and incubated for overnight. And then ^{n}Mal-*g*-ASO-Cy3 were added and incubated at 37 ºC for 2 h. Cell fluorescent intensity was determined by flow cytometry.

In addition, flow cytometry were also used to investigate the influence of incubation time and concentration on cell uptake by incubating the cells mentioned above either with ^{7}Mal-*g*-ASO-Cy3 (1 μM) for different time (0.5 h, 1 h, 2 h, 3 h, 4 h, and 6 h) or varied concentrations (0.1 μM and 1 μM) for 2 h.

Endocytosis inhibition assay. 4×10^4 cells (per well) were seeded at 24-well plate and incubated for overnight, followed by refreshing with 500 μL opti-MEM culture medium. Then different endocytosis inhibitors including wortmannin (50 nM), methyl-β-cyclodextrin (Me-β-CD) (1 mM) and sucrose (146 mM) were added and incubated at 37 ºC for 0.5 h to inhibit micropinocytosis, caveolin and clathrin mediated endocytosis pathways, respectively. Thereafter, the medium was removed and cells were washed for two times. ^{7}Mal-*g*-ASO-Cy3 was added and incubated at 37 ºC for 2 h. Cells without treatment were used as control group. The cell fluorescent intensity was determined by flow cytometry after cells were harvested.

Thiol inhibition assay. Three thiol-consuming agents including N-ethylmaleimide (NEM, 1 μM), iodoacetate (1 mM) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 1 mM) were used to consume the cell membrane thiol. 4×10^4 cells (per well) were seeded at 24-well plate and incubated for overnight, followed by refreshing with 500 μL opti-MEM culture medium. Thiol consuming agents were added and incubated at 37 ºC for 0.5 h. Afterwards, the medium was removed and cells were washed for two times. And then ^{7}Mal-*g*-ASO-Cy3 was added and incubated at 37 ºC for 2 h. Cells without treatment were used as control group. The cell fluorescence intensity was determined by flow cytometry after cells were harvested.

Confocal laser scanning microscope of Mal-*g*-ASO. 5×10^4 MCF-7 cells (per well) were seeded in 24 well plates with a clean coverslip at bottom and cultured for overnight, followed by refreshing the culture medium and addition of the ^{7}Mal-*g*-ASO-Cy3 (1 μM) and LysoTracker DND-26 (1 μM) to label lysosomes at corresponding time. The LysoTracker was incubated for 2 h and ^{7}Mal-*g*-ASO-Cy3 was incubated for 0.25 h, 0.5 h, 1 h and 2 h, respectively. Afterwards, the culture medium was removed and cells were washed with PBS for 3 times, followed by addition of 4% formaldehyde to fix cell for 20 min. Then the formaldehyde was removed and cells were washed with PBS for 3 times, followed by addition of Hoechst 33342 to stain cell nucleus. After incubating for 10 min, the Hoechst solution was removed and cells were washed with PBS for 3 times. Finally, the slides were mounted on glass slides and visualized under laser scanning confocal microscope (Leica TCS SP8 STED 3X).

For early endosomes or late endosomes staining, trackers (Early Endosomes-GFP BacMam 2.0 or Late Endosome-GFP BacMam 2.0) were added according to the protocol and transfected into MCF-7 cells for overnight, respectively. And then the medium was removed and the cells were washed with PBS, followed by adding 300 μL opti-MEM culture medium. Thereafter, the ^{7}Mal-*g*-ASO-Cy3 (1 μM) was added and incubated for another 0.25 h, 0.5 h, 1 h and 2 h. The next steps were the same as above. The final slides were visualized under laser scanning confocal microscope (Leica TCS SP8 STED 3X).

Intracellular gene silencing of Mal-*g*-ASO. The gene silencing effect of Mal-*g*-ASO was investigated by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot assay to quantify the amount of mRNA and protein respectively using MCF-7 cells. 1.5×10^5 MCF-7 cells (per well) were seeded at 6-well plates and incubated in DMEM culture medium for overnight, followed by replacing the culture medium to 1 mL opti-MEM culture and addition of free PS-ASO, ^{7}Mal-*g*-ASO, and PS-ASO transfected by Lipofectamine 2k (Lipo 2k/PS-ASO) with the concentration of ASO of 5 μM. Cells without treatment was used as negative control group. After incubating for 24 h, 1 mL DMEM was added and incubated for another 24 h. For qRT-PCR assay, the cells were harvested and total RNA was extracted by TRIzol and further perform reverse-transcription. Bcl-2 mRNA quantification experiments were performed using ABI 7300 Real-Time PCR Detection System. The thermal cycling conditions for PCR program were: 95 ºC for 10 min, 40 cycles of 95 ºC for 15 sec and 60 ºC for 60 sec.

For western blot assay, the cells were washed with PBS buffer after incubation and then the PBS was removed. Thereafter, cells were lysed by adding RIPA lysis buffer containing protease inhibitor and incubated for 30 min at 4 ºC. Thereafter, the cell lysates were harvested and centrifuged at 12000 rpm at 4 ºC to obtain total protein. After protein quantification by bicinchoninic acid (BCA) protein assay kit (Servicebio), the loading buffer was mixed with protein and boiled for 10 min. Thereafter, 25 μg of proteins were separated by SDS-PACE and transferred to PVDF membranes (0.22 μm, Millipore). Subsequently, the total membranes were blocked with 5% blotting grade milk, and then incubated with primary and secondary antibodies successively. Then the protein bands were stained by ECL chemiluminescent reagents (Servicebio) for 2 min and then imaged and quantified by Alpha software.

Cell apoptosis assay. According to the literatures, downregulation of Bcl-2 protein would induce cancer cell apoptosis.[2] The cell apoptosis of Mal-*g*-ASO was estimated using Annexin V-FITC apoptosis kit. 5×10^4 MCF-7 cells (per well) were seeded at 24-well plates and incubated at 37 ºC for overnight, followed by replacing the culture medium to 300 μL opti-MEM culture and addition of free PS-ASO, ^{7}Mal-*g*-ASO, and PS-ASO transfected by Lipofectamine 2k (Lipo 2k/PS-ASO) with the concentration of ASO of 5 μM. Cells without treatment were used as negative control group. After incubating for 24 h, 1 mL DMEM was added and incubated for another 24 h. Finally, the culture medium was collected and cells were harvested and stained by Annexin V-FITC apoptosis kit according to the protocol. The cell apoptosis rate was then determined by flow cytometry (LSRFortessa, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

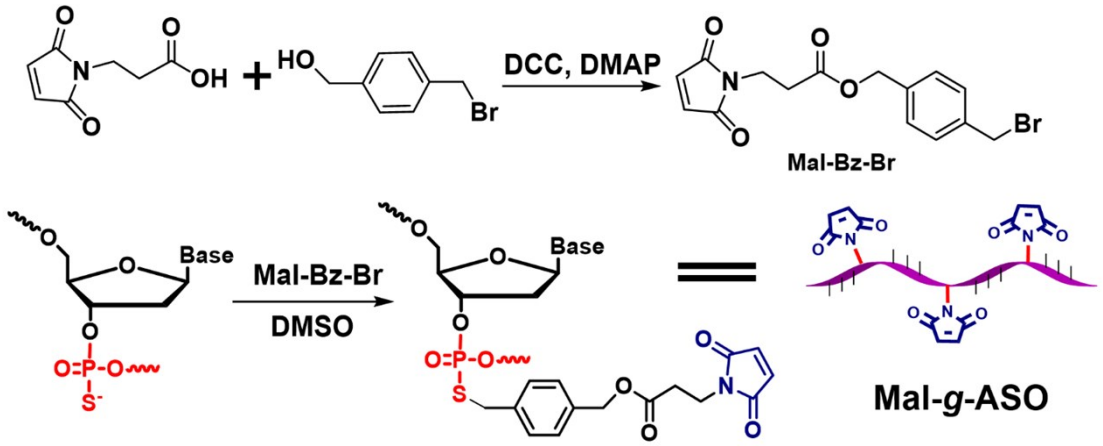

Fig. S1 Syntheses of Mal-Bz-Br and Mal-*g*-ASO.

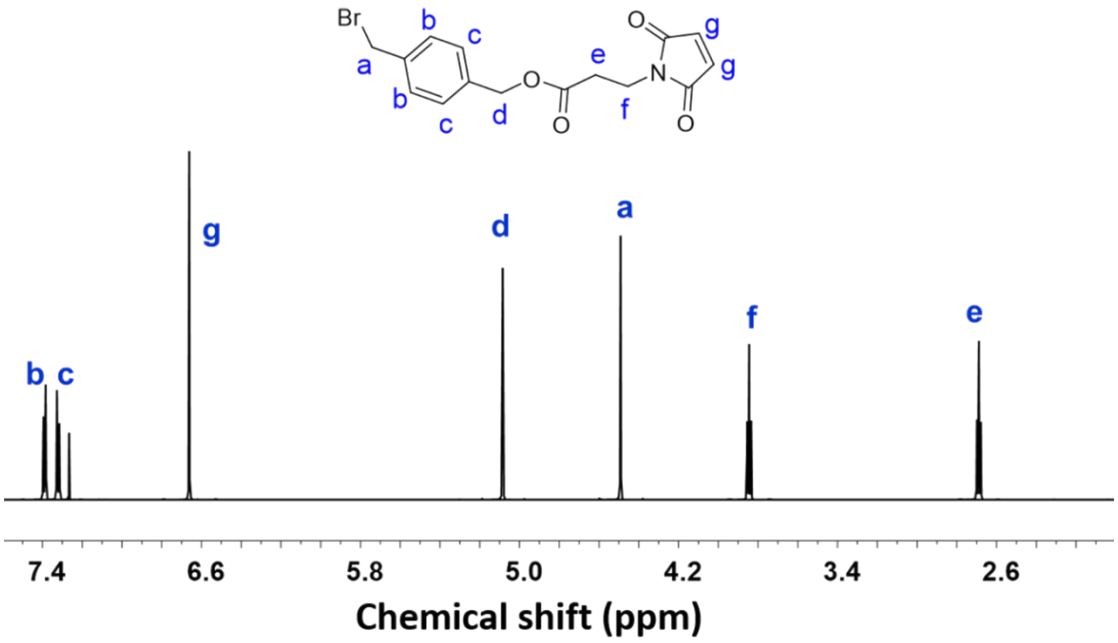

Fig. S2 ^{1}H-NMR spectrum of Mal-Bz-Br.

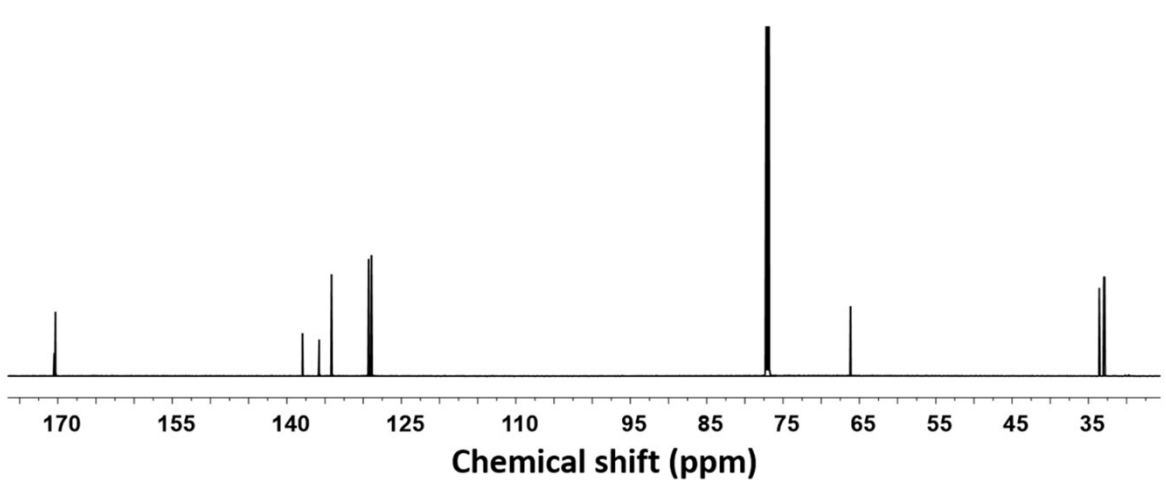

Fig. S3 ^{13}C-NMR spectrum of Mal-Bz-Br.

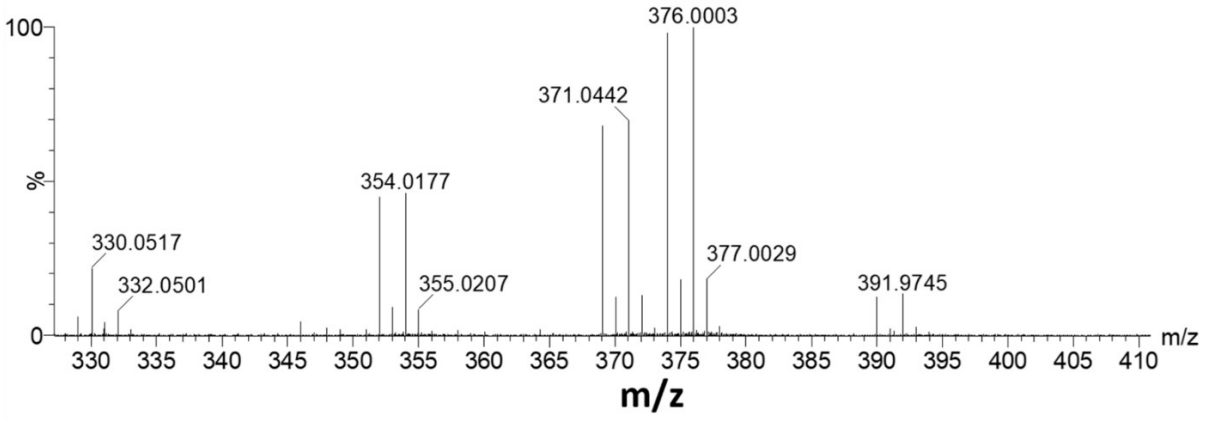

Fig. S4 MS spectrum of Mal-Bz-Br. MS showed an m/z value of 376, which was attributed to the $[M + Na]^+$ ion.

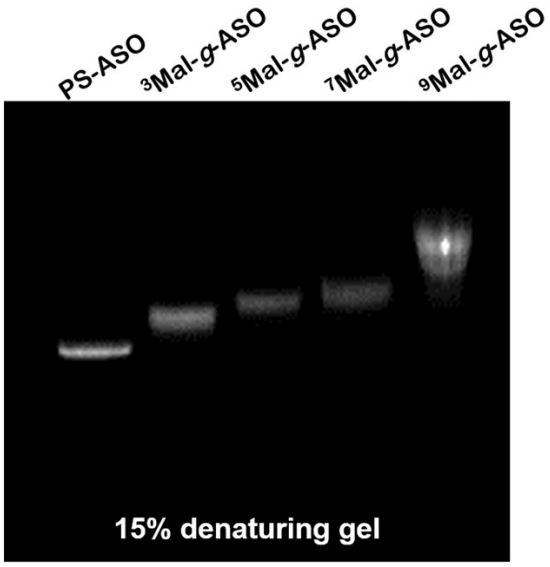

Fig. S5 15% denaturing PAGE gel electrophoresis image of PS-ASO and ^{n}Mal-*g*-ASO. All the PS-ASOs used in this experiment were labeled by fluorescence probe Cy3.

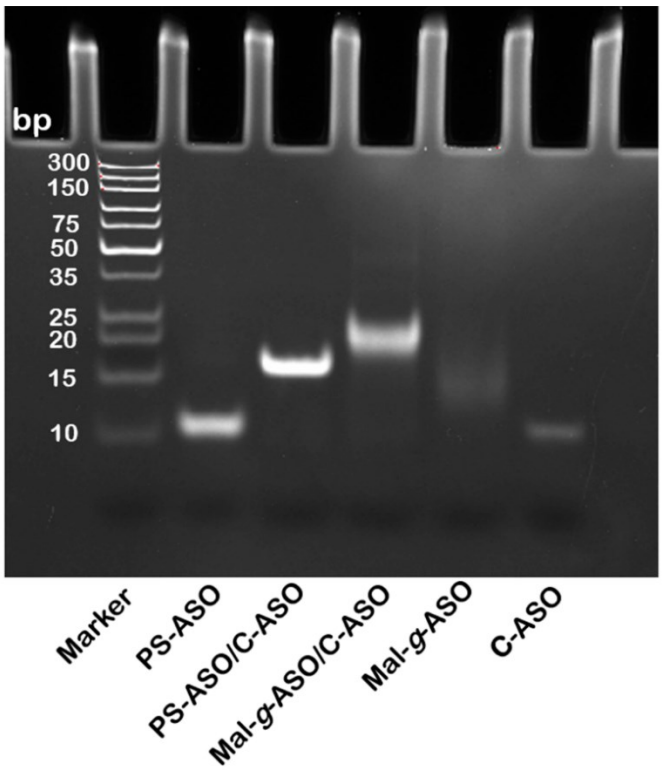

Fig. S6 15% native PAGE gel electrophoresis image of in vitro molecular recognition of Mal-*g*-ASO.

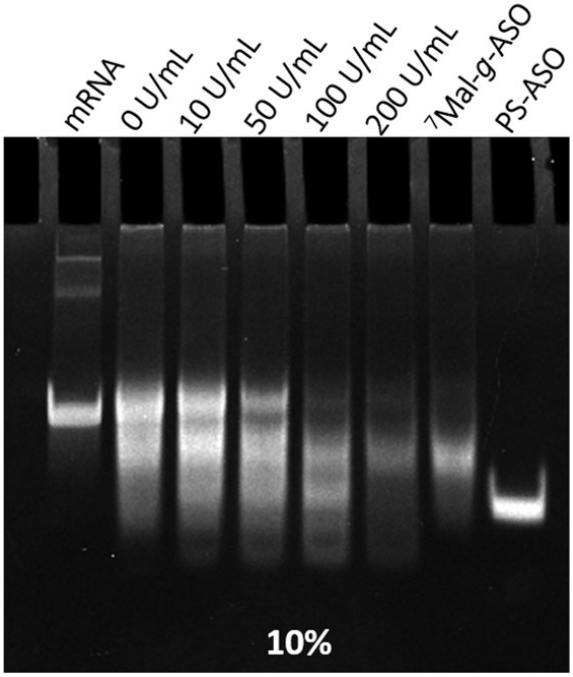

Fig. S7 RNase H mediated degradation of Mal-*g*-ASO/mRNA fragment hybrid after incubation in different concentration of RNase H at 37 ℃ for 1 h. The Mal-*g*-ASO/mRNA fragment hybrid was prepared by heating to 85 ℃ for 10 min and naturally cooled to room temperature.

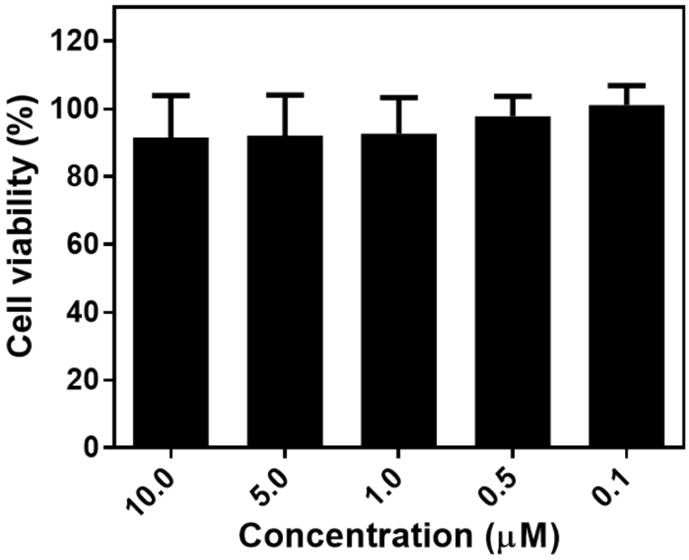

Fig. S8 The cell cytotoxicity of Mal-*g*-ASO to L929 cells.

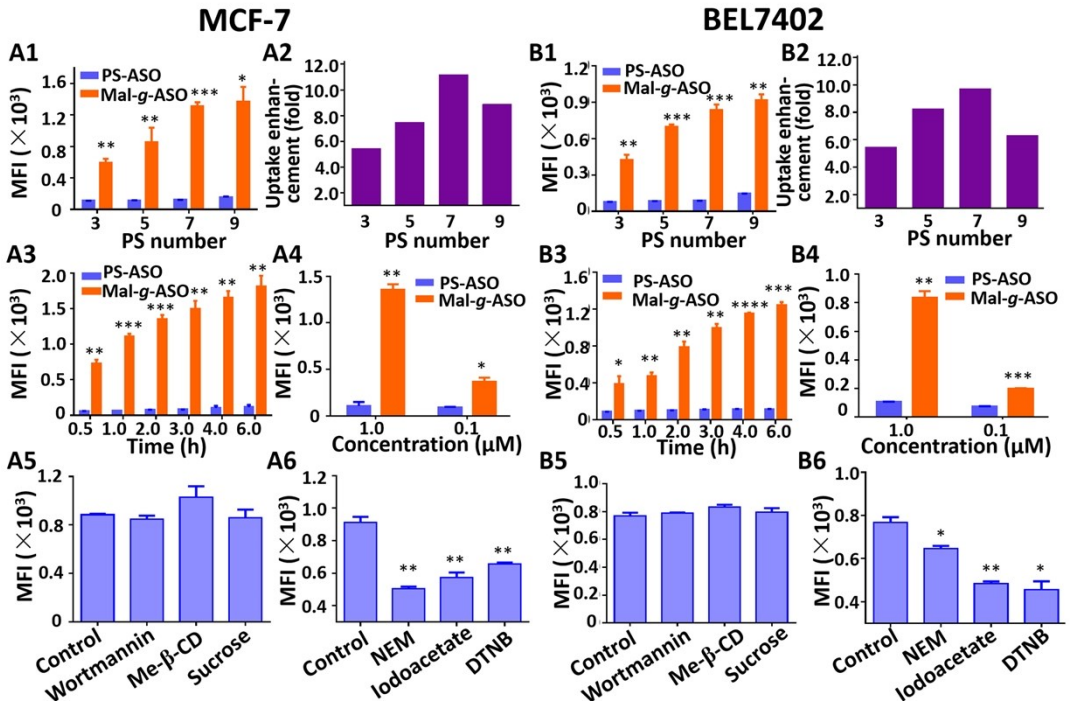

Fig. S9 The cellular uptake of Mal-*g*-ASO-Cy3 in MCF-7 cells (A) and BEL7402 cells (B) as evaluated by flow cytometry analysis. (A1 and B1), effect of maleimide number on Mal-*g*-ASO-Cy3 to the cellular uptake efficiency; (A2 and B2), the cellular uptake enhancement by maleimide grafting as determined by MFI ratios after incubating with ^{n}Mal-*g*-ASO-Cy3 and ^{n}PS-ASO; (A3 and B3), influence of incubation time on cellular uptake efficiency of ^{7}Mal-*g*-ASO-Cy3; (A4 and B4), influence of oligonucleotide concentration on cellular uptake efficiency of ^{7}Mal-*g*-ASO-Cy3; (A5 and B5), cellular uptake of ^{7}Mal-*g*-ASO-Cy3 with pre-treatment using different endocytosis inhibitors; (A6 and B6), cellular uptake of ^{7}Mal-*g*-ASO-Cy3 with pre-treatment using different thiol consuming agents. Statistical significance compared with PS-ASO or control group: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

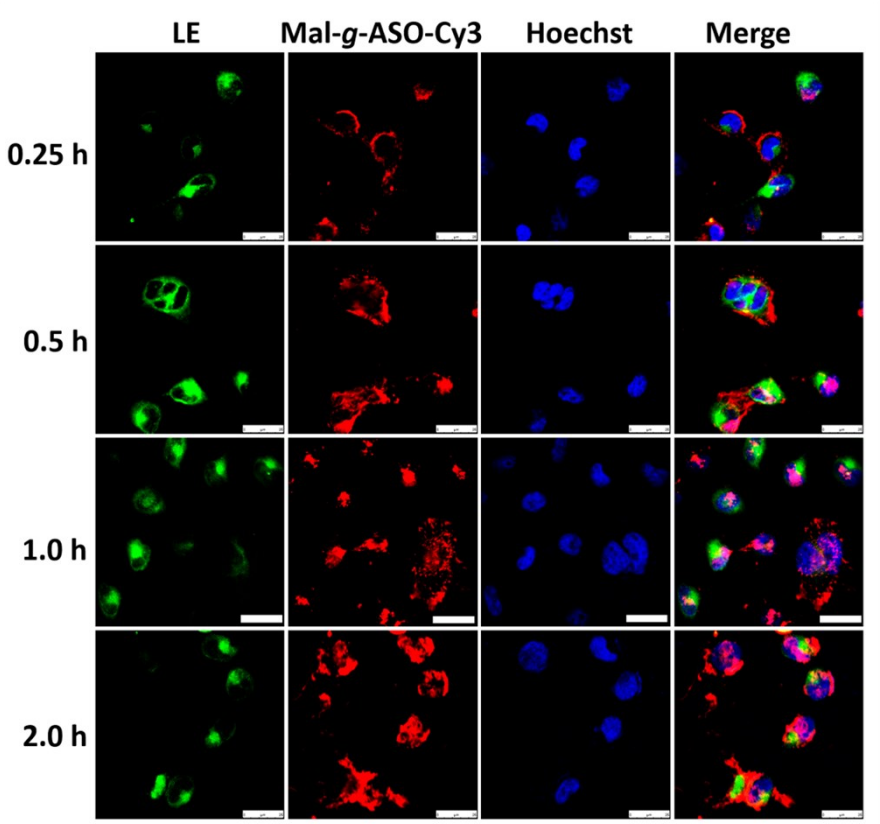

Fig. S10 Colocalization between late endosome (LE) and ^{7}Mal-*g*-ASO-Cy3 in MCF-7 cells imaged by confocal laser scanning microscopy after incubation for different time. The concentration of ^{7}Mal-*g*-ASO-Cy3 was 1 μM. Scale bar, 25 μm.

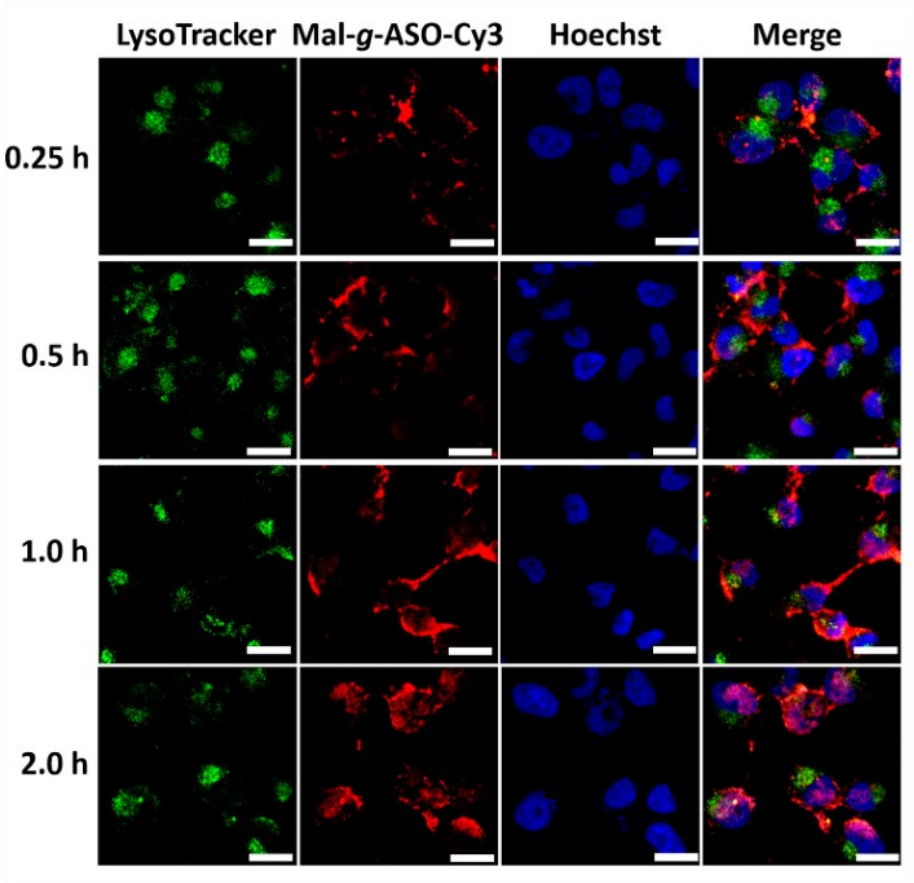

Fig. S11 Colocalization between lysosomes (LysoTracker) and ^{7}Mal-*g*-ASO-Cy3 in MCF-7 cells imaged by confocal laser scanning microscopy after incubation for different time. The concentration of ^{7}Mal-*g*-ASO-Cy3 was 1 μM. Scale bar, 25 μm.

Table S2. The cellular uptake enhancement (fold) of ^{7}Mal-g-ASO-Cy3 in different cells. (n=3)

Cells	PS-ASO-Cy3 (MFI)	^{7}Mal-g-ASO-Cy3 (MFI)	Uptake enhancement (fold)
MCF-7	95.9 ± 2.1	1395.7 ± 308.4	14.5
HeLa	99.4 ± 9.8	1238.5 ± 47.0	12.5
A549	106.3 ± 11.7	1615.0 ± 269.2	15.2
BEL7402	92.1 ± 13.7	1058.3 ± 245.9	11.5
HUVEC	142.3 ± 1.8	617.7 ± 21.4	4.3
L929	65.4 ± 1.3	394.5 ± 71.3	6.0
MRC5	127.0 ± 0.7	215.5 ± 11.3	1.7
BRL3A	76.9 ± 0.4	583.0 ± 19.8	7.6

References

1 Y. Guo, J. Zhang, F. Ding, G. Pan, J. Li, J. Feng, X. Zhu and C. Zhang, *Adv. Mater.*, 2019, **31**, 1807533.

2 T. Oltersdorf, S. W. Elmore, A. R. Shoemaker, R. C. Armstrong, D. J. Augeri, B. A. Belli, M. Bruncko, T. L. Deckwerth, J. Dinges, P. J. Hajduk, M. K.Joseph, S. Kitada, S. J. Korsmeyer, A. R. Kunzer, A. Letai, C. Li, M. J. Mitten, D. G. Nettesheim, S. Ng, P. M. Nimmer, J. M. O'Connor, A. Oleksijew, A. M. Petros, J. C. Reed, W. Shen, S. K. Tahir, C. B. Thompson, K. J. Tomaselli, B. Wang, M. D. Wendt, H. Zhang, S. W. Fesik and S. H. Rosenberg, *Nature*, 2005, **435**, 677.