Research article

Title:

Anti-cell growth and anti-cancer stem cell activities of the non-canonical hedgehog inhibitor GANT61 in triple-negative breast cancer cells

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Abstract

Background:

Triple-negative breast cancer (TNBC) exhibits biologically aggressive behavior and has a poor prognosis. Novel molecular targeting agents are needed to control TNBC. Recent studies revealed that the non-canonical hedgehog (Hh) signaling pathway plays important roles in the regulation of cancer stem cells (CSCs) in breast cancer. Therefore, the anti-cell growth and anti-CSC effects of the non-canonical Hh inhibitor GANT61 were investigated in TNBC cells.

Methods:

The effects of GANT61 on cell growth, cell cycle progression, apoptosis, and the proportion of CSCs were investigated in three TNBC cell lines. Four ER-positive breast cancer cell lines were also used for comparisons. The expression levels of effector molecules in the Hh pathway: glioma-associated oncogene (GLI) 1 and GLI2, were measured. The combined effects of GANT61 and paclitaxel on anti-cell growth and anti-CSC activities were also investigated.

Results:

Basal expression levels of GLI1 and GLI2 were significantly higher in TNBC cells than in ER-positive breast cancer cells. GANT61 dose-dependently decreased cell growth in association with G1-S cell cycle retardation and increased apoptosis. GANT61 significantly decreased the CSC proportion in all TNBC cell lines. Paclitaxel decreased cell growth, but not the CSC proportion. Combined treatments of GANT61 and paclitaxel more than additively enhanced anti-cell growth and/or anti-CSC activities.

Conclusions:

The non-canonical Hh inhibitor GANT61 decreased not only cell growth, but also the

CSC population in TNBC cells. GANT61 enhanced the anti-cell growth activity of paclitaxel in these cells. These results suggest for the first time that GANT61 has potential as a therapeutic agent in the treatment of patients with TNBC. (257 words)

Keywords:

Triple-negative breast cancer, Cancer stem cells, Hedgehog pathway, GANT61, Paclitaxel

Background

Triple-negative breast cancer (TNBC, estrogen receptor [ER]-negative, progesterone receptor-negative, and human epidermal growth factor receptor [HER] 2-negative breast cancer) exhibits biologically aggressive behavior and has a poor prognosis. Since neither endocrine therapy nor anti-HER2 therapy is effective in the treatment of patients with TNBC, cytotoxic chemotherapeutic agents such as taxanes and anthracyclines have been clinically used; however, their efficacies are limited. Therefore, novel molecular targeting agents are needed to control TNBC [1].

Recent preclinical and clinical studies have indicated that tumor-initiating cell or cancer stem cell (CSC) populations exist in breast cancer, and CSCs play important roles in metastasis, recurrence, and resistance to anticancer drugs and radiation therapy. The eradication of CSCs may be the key to obtaining the so-called “total cancer cell kill” or prolongation of successful anticancer therapy [2]. TNBC has been suggested to contain a larger CSC population and more epithelial-mesenchymal transition phenotypes than ER-positive breast cancer cells [3, 4]. These findings may explain why TNBC is more aggressive and metastatic than ER-positive breast cancers.

The molecular mechanisms responsible for the regulation of the CSC proportion have been extensively studied in recent years, but remain unclear [5]. Two studies recently demonstrated the important roles of the hedgehog (Hh) pathway in the regulation of CSCs in TNBC. Colavito SA et al. showed that the non-canonical activation of GLI1 induced by the nuclear factor kappa-light-chain-enhancer of activated B cells pathway was involved in the maintenance of CSCs in breast cancer cells categorized as one of the TNBC subtypes, the claudin-low subtype [6]. Han B et al.

indicated that the non-canonical activation of GLI2 induced by forkhead box C1 regulated the properties of CSCs in breast cancer cells categorized as one of the TNBC subtypes, the basal-like subtype [7]. These findings suggest that the non-canonical Hh pathway is a pivotal player in the regulation of CSCs in TNBC.

The Hh signaling pathway plays an important role in tumor initiation and progression. In a cohort study with invasive ductal carcinoma of the breast, activation of Hh pathway was suggested to associate with increased risk of metastasis, breast cancer-specific death, and a basal-like phenotype [8]. Aberrant Hh signaling has been detected in various human cancers. The Hh pathway involves complex signaling through canonical and non-canonical signaling pathways. Targeting Hh signaling has been investigated with canonical Hh inhibitors such as Smoothened (SMO) inhibitors. Resistance to SMO inhibitors was previously reported in patients with basal cell carcinoma. One of the most promising agents that inhibits Hh signaling is the GLI1/2 inhibitor GANT61, which has been investigated in various cancers [9].

Therefore, we conducted the present study in order to test the hypothesis that the non-canonical Hh inhibitor GANT61 may decrease the activation of GLI1/2 and effectively reduce the proportion of CSCs among TNBC cells. Furthermore, GANT61 is known to exhibit potent anti-cell growth activity in a number of cancers [9]. Therefore, combined treatments of GANT61 and paclitaxel, which is commonly used in the treatment of TNBC, were also tested in TNBC cells.

Methods

Reagents

GANT61 was obtained from CHEMSCENE, LLC (Monmouth Junction, NJ, USA). Paclitaxel was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Breast cancer cell lines and cell cultures

The MDA-MB-231 cell line was kindly provided by the late Dr. Robert B. Dickson (Lombardi Cancer Research Center, Washington DC, USA). The MDA-MB-157 and HCC1937 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). We and others previously demonstrated that MDA-MB-231 and MDA-MB-157 cell lines are categorized as one of the TNBC subtypes, the basal B subtype or claudin-low subtype [10, 11]. The HCC1937 cell line was derived from a patient with a germ-line BRCA1 mutation and categorized as one of the TNBC subtypes, the basal A subtype [12]. Regarding ER-positive breast cancer cell lines, KPL-1 and KPL-3C breast cancer cell lines were established in our laboratory [13, 14]. MCF-7 and T-47D cell lines were kindly provided by the late Dr. Robert B. Dickson (Lombardi Cancer Research Center). All cell lines were maintained in Dulbecco's modified Eagle's medium (D-MEM, Sigma Co.) supplemented with 10 % fetal bovine serum (FBS).

Antitumor activity

In order to investigate the effects of GANT61 and/or paclitaxel on cell growth, breast cancer cells (1 – 5 x 10^4 cells per well) were seeded on 24-well plates (SB Medical,

Tokyo, Japan) and grown in D-MEM supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere for two days. After washing with phosphate-buffered saline (PBS, Nissui Co., Tokyo, Japan), cells were treated with D-MEM supplemented with 10% FBS plus the indicated concentrations of GANT61 and/or paclitaxel for three days. In the paclitaxel treatments, cells were exposed to paclitaxel for 4 hours, washed with PBS, and cultured thereafter. After these treatments, cells were harvested and counted with a Coulter counter (Coulter Electronics, Harpenden, UK). Reproducibility was confirmed in at least two separate experiments.

In order to evaluate the antitumor effects of combined treatments, a combination index based on the 50% inhibitory concentration (IC_{50}) was calculated according to the following formula: combination index = IC_{50} with the combined treatment/ IC_{50} with the single treatment. A combination index < 0.5 was considered to be evidence of a more than additive interaction [15].

Cell cycle and apoptosis assays

In order to investigate the effects of agents on cell cycle progression, harvested cells were stained with propidium iodide using the CycleTest Plus DNA Reagent kit (Becton–Dickinson, San Jose, CA, USA). Apoptotic cells were stained with an Annexin-V-FLUOS staining kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's recommendations. Flow cytometry was performed with a FACSCalibur flow cytometer (Becton–Dickinson), and the DNA histogram obtained was analyzed using CELLQuest version 6.0 (Becton–Dickinson). Reproducibility was confirmed in at least two separate experiments.

CSC analysis by the Aldefluor assay

The ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) was used to isolate the cell population exhibiting strong aldehyde dehydrogenase (ALDH) activity. Harvested cells were suspended in Aldefluor assay buffer containing ALDH substrate (BODIPYTM-aminoacetaldehyde, 1 μmol/l per 1 x 10^6 cells) in duplicate and incubated at 37°C for 30 min. As a negative control, cells were treated with 50 mmol/l diethylaminobenzaldehyde, a specific ALDH inhibitor [16].

CSC analysis by the mammosphere assay

Breast cancer cells (0.3–1.5 x 10^5 cells per well) were seeded on 35-mm dishes (SB Medical) and grown in D-MEM supplemented with 10% FBS at 37°C in a 5%CO_2 atmosphere for two days. After washing with PBS, cells were treated with D-MEM supplemented with 10% FBS plus the indicated concentrations of GANT61 and/or paclitaxel for six days. In the paclitaxel treatment, cells were exposed to paclitaxel for 4 hours, washed with PBS, and cultured thereafter. In the GANT61 treatment, cells were exposed to GANT61 for three days, washed with PBS, and cultured for three days. These cells were then dispersed, and single-cell suspensions (5 x 10^3 cells/well) were incubated in MammoCultTM basal medium (STEMCELL Technologies Co., Vancouver, Canada) supplemented with 10% MammoCultTM proliferation supplements (STEMCELL Technologies Co.) in non-adhesive 6-well plates (CORNING Co., NY, USA) in duplicate for seven days. According to the instruction provided from

STEMCELL Technologies Co., mammospheres larger than 60 μm were counted with an Olympus phase-contrast microscope [17].

RNA isolation and quantitative reverse-transcription (RT) polymerase chain reaction (PCR)

Breast cancer cells were seeded at 2 x 10^5 cells/well in 6-well plates and incubated at 37°C to allow cell attachment. Cells were then treated with or without GANT61 for the indicated duration of time. After the incubation, total RNA was extracted from cells using an RNeasy MiniKit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions, and cDNA synthesis was performed with a ReverTra Ace qPCR RT kit (TOYOBO, Tokyo, Japan). A quantitative real-time PCR analysis of *GLI1* and *GLI2* mRNA was performed on cDNA using TaqMan gene expression assays according to the manufacturer's instructions (Applied Biosystems, Life Technologies, Waltham, MA, USA) and a 7500 Real-Time PCR System (Applied Biosystems). Each amplification reaction was performed in duplicate, and the average of the two threshold cycles was used to calculate the amount of transcripts in the sample. mRNA quantification was expressed, in arbitrary units, as the ratio of the sample quantity to the calibrator or to the mean values of control samples. All values were normalized to an endogenous control, *ACTB.*

In a study to measure the baseline expression levels of the molecules, expression levels in MCF-7 cells were defined as 1. In a study to measure the expression levels of the molecules modified by the GANT61 treatments, expression levels in the controls were defined as 1.

Western blot analysis

Cells were lysed for protein extraction using Pierce RIPA Buffer with protease inhibitor and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). The total protein concentration was measured using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Isolated proteins were separated by 5-20% SDS-PAGE or 7.5% SDS-PAGE and transferred to an Immobilon-FL (Merck Millipore Corporation, Billerica, MA, USA) or transferred to an Amersham Hybond P PVDF 0.45 (GE Healthcare Japan, Tokyo, Japan). Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) or blocking buffer (5% BSA in 1x Tris buffer Saline with 0.1% tween 20) at room temperature for one hour and then subjected to immunoblots using primary antibodies at 4°C overnight, followed by an incubation with secondary antibodies at room temperature for one hour. Labeled protein was visualized using the Odyssey CLx Imaging System (LI-COR Biosciences) or ECL Prime Western Blotting Detection Reagent (GE Healthcare Japan) with the expression of β-actin as the internal standard.

Rabbit antibodies against Gli1 (polyclonal antibody #2534), Gli2 (polyclonal antibody #2585) and survivin (mAb #2808) were purchased from Cell Signaling Technologies (Danvers, MA, USA). Mouse polyclonal antibody against β-actin was from Sigma Aldrich. Secondary antibodies, goat anti-rabbit lgG-HRP and goat anti-mouse lgG-HRP were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA), and IRDye800CW Goat Anti-Rabbit IgG and IRDye680RD Goat Anti-Mouse IgG were purchased from LI-COR Biosciences.

Statistical analysis

All values are expressed as the mean ± SE. An analysis of variance with StatView computer software (ATMS Co., Tokyo, Japan) was used to compare differences between two groups. A two-sided P value less than 0.05 was considered significant.

Results

Expression levels of Hh signaling molecules in breast cancer cells

Basal mRNA and protein expression levels of the Hh signaling molecules, GLI1 and GLI2 were measured by quantitative RT-PCR and western blotting, respectively. The expression levels of GLI1 and GLI2 were very high in MDA-MB-157 cells, while those of GLI2 were higher in MDA-MB-231 and HCC1937 cells than in ER-positive breast cancer cell lines. The expression levels of GLI2 were higher in TNBC cell lines than in ER-positive cell lines (Figures 1A-1B, Figures S1A-S1B in electronic supplementary material [ESM] file 1).

Anti-cell growth activity of GANT61 in breast cancer cells

Growth inhibitory curves of GANT61 in three TNBC and four ER-positive breast cancer cell lines are shown in Figure 2. These curves were similar among the cell lines tested, except for the MDA-MB-231 cell line. This cell line was more sensitive to

GANT61 than the other lines. The IC_{50}s of GANT61 are shown in Table S1 in ESM file 2.

Effects of GANT61 on cell cycle progression and apoptosis

Lower concentrations (1 - 10 μM) of GANT61 modestly increased the cell proportion in the G1 phase, decreased it in the S phase, and caused G1-S cell cycle retardation in MDA-MB-231 cells (Figure S2A in ESM file 1). In contrast, higher concentrations of GANT61 (10 and/or 20 μM) increased the cell proportion in the sub G1 phase in all TNBC cell lines (Figures S2A-S2C in ESM file 1).

In line with the results of the cell cycle analysis, the proportion of apoptotic cells measured with the Annexin V assay was increased in association with suppression of an anti-apoptotic effector, survivin expression [18] by treatments with higher concentrations of GANT61 (10 and/or 20 μM) in all TNBC cell lines (Figures 3A-3C, Figure S3 in ESM file 1).

Effects of GANT61 on the putative CSC proportion

GANT61 dose-dependently decreased the proportion of ALDH-positive cells, which is known as the CSC population, in all TNBC cell lines (Figures 4A, 4C, and 4E). Representative results of the Aldefluor assay are shown in Figures 4B, 4D, and 4F.

In line with the results of the Aldefluor assay, GANT61 dose-dependently decreased the number of mammospheres, which are known to originate from CSCs, in all TNBC cell lines (Figures 5A, 5C, and 5E). Representative results of the

mammosphere assay are shown in Figures 5B, 5D, and 5F. Mammosphere size was also dose-dependently decreased by GANT61 in MDA-MB-231 and MDA-MB-157 cells. Both the number of mammospheres and their size were smaller in HCC1937 cells.

Effects of GANT61 on GLI1 and GLI2 mRNA expression levels

The time courses of changes induced in GLI1 and GLI2 mRNA expression levels by 10 μM GANT61 were investigated in the MDA-MB-157 cells, which expressed the highest mRNA levels of GLI1 and GLI2. GANT61 maximally decreased both GLI1 and GLI2 mRNA expression levels 24 hours after the start of the treatment (Figures S4A and S4B in ESM file 1). In order to investigate dose-dependent decreases induced in GLI1 and GLI2 expression levels by GANT61, MDA-MB-157 cells were treated with 1 – 20 μM GANT61 for 24 hours. As expected, GANT61 dose-dependently decreased their expression levels in MDA-MB-157 cells (Figures S4C and S4D in ESM file 2).

Combined anti-cell growth activity of GANT61 and paclitaxel

The combined treatments of paclitaxel and GANT61 showed more than additive anti-cell growth interactions in MDA-MB-231 and HCC1937 cells and close to additive interactions in MDA-MB-157 cells (Figures 6A-6C). Combination indexes for the IC_{50} were 0.37±0.03 for MDA-MB-231, 0.51±0.25 for MDA-MB-157, and 0.10±0.03 for HCC1937.

Combined anti-CSC activity of GANT61 and paclitaxel

Lower concentrations of paclitaxel (1.0 and 2.5 nM) did not significantly change the CSC proportion measured by the mammosphere assay in MDA-MB-231 cells and slightly increased it in MDA-MB-157 cells (Figures 7A-7B). In contrast, higher concentrations of paclitaxel (10 and 50 nM) significantly decreased the CSC proportion in HCC1937 cells (Figure 7C). The addition of GANT61 to higher concentrations of paclitaxel significantly decreased the CSC proportion in all TNBC cell lines (Figures 7A-7C).

Discussion

Recent experimental studies using the non-canonical Hh inhibitor GANT61 as an anticancer agent have indicated that this agent targets most of the classical hallmarks of cancer, i.e., cell viability, proliferation, apoptosis, DNA damage repair, epithelial-mesenchymal transition, autophagy, CSCs, and immune responses in various types of malignancies [9]. A very recent study revealed that GANT61 inhibited the growth of various breast cancer cell lines *in vitro* and *in vivo* [19]. However, the more than additive anti-cell growth effects of GANT61 and paclitaxel, which have frequently been used in the treatment of patients with TNBC, have never been demonstrated in TNBC cells. Furthermore, the combined effects of GANT61 and/or paclitaxel on the proportion of CSCs in TNBC cells has not yet been reported. The results of the present study have shown for the first time that GANT61 significantly enhanced the anti-cell growth activity of paclitaxel, and also effectively decreased the proportion of CSCs in TNBC cells.

The Hh pathway plays important roles in controlling cell proliferation, cell fate, and patterning as well as stem cell maintenance, self-renewal, and tissue repair. Hh signaling is transduced by two distinct mechanisms, known as the canonical and non-canonical pathways. In addition to the canonical Hh signaling pathway, a non-canonical Hh pathway was recently reported. This mechanism involves the activation of Hh pathway components by other signaling cascades such as oncogenic pathways [9, 20].

GANT61 was discovered in a cell-based screening system for small molecule inhibitors of GLI-mediated transcription. GANT61 was shown to selectively inhibit GLI1- and GLI2-mediated gene transactivation [9]. GANT61 also decreased the expression levels of the target genes GLI1 and Patched 1 and reduced transcriptional activity using GLI reporter assays in various cell types [21]. In the present study, it was demonstrated that GANT61 time- and dose-dependently decreased GLI1 and GLI2 mRNA expression levels in MDA-MB-157 cells, which express very high levels of GLI1 and GLI2 (Figure S4 in ESM file 1).

The cytotoxic effects of GANT61 have been investigated in a number of cancer cell types, with IC_{50} values ranging between 5 and 15 μM in most cancer cell lines [9]. In line with previous findings, the IC_{50}s of GANT61 for the three TNBC cell lines and four ER-positive breast cancer cell lines tested were approximately 10 μM in the present study. GANT61 has been suggested to inhibit proliferation through its effects on cell cycle progression. A previous study reported that GANT61 induces G1 arrest, consistent with decreased expression levels of the Hh target gene Cyclin D and/or increased expression levels of p21 [22]. In the present study, lower doses of GANT61 were shown to dose-dependently induce G1-S cell cycle retardation in MDA-MB-231

cells (Figure S2). It has also been suggested that the inhibition of Hh signaling may cause apoptosis either through the activation of Fas signaling or by decreasing protein levels of anti-apoptotic Bcl-2 or survivin [18, 23]. As expected, GANT61 dose-dependently increased apoptosis in all TNBC cell lines tested in the present study (Figure 3).

Several studies have indicated that Hh signaling plays a pivotal role in the regulation of CSCs by controlling the transcription of genes implicated in cell fate and stemness [24-26]. A recent study revealed that estrogen activates Hh signaling through the non-canonical pathway, increases GLI1 expression, promotes the activity of GLI1 target genes, and increases the CSC population in ER-positive breast cancer cells [27]. In addition, two recent studies reported important roles for the non-canonical Hh pathway in the regulation of CSCs in TNBC cells. Colavito SA et al. showed that the non-canonical activation of GLI1 induced by the nuclear factor kappa-light-chain-enhancer of activated B cells pathway was involved in the maintenance of CSCs in claudin-low breast cancer cells [6]. Han B et al. indicated that the non-canonical activation of GLI2 induced by forkhead box C1 regulated CSC properties in basal-like breast cancer cells [7]. These findings suggest that the non-canonical Hh pathway is a common player in the regulation of breast CSCs.

A previous study suggested that breast CSCs play a specific role in the development of resistance to cytotoxic chemotherapy in breast cancer [2]. We hypothesized that a combined treatment with paclitaxel and the CSC-regulating agent GANT61 may have an additive/synergistic anti-cancer effect on TNBC cells. As expected, combined treatments of GANT61 and paclitaxel additively inhibited the growth of all three TNBC cell lines tested in the present study (Figure 6).

We used two different methods, the Aldefluor assay and mammosphere assay, to detect and quantify the proportion of CSCs in the present study. Basal CSC proportions measured by the Aldefluor assay were approximately 2% for the MDA-MB-231 and MDA-MB-157 cell lines and approximately 20% for the HCC1937 cell line (Figure 4). In contrast, basal CSC proportions measured by the mammosphere assay were approximately 3% for MDA-MB-231, 7% for MDA-MB-157, and 2% for HCC1937 (Figure 5). Based on these results, these two different methods appear to be capable of detecting and quantifying two distinct cell populations. We consider that the mammosphere assay which measures ability of self-renewal of cells is more important in terms of the CSC function than the Aldefluor assay. Further studies are needed in order to elucidate differences in the biological characteristics of these two cell populations detected by the two different methods.

Although GANT61 and paclitaxel additively inhibited the growth of all TNBC cell lines, the combined treatments with paclitaxel and GANT61 did not additively reduce the proportion of CSCs in any of the TNBC cell lines tested in the present study (Figure 7). Paclitaxel increased the proportion of CSCs in MDA-MB-157 cells, decreased it in HCC1937 cells, and did not change it in MDA-MB-231 cells (Figure 7). Therefore, we speculate that the molecular mechanisms responsible for the anti-cell growth and anti-CSC effects of paclitaxel and GANT61 may differ.

In conclusion, the results of the present study strongly support the hypothetical mechanisms responsible for the regulation of the CSC proportion being mediated through the non-canonical Hh signaling pathway in TNBC cells. GANT61 dose-dependently reduced the proportion of CSCs in TNBC cell lines. GANT61 also inhibited the growth of the cell lines tested in association with G1 arrest and the

induction of apoptosis. The combined treatments of GANT61 and paclitaxel additively inhibited cell growth in TNBC cell lines. These results suggest for the first time that non-canonical Hh inhibitors have potential as therapeutics in the treatment of patients with TNBC.

Conflict of interest

J. Kurebayashi received advisory/consultation fees and research funding from Takeda Pharmaceutical Co., Limited. J. Kurebayashi received research funding from Takeda Pharmaceutical Co., Esai Co., Chugai Co., and AstraZeneca Pharmaceuticals. The other authors declare that they have no conflict of interest.

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Figure legends

Figure 1

Basal mRNA expression levels of GLI1 (A) and GLI2 (B) in three TNBC cell lines and four ER-positive breast cancer cell lines. Expression levels were measured as described in the Materials and Methods. Values were analyzed after normalization to the controls and expressed as means ± SEs. The expression level in each molecule in MCF-7 cells was defined as 1. **, $P < 0.01$ significantly different from the control.

Figure 2

Anti-cell growth effects of GANT61 in three TNBC cell lines and four ER-positive cell lines. All cell lines were treated with GANT61 for three days. Cell numbers were measured using a Coulter counter. Values are expressed as means ± SEs of % of the control.

Figure 3

Effects of GANT61 on apoptosis in MDA-MB-231 cells (A), MDA-MB-157 cells (B), and HCC1937 cells (C). Cells were treated with GANT61 for two days. The percentages of apoptotic cells were analyzed as described in the Materials and Methods. Values are means ± SEs. White bars, control; the lightest grey bars, 1 μM GANT61; the second lightest gray bars, 5 μM GANT61; the third lightest gray bars, 10 μM GANT61; and the darkest grey bars, 20 μM GANT61. * $P < 0.05$; **$P < 0.01$ significantly different from control cells.

Figure 4

Effects of GANT61 on the proportion of ALDH-positive cells in MDA-MB-231 cells (A), MDA-MB-157 cells (C), and HCC1937 cells (E). Cells were treated with GANT61 for three days. The percentages of ALDH-positive cells were analyzed by the Aldefluor assay as described in the Materials and Methods. Values are expressed as means ± SEs. White bars, control; the lightest grey bars, 1 μM GANT61; the second lightest gray bars, 5 μM GANT61; the third lightest gray bars, 10 μM GANT61; and the darkest grey bars, 20 μM GANT61. * $P < 0.05$; **$P < 0.01$ significantly different from control cells. Representative results of the Aldefluor assay are shown in Figures 5B, 5D, and 5F. The dots in the R3 area were ALDH-positive cells.

Figure 5

Effects of GANT61 on the number of mammospheres/1,000 cells seeded in MDA-MB-231 cells (A), MDA-MB-157 cells (C), and HCC1937 cells (E). Cells were treated with GANT61 for three days. The number of mammospheres were analyzed as described in the Materials and Methods. Values are expressed as means ± SEs. White bars, control; the lightest grey bars, 1 μM GANT61; the second lightest gray bars, 5 μM GANT61; the third lightest gray bars, 10 μM GANT61; and the darkest grey bars, 20 μM GANT61. * $P < 0.05$; **$P < 0.01$ significantly different from control cells. Representative results of the Aldefluor assay are shown in Figures 6B, 6D, and 6F.

Figure 6

Growth inhibitory curves of combined treatments of GANT61 and paclitaxel in MDA-MB-231 cells (A), MDA-MB-157 cells (B), and HCC1937 cells (C). Cells were treated with GANT61 for three days and paclitaxel for 4 hours. Values are expressed as

means ± SEs.

Figure 7

Effects of GANT61 and paclitaxel on the number of mammospheres/1,000 cells seeded in MDA-MB-231 cells (A), MDA-MB-157 cells (B), and HCC1937 cells (C). Cells were treated with GANT61 for three days and paclitaxel for four hours. The number of mammospheres was analyzed as described in the Materials and Methods. Values are expressed as means ± SEs. * $P < 0.05$; **$P < 0.01$ significantly different from control cells.

Electronic Supplementary Material

File 1

Figure Legends

Figure S1

Basal protein expression levels of GLI1 (A) and GLI2 (B) in three TNBC cell lines and four ER-positive breast cancer cell lines. Expression levels were measured using western blotting as described in the Materials and Methods. The deduced molecular weights of GLI1 and Gli2 were approximately 160 kDa and 220 kDa according to the manufacturer's instructions, respectively.

Figure S2

Effects of GANT61 on cell cycle progression in MDA-MB-231 cells (A), MDA-MB-157 cells (B), and HCC1937 cells (C). Cells were treated with GANT61 for two days. The percentages of cells at each cell cycle phase were analyzed as described in the Materials and Methods. Values are expressed as means ± SEs. White bars, control; the lightest grey bars, 1 μM GANT61; the second lightest gray bars, 5 μM GANT61; the third lightest gray bars, 10 μM GANT61; and the darkest grey bars, 20 μM GANT61. * $P < 0.05$; **$P < 0.01$ significantly different from control cells.

Figure S3

Effects of GANT61 (0 – 20 μ M) on the expression levels of survivin in MDA-MB-231 cells. The cells were treated with the indicated concentrations of GANT61 for two days. The expression levels were tested using western blotting as described in the Methods. The deduced molecular weight of survivin was approximately 16 kDa according to the manufacture's instruction.

Figure S4

Time courses of changes induced in mRNA expression levels of GLI1 (A) and GLI2 (B) by GANT61 in MDA-MB-157 cells. The expression levels of GLI1 and GLI2 in controls at the respective time points were defined as 1. In addition, dose-dependent decreases in the mRNA expression levels of GLI1 (C) and GLI2 (D) were investigated in MDA-MB-157 cells. Cells were treated with 1 – 20 μM GANT61 for 24 hours. Values are expressed as means ± SEs. * $P < 0.05$; **$P < 0.01$; and #, $P < 0.10$ significantly different from the respective controls.

Electronic Supplementary Material

File 2

Table S1

Subtypes and IC_{50}s of GANT61 in three TNBC cell lines and four ER-positive breast cancer cell lines. All cell lines were treated with 0 – 20 μM GANT61 for three days. Values are expressed as means ± SEs.

Figure S1A The protein expression levels of GLI1 in ER-positive and ER-negative breast cancer cell lines

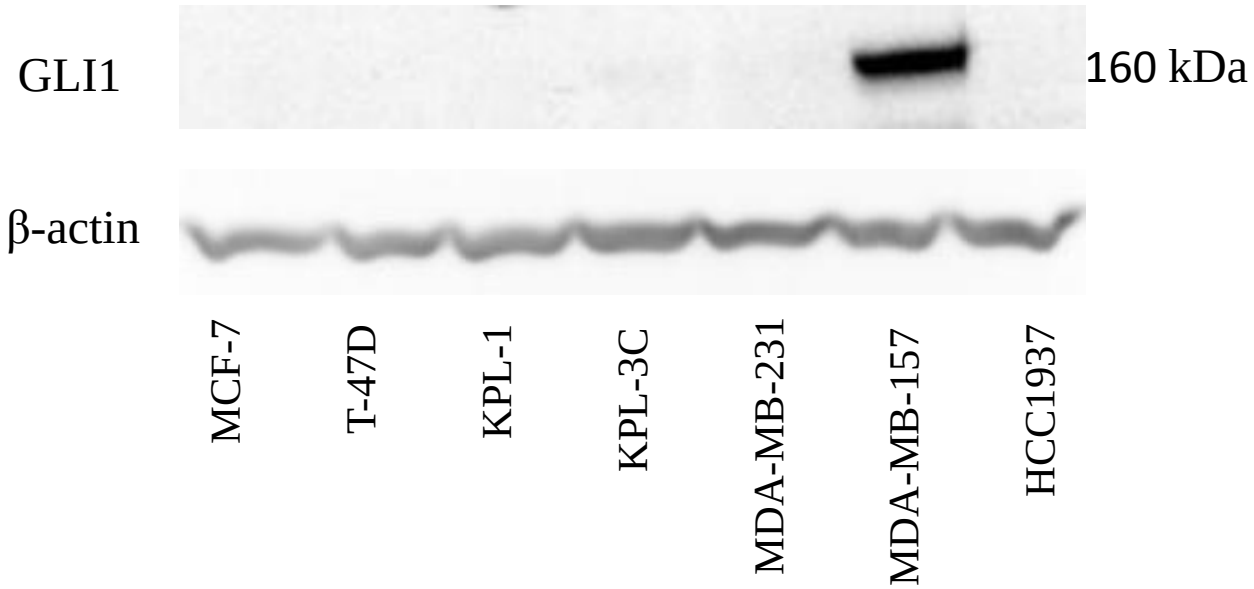


Figure S1B The protein expression levels of GLI2 in ER-positive and ER-negative breast cancer cell lines

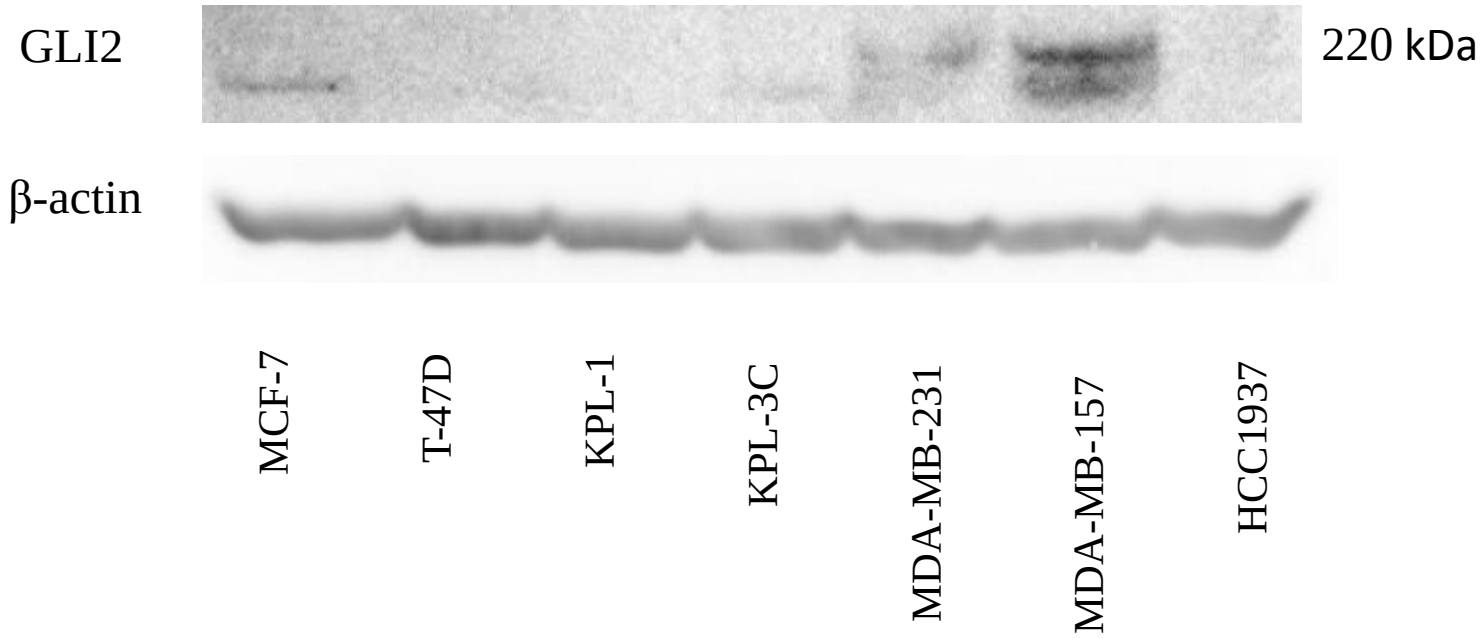


Figure S2A

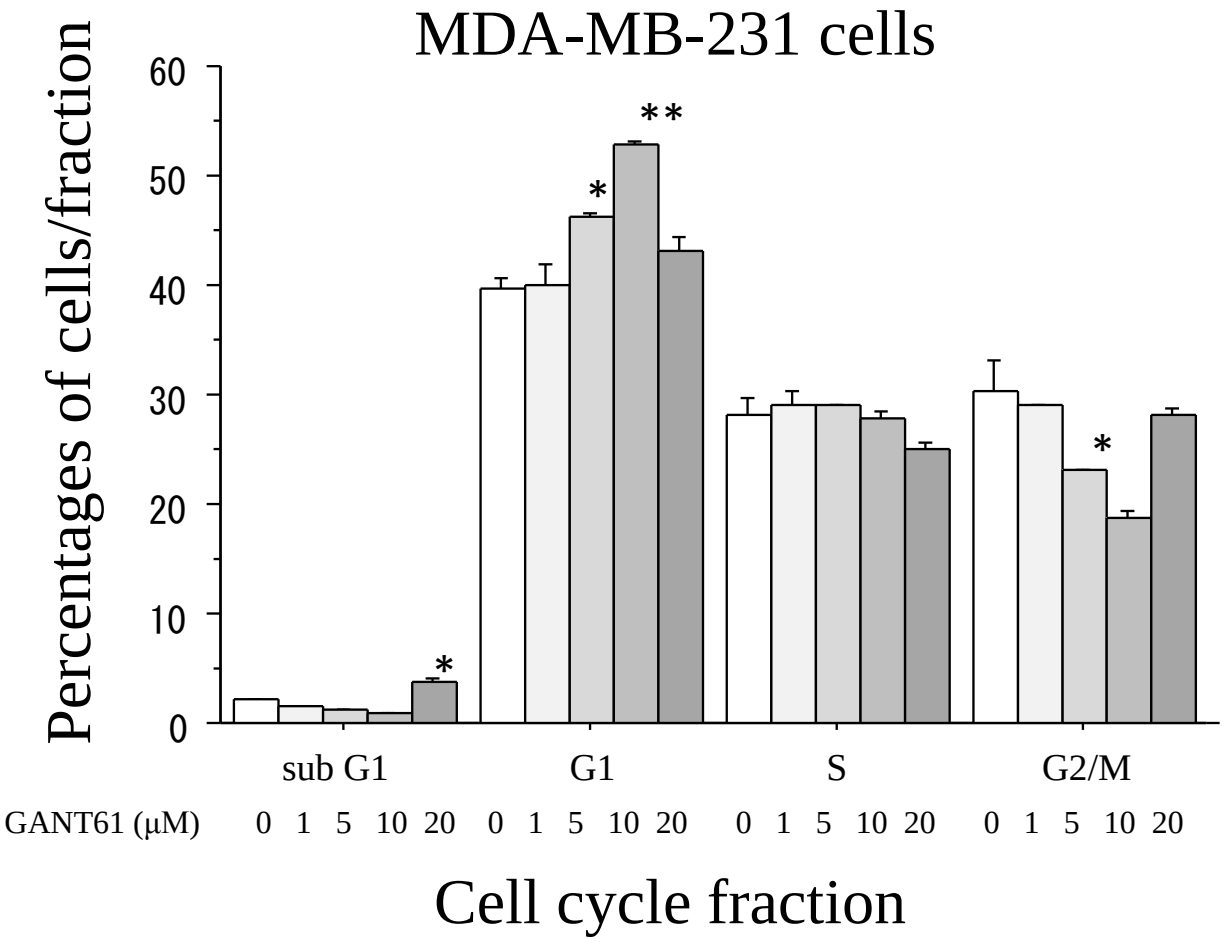

MDA-MB-231 cells
Percentages of cells/fraction
60
50
40
30
20
10
0
**
*
*
*
sub G1
G1
S
G2/M
GANT61 (μM) 0 1 5 10 20 0 1 5 10 20 0 1 5 10 20 0 1 5 10 20
Cell cycle fraction

Figure S2B

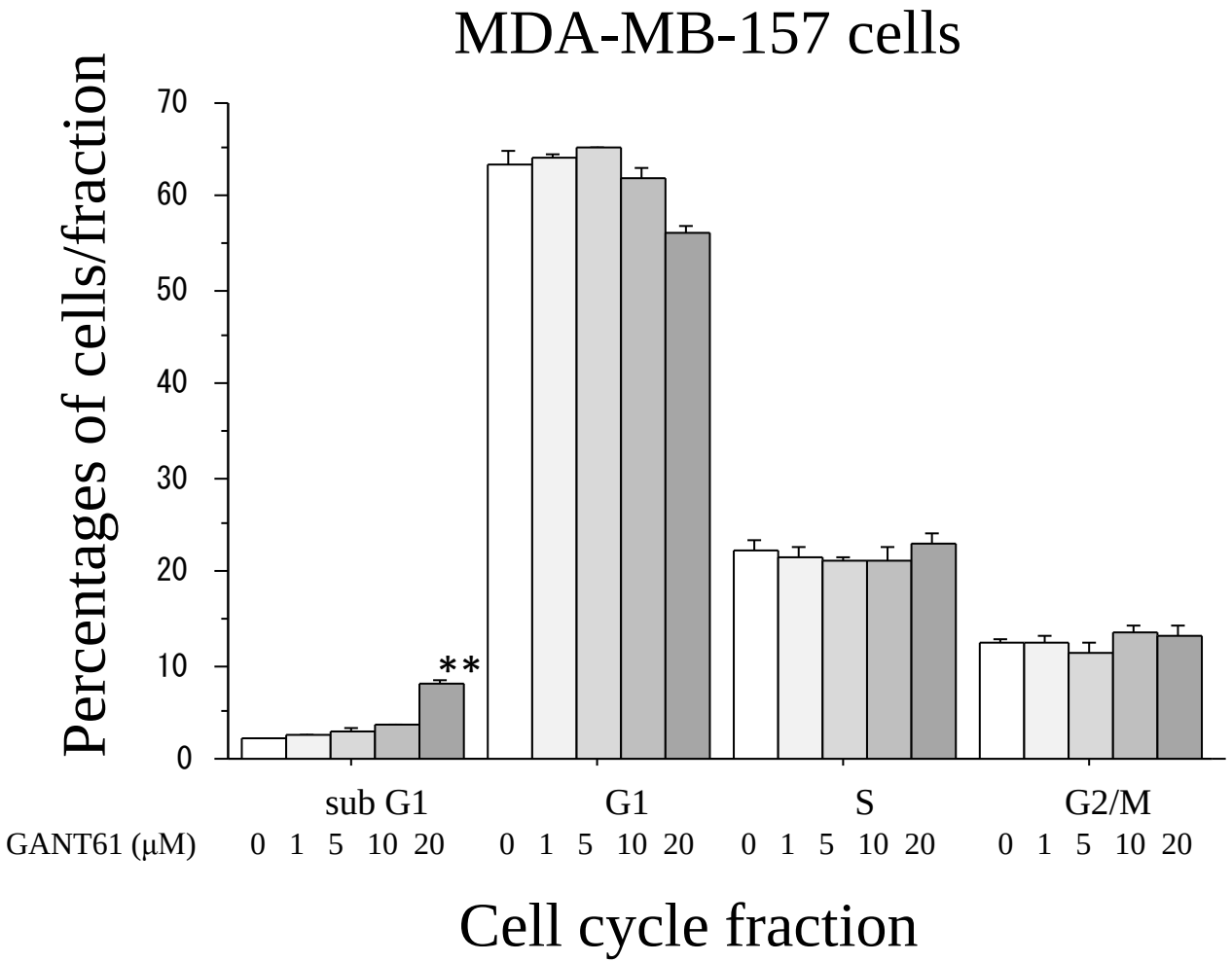

MDA-MB-157 cells
Percentages of cells/fraction
70
60
50
40
30
20
10
0
**
sub G1
G1
S
G2/M
GANT61 (μM)
0 1 5 10 20
0 1 5 10 20
0 1 5 10 20
0 1 5 10 20
Cell cycle fraction

Figure S2C

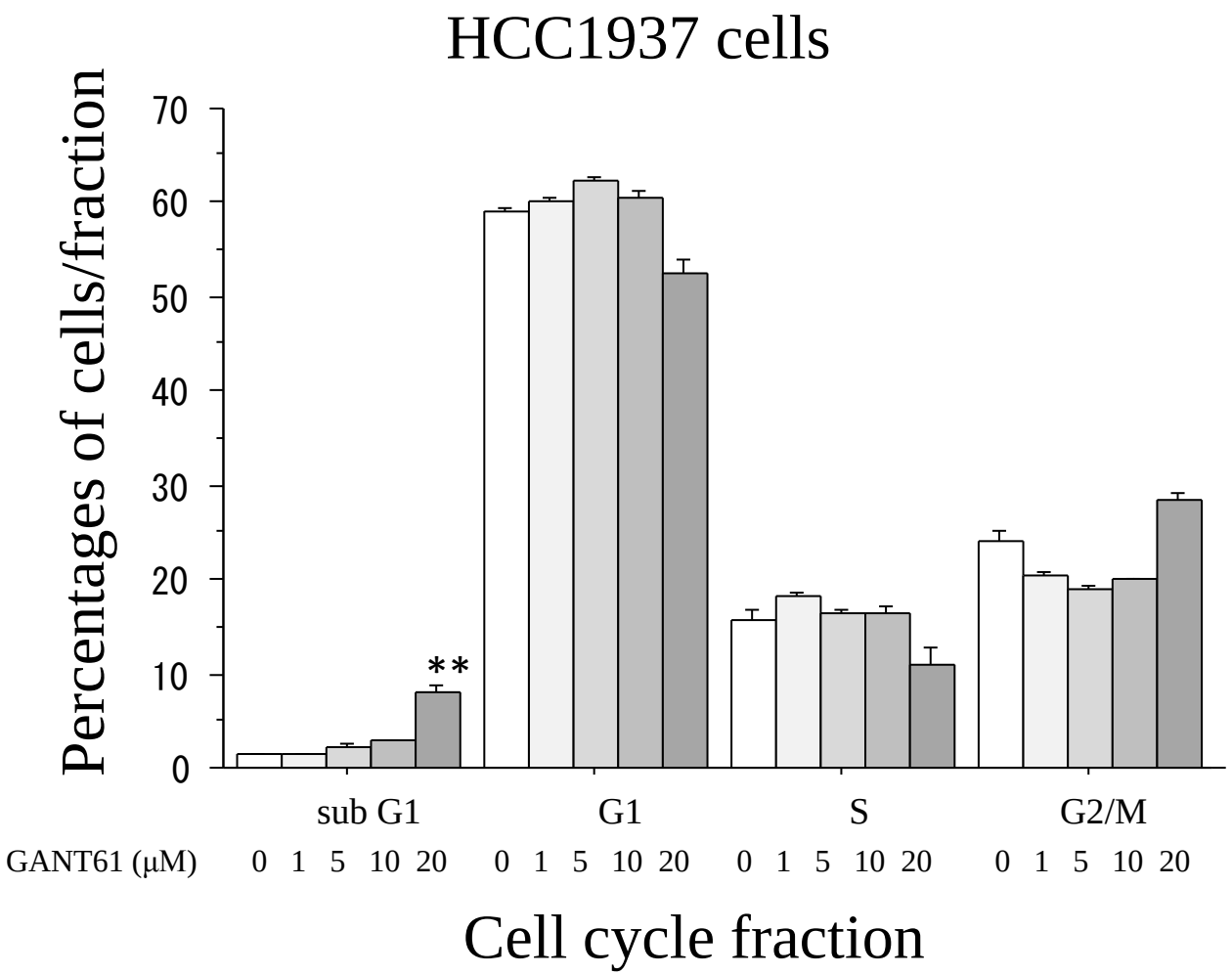

HCC1937 cells
Percentages of cells/fraction
70
60
50
40
30
20
10
0
**
sub G1
G1
S
G2/M
GANT61 (μM)
0 1 5 10 20
0 1 5 10 20
0 1 5 10 20
0 1 5 10 20
Cell cycle fraction

Figure S3

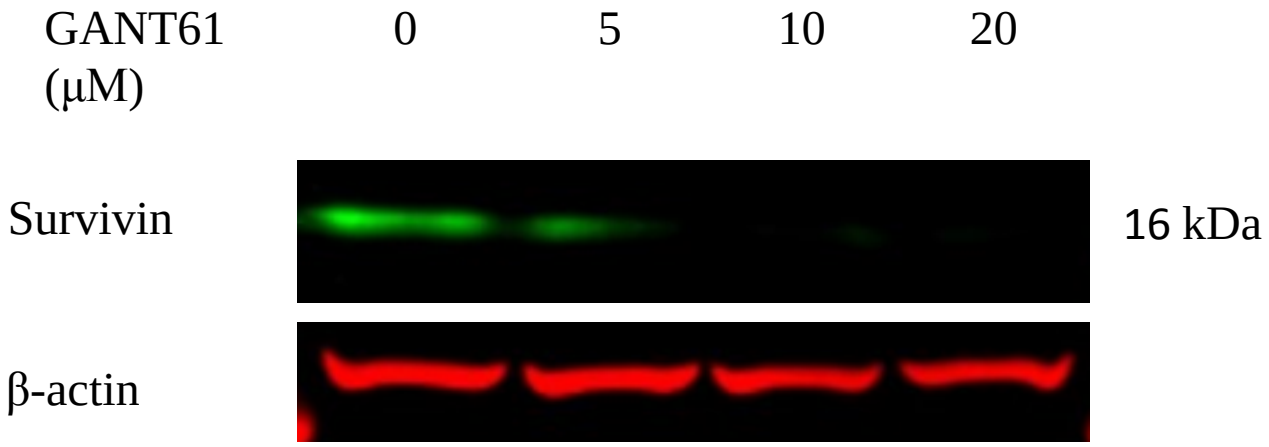


Figure S4A

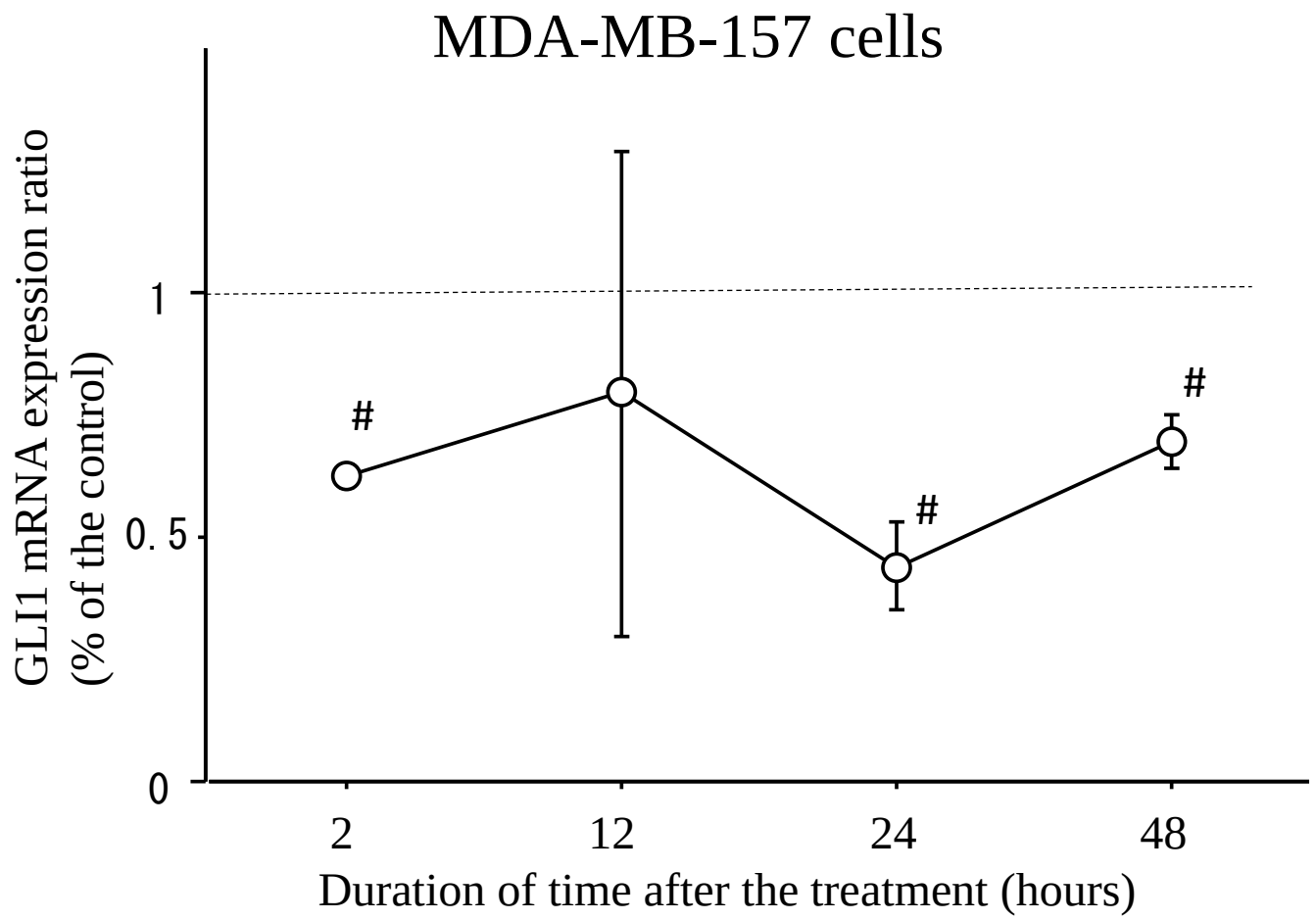

MDA-MB-157 cells
GLI1 mRNA expression ratio
(% of the control)
1
0. 5
0
#
#
#
2
12
24
48
Duration of time after the treatment (hours)

Figure S4B

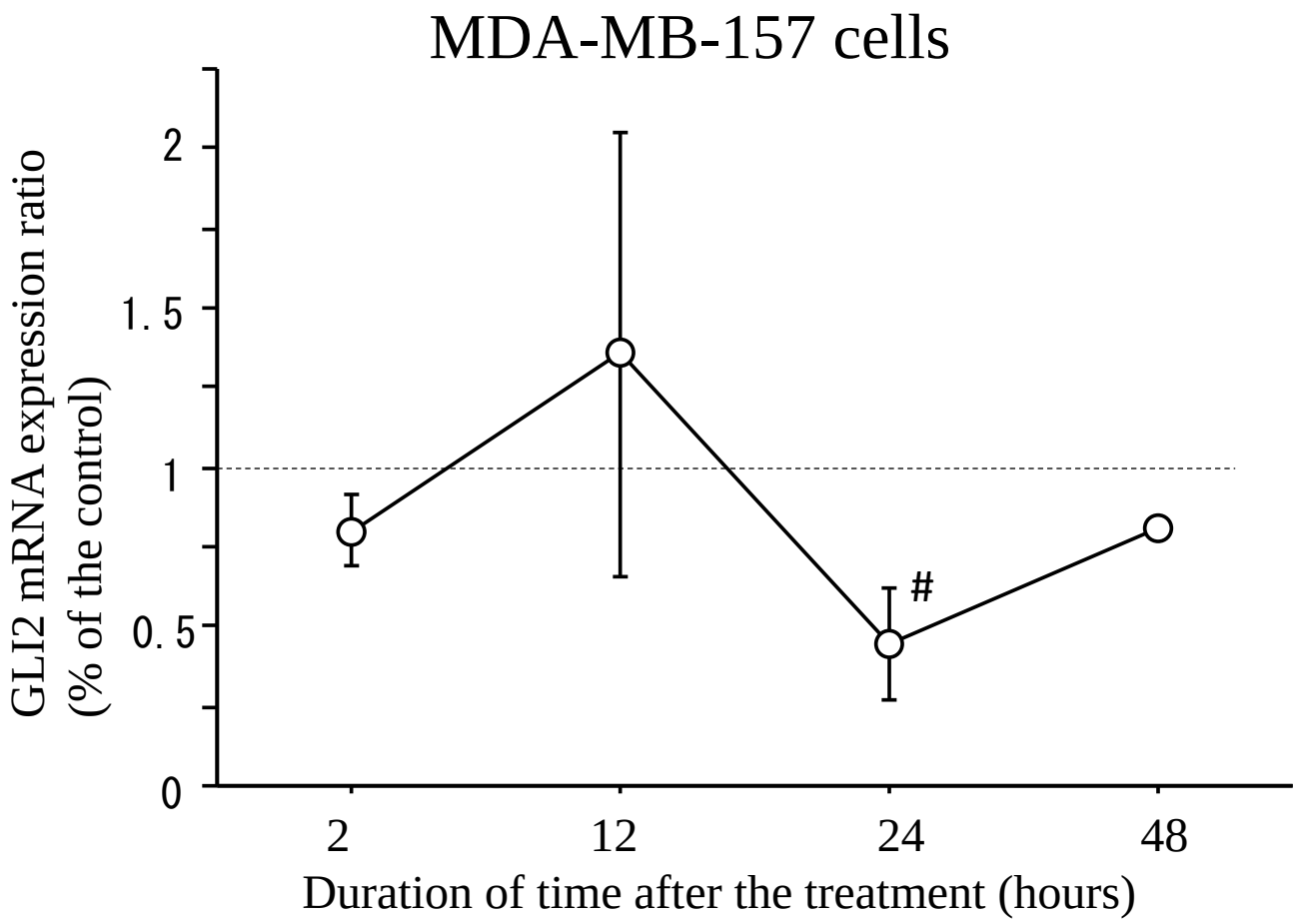

MDA-MB-157 cells
GLI2 mRNA expression ratio
(% of the control)
2
1.5
1
0.5
0
#
2
12
24
48
Duration of time after the treatment (hours)

Figure S4C

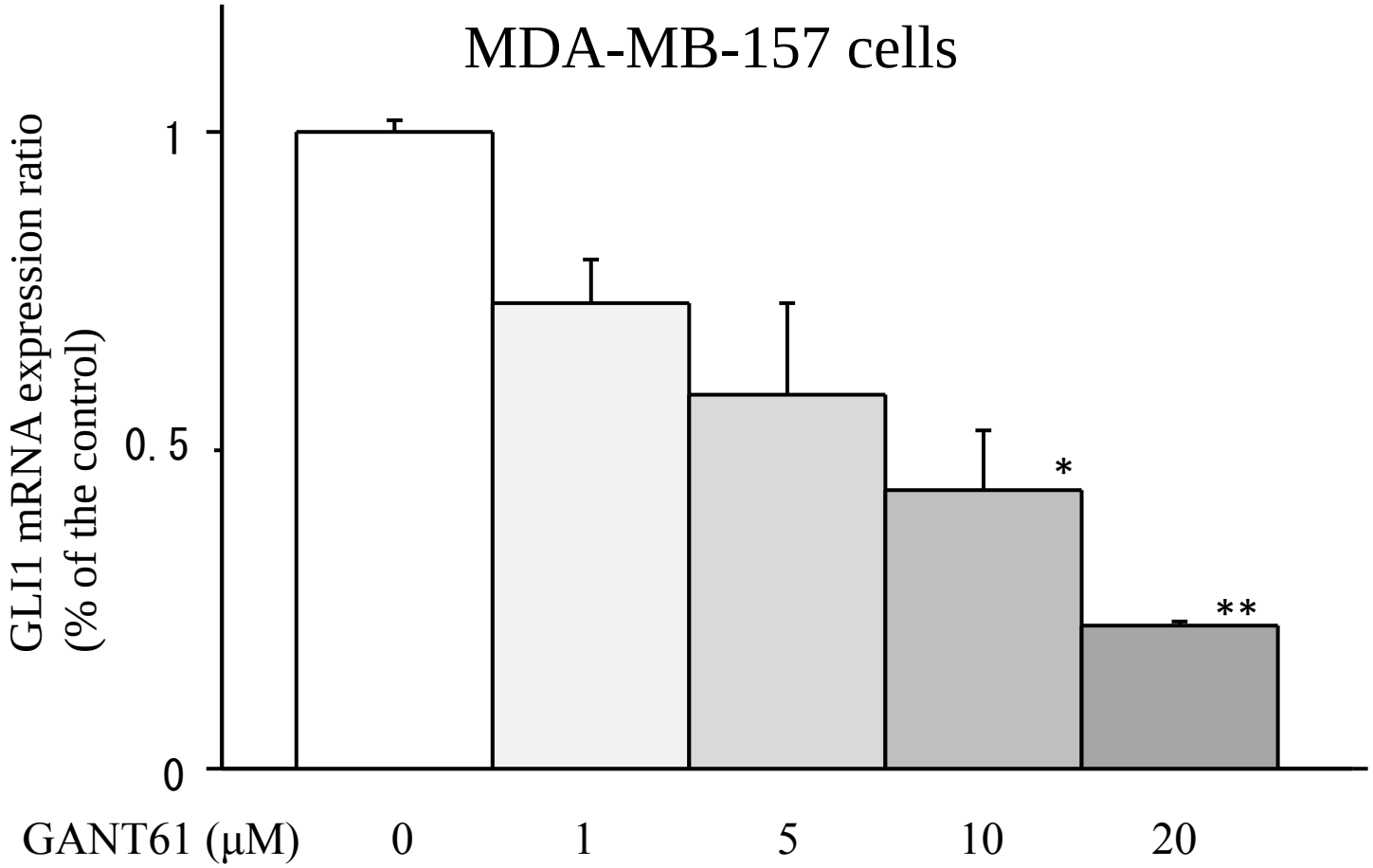

MDA-MB-157 cells
GLI1 mRNA expression ratio
(% of the control)
1
0.5
0
*
**
GANT61 (μM)
0
1
5
10
20

Figure S4D

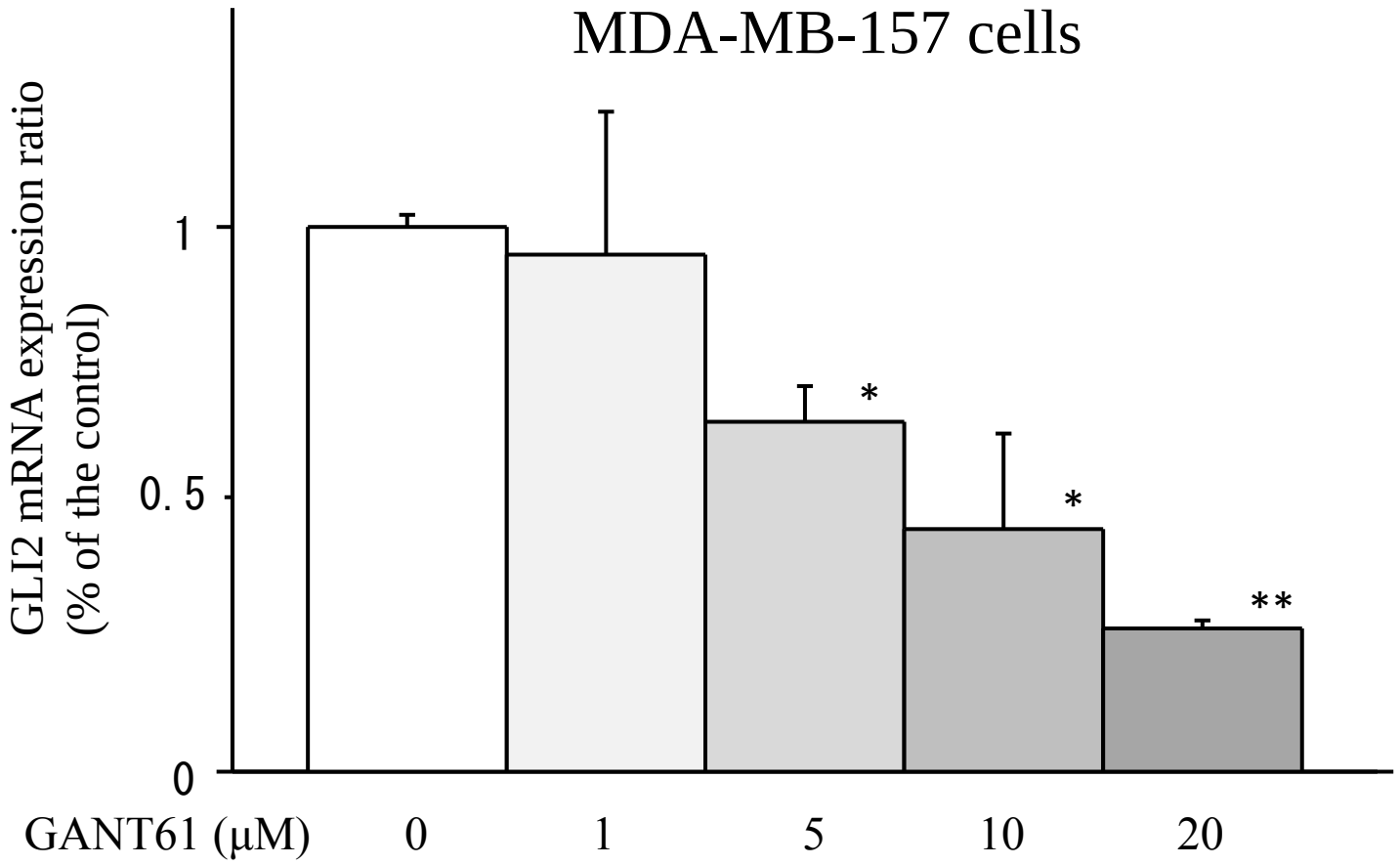

MDA-MB-157 cells
GLI2 mRNA expression ratio
(% of the control)
1
0.5
0
*
*
**
GANT61 (μM)
0
1
5
10
20

Table 1

Cell line	Subtype	50% IC (μM)
MDA-MB-231	TNBC (claudin-low)	6.74±1.08
MDA-MB-157	TNBC (claudin-low)	10.71±0.79
HCC1937	TNBC (basal-like)	9.64±1.19
MCF-7	Luminal	12.78±1.23
T-47D	Luminal	14.09±1.16
KPL-1	Luminal	13.50±0.46
KPL-3C	Luminal	12.35±0.48

Figure 1A

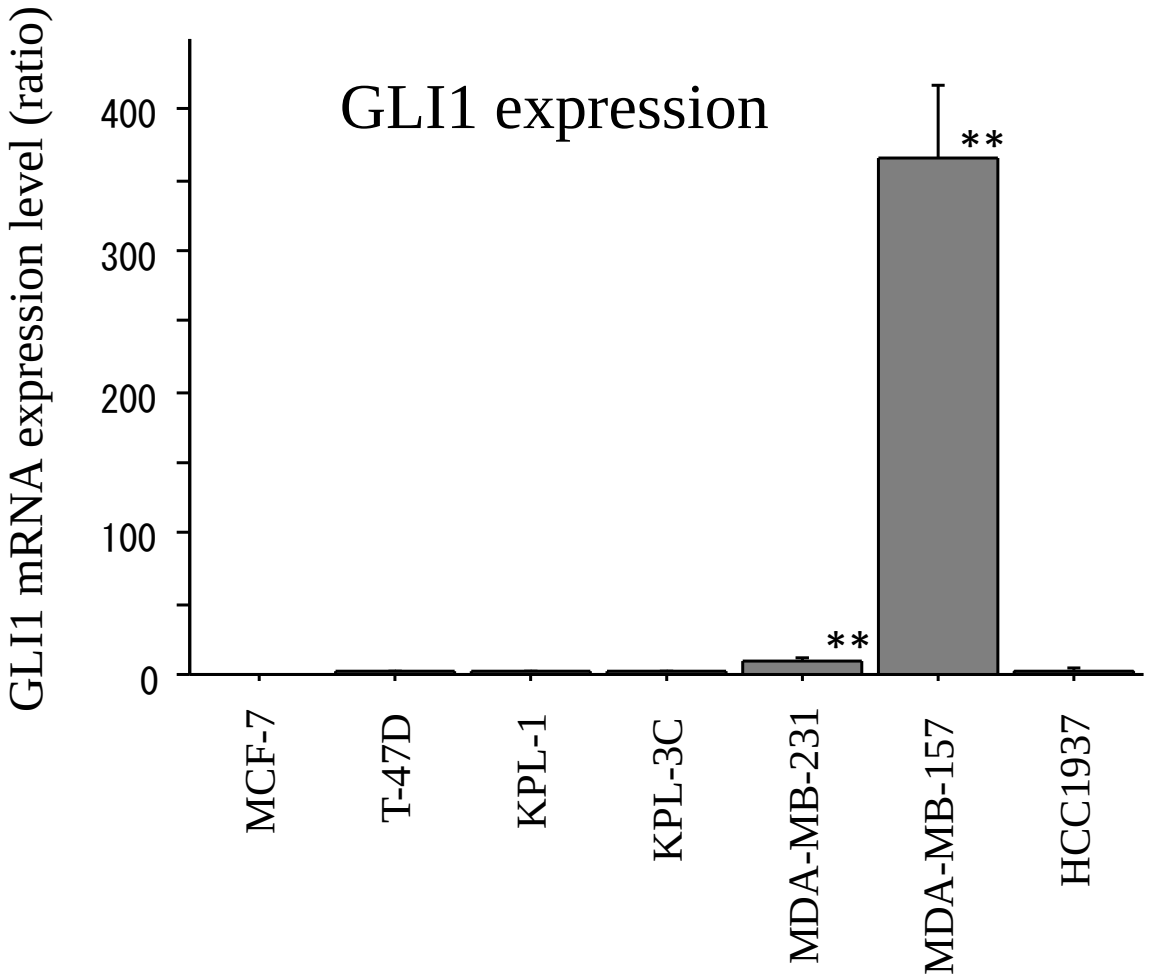

GLI1 expression
GLI1 mRNA expression level (ratio)
400
300
200
100
0
**
**
MCF-7
T-47D
KPL-1
KPL-3C
MDA-MB-231
MDA-MB-157
HCC1937

Figure 1B

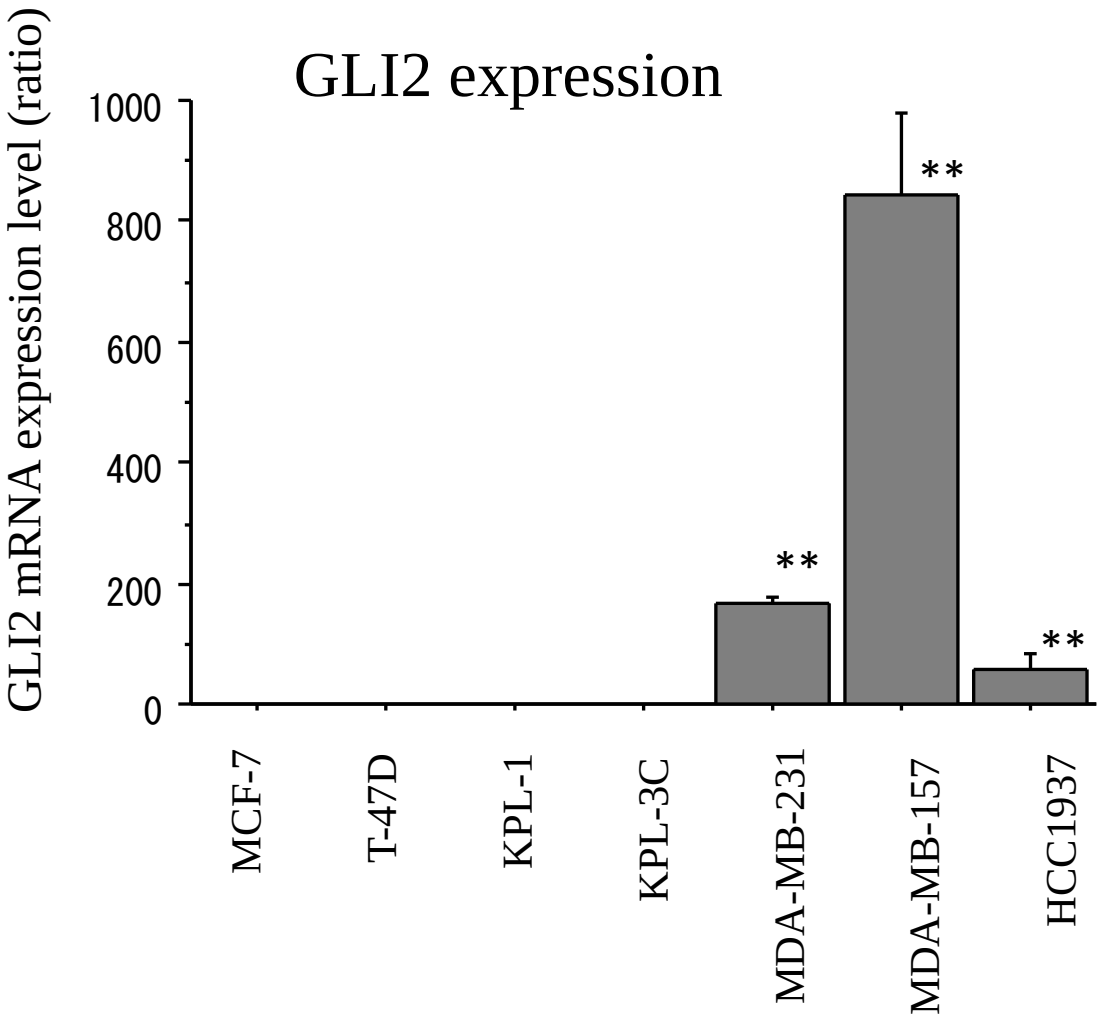

GLI2 expression
GLI2 mRNA expression level (ratio)
1000
800
600
400
200
0
**
**
**
MCF-7
T-47D
KPL-1
KPL-3C
MDA-MB-231
MDA-MB-157
HCC1937

Figure 2

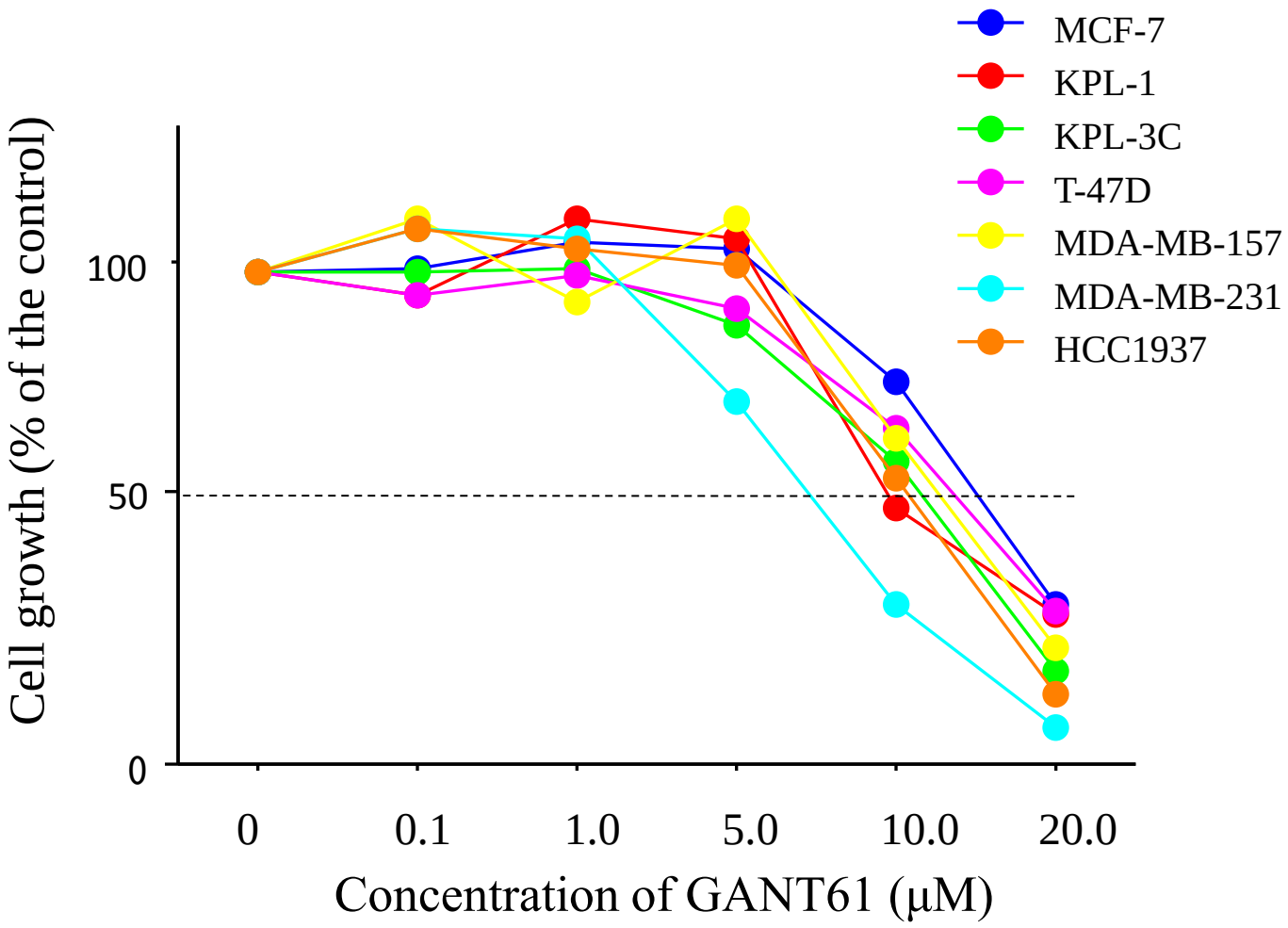

MCF-7
KPL-1
KPL-3C
T-47D
MDA-MB-157
MDA-MB-231
HCC1937
Cell growth (% of the control)
100
50
0
0
0.1
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 3A

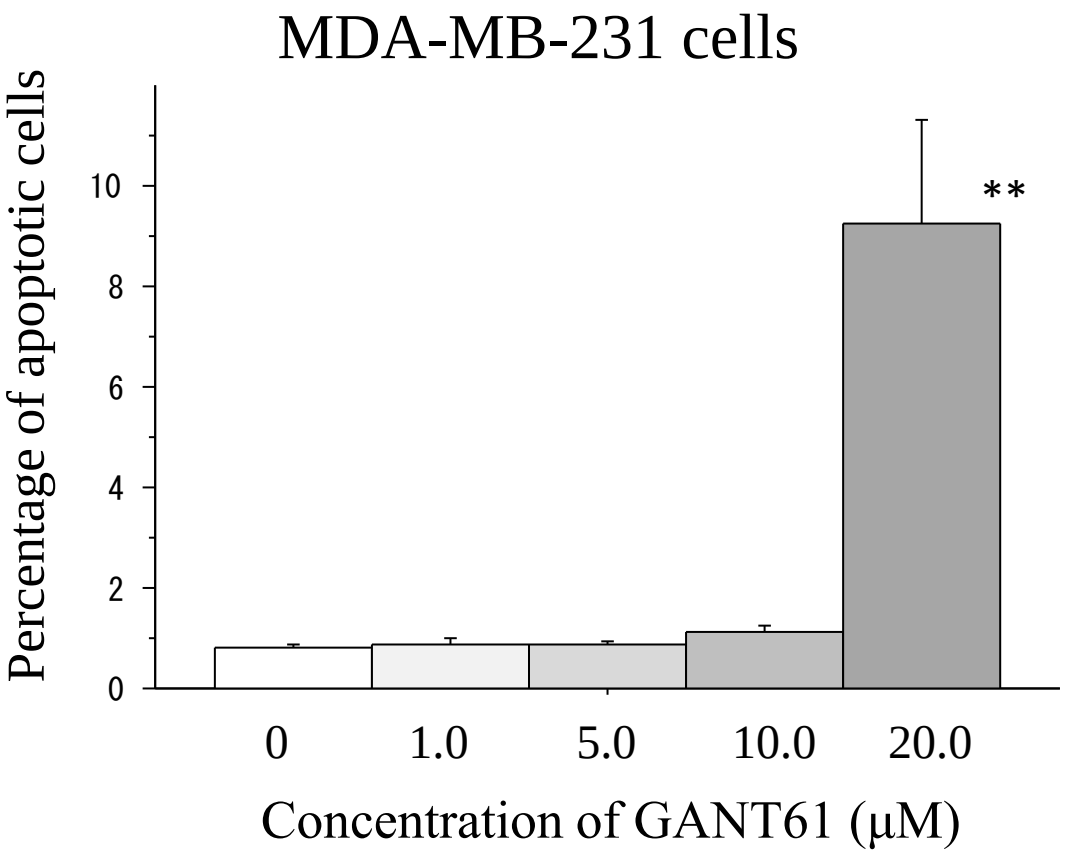

MDA-MB-231 cells
Percentage of apoptotic cells
0
2
4
6
8
10
**
0
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 3B

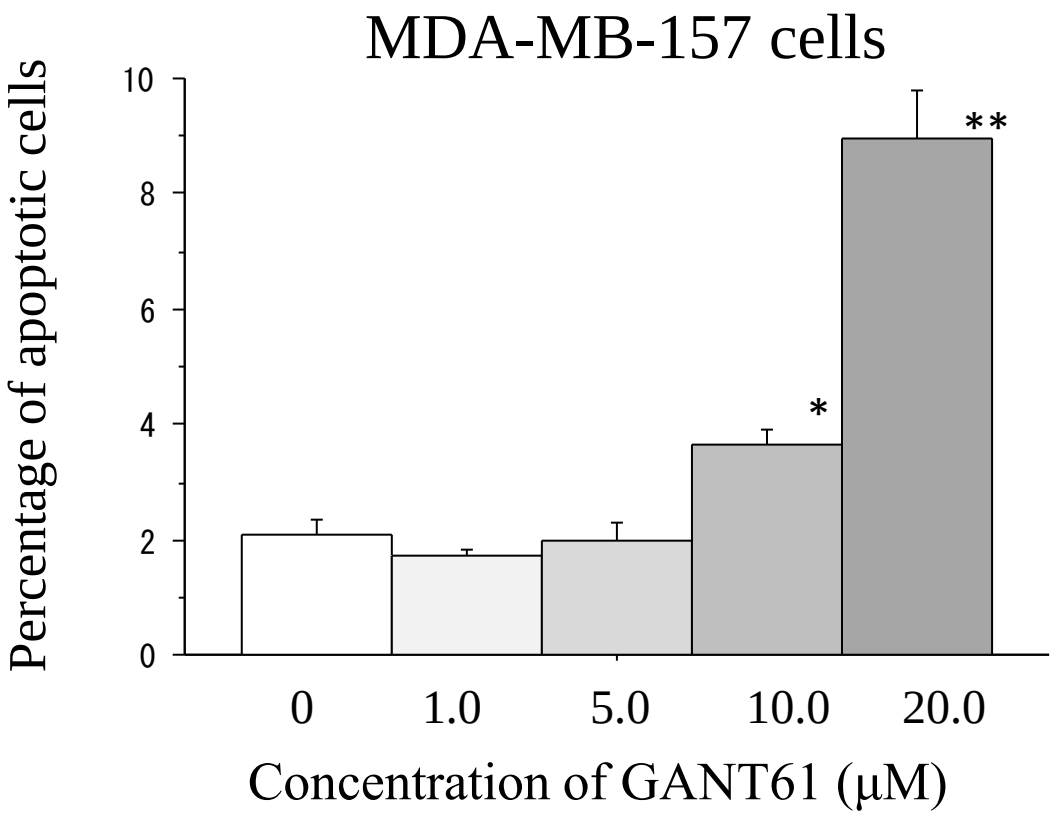

MDA-MB-157 cells
Percentage of apoptotic cells
10
8
6
4
2
0
*
**
0
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 3C

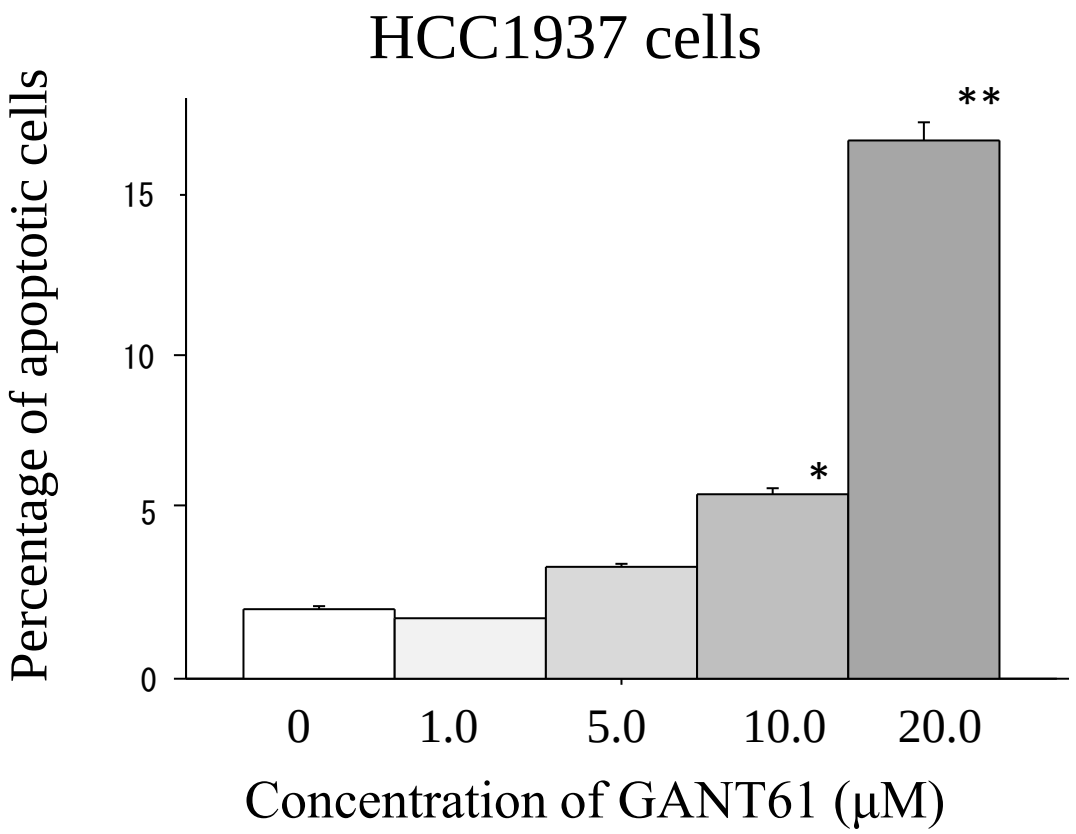

HCC1937 cells
Percentage of apoptotic cells
15
10
5
0
**
*
0
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 4A

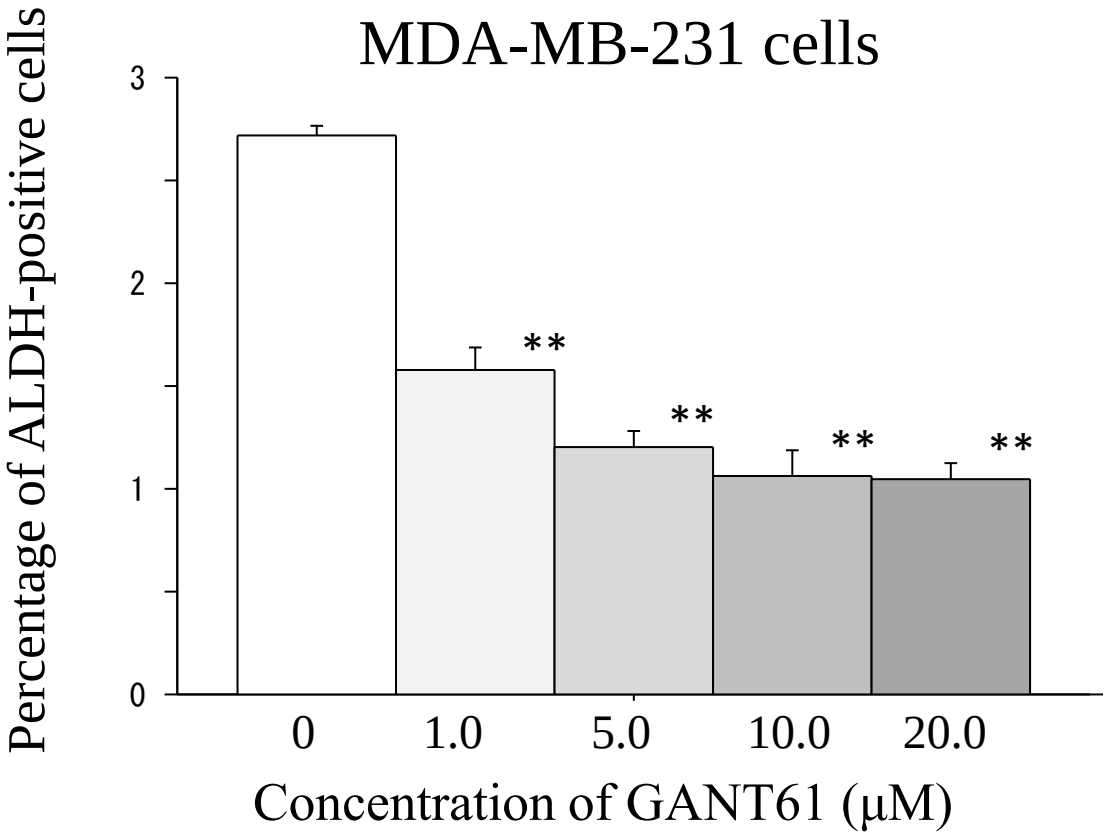
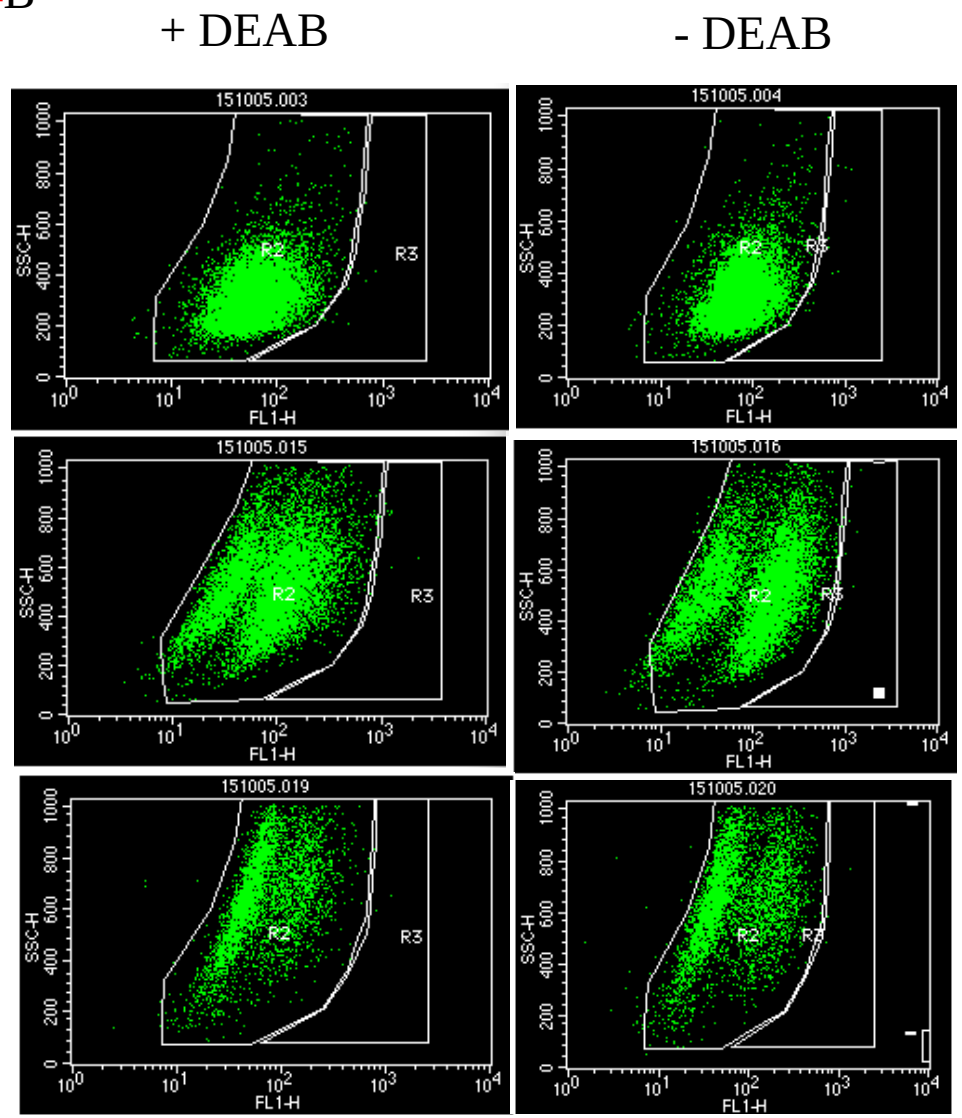

MDA-MB-231 cells
Percentage of ALDH-positive cells
3
2
1
0
**
**
**
**
0
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 4B



MDA-MB-231 cells

Control

Treated with 10 μM GANT61

Treated with 20 μM GANT61

Figure 4C

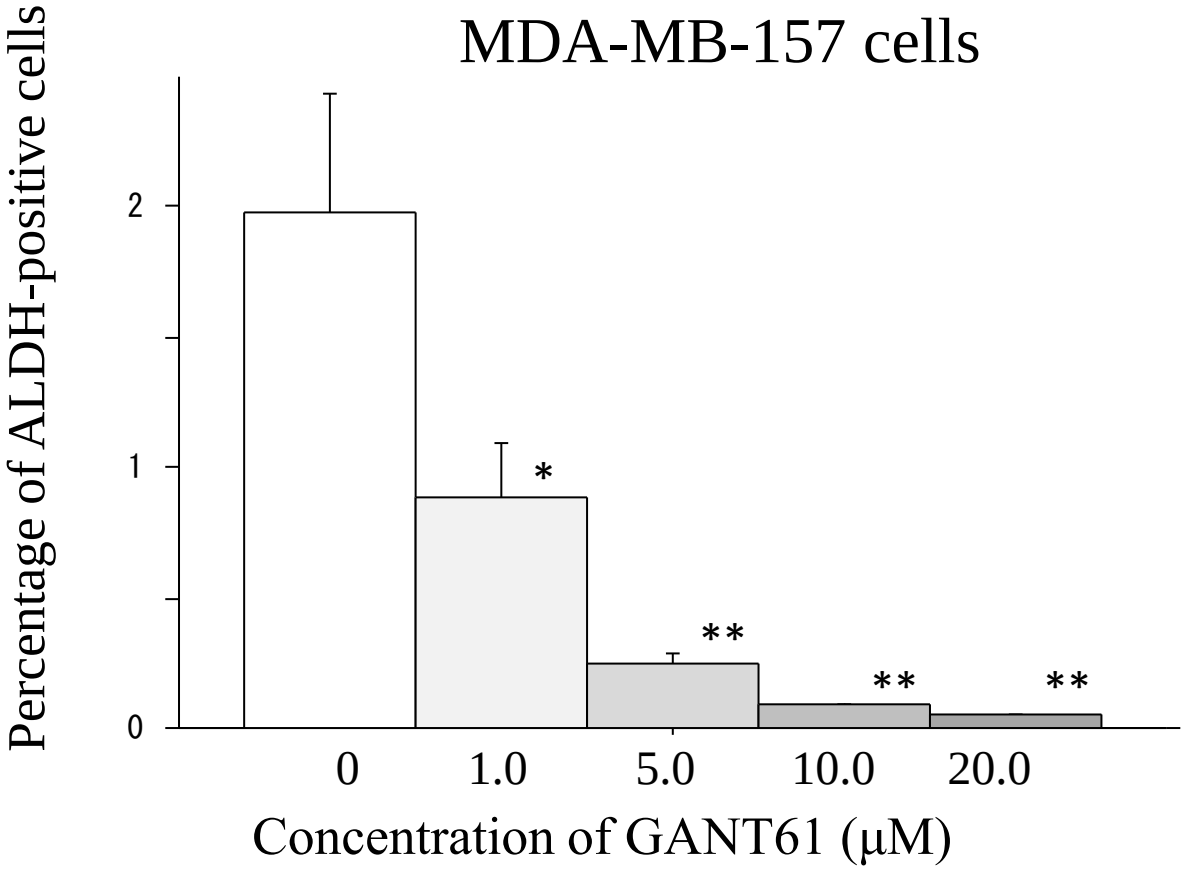
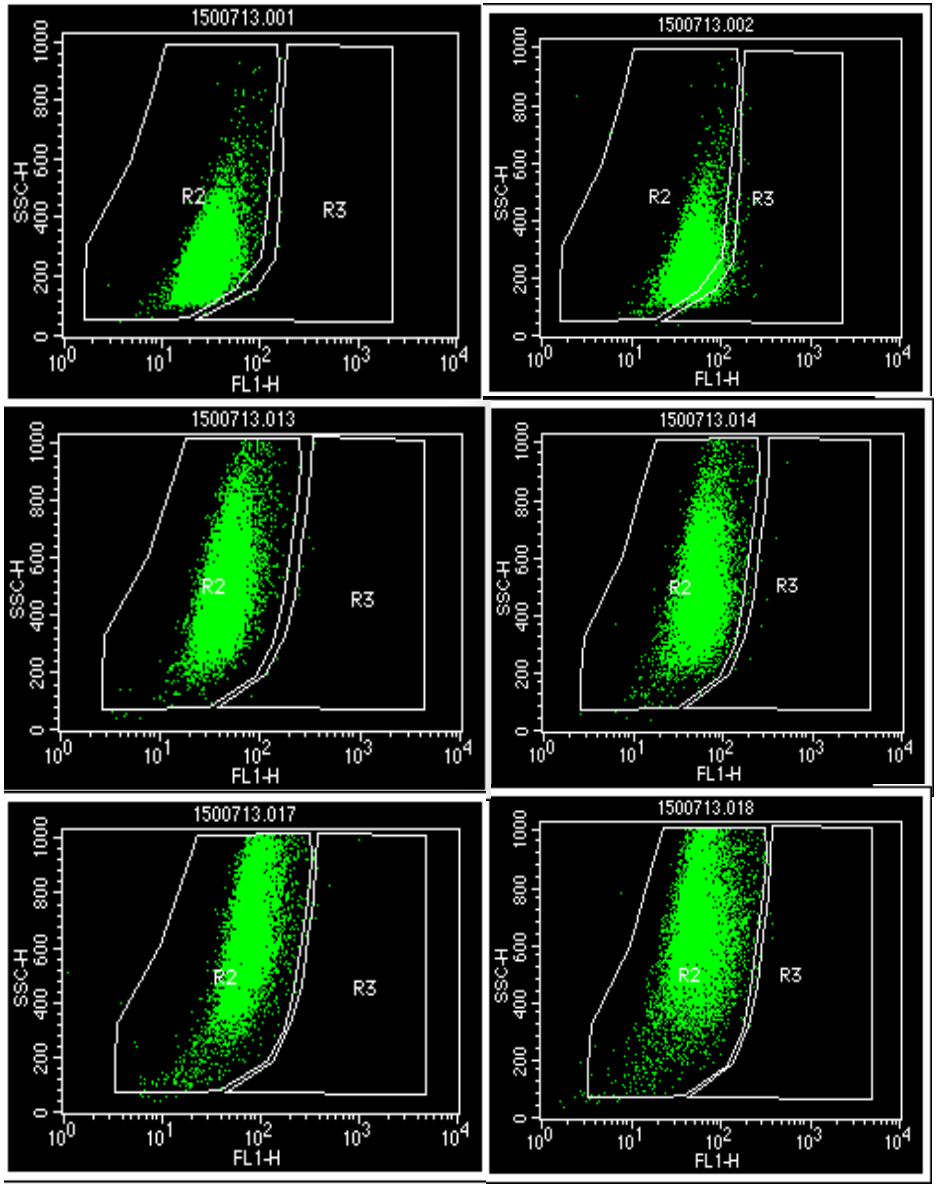

MDA-MB-157 cells
Percentage of ALDH-positive cells
2
1
0
*
**
**
**
0
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 4D

MDA-MB-157 cells

+ DEAB

- DEAB



Control

Treated with 10 μM GANT61

Treated with 20 μM GANT61

Figure 4E

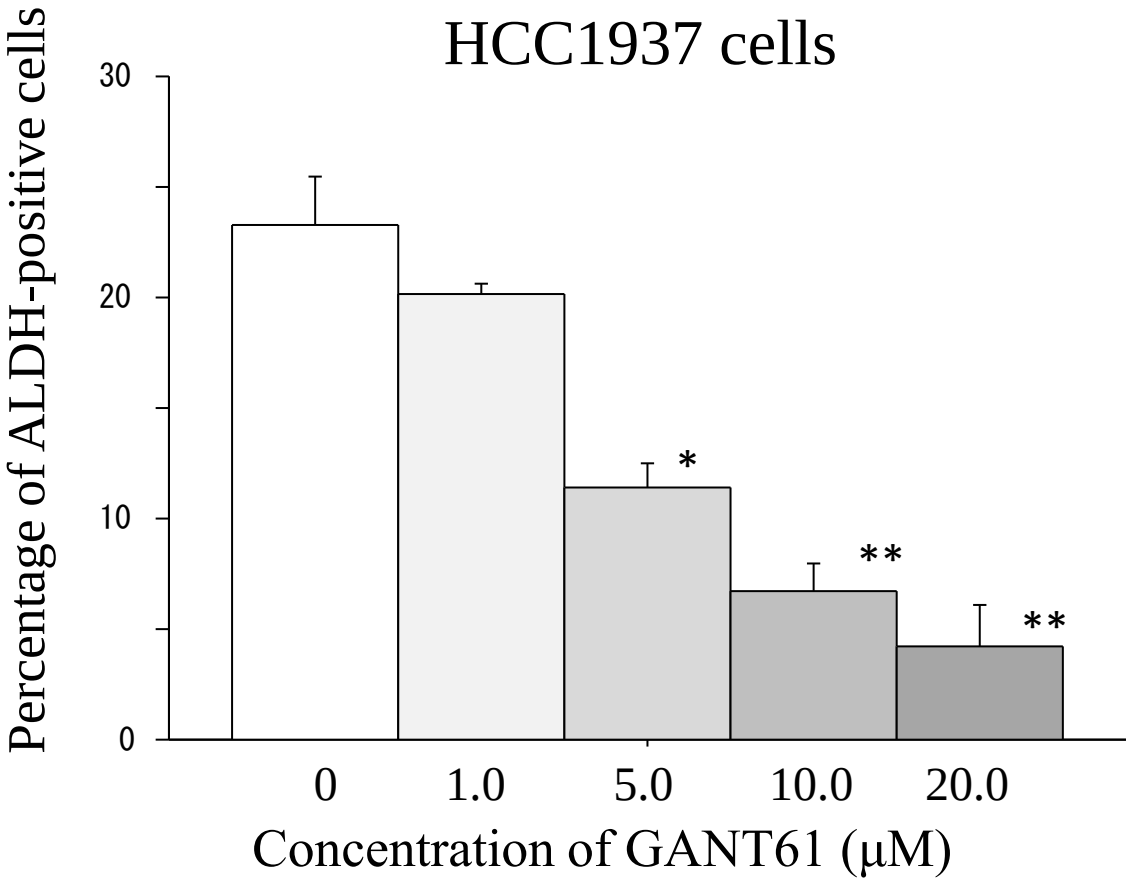
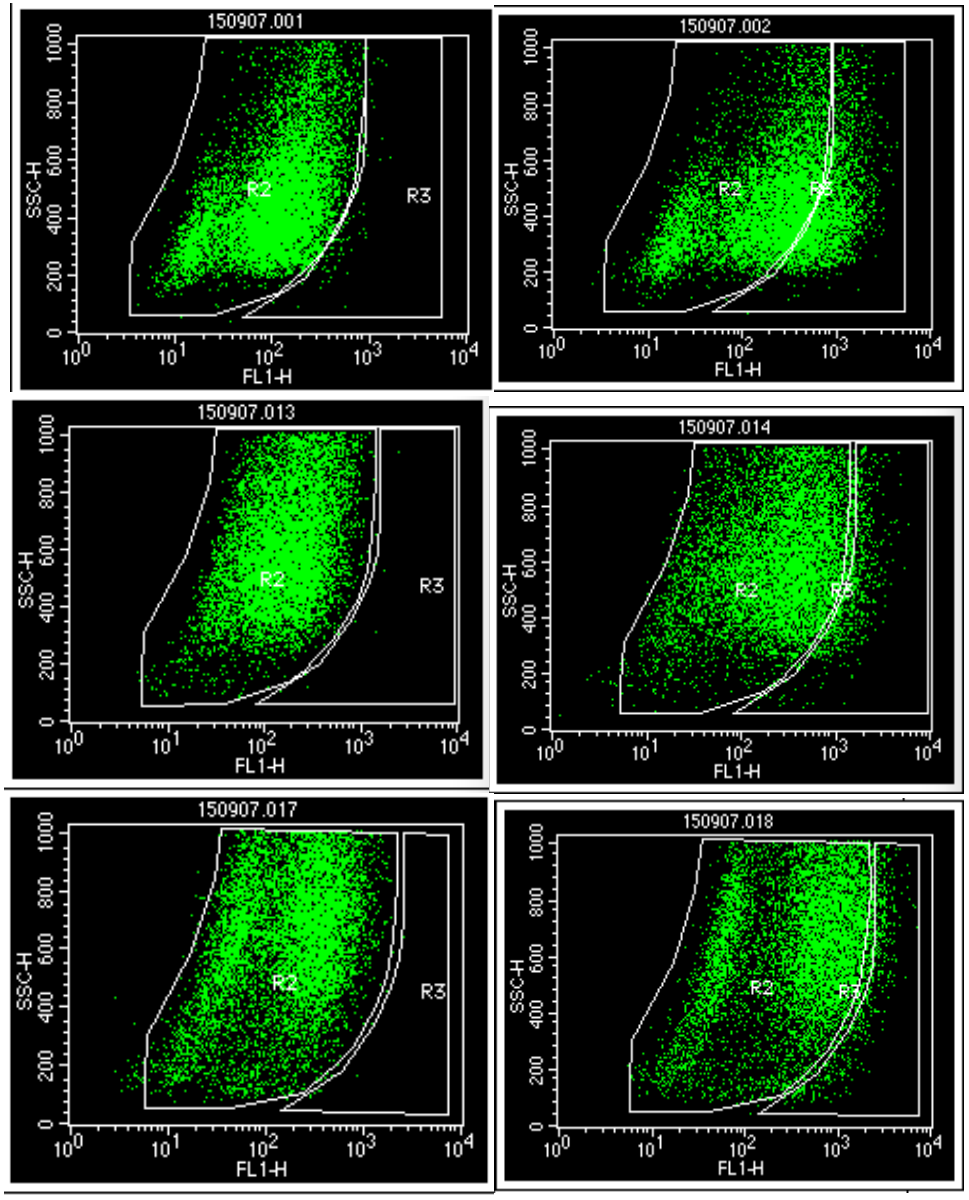

HCC1937 cells
Percentage of ALDH-positive cells
30
20
10
0
*
**
**
0
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 4F

HCC1937 cells

+ DEAB

- DEAB



Control

Treated with 10 μM GANT61

Treated with 20 μM GANT61

Figure 5A

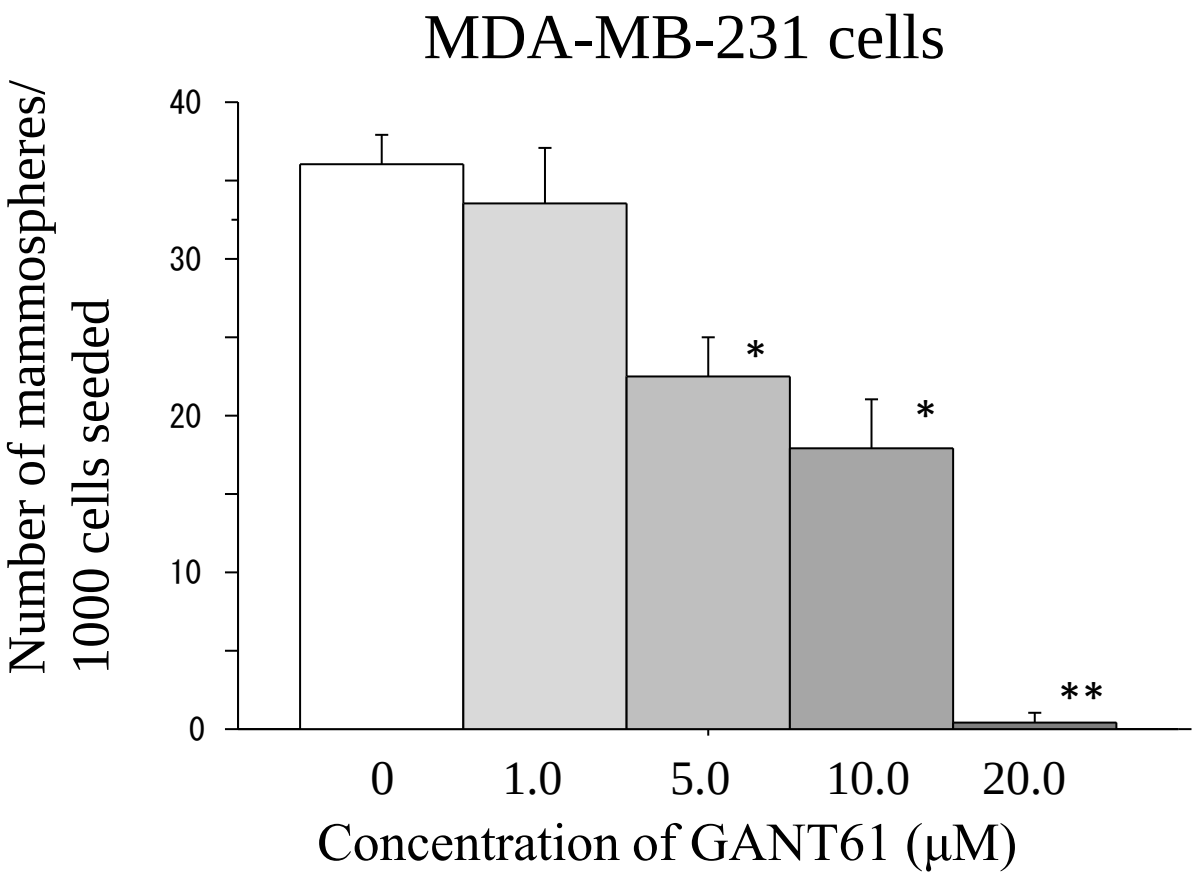
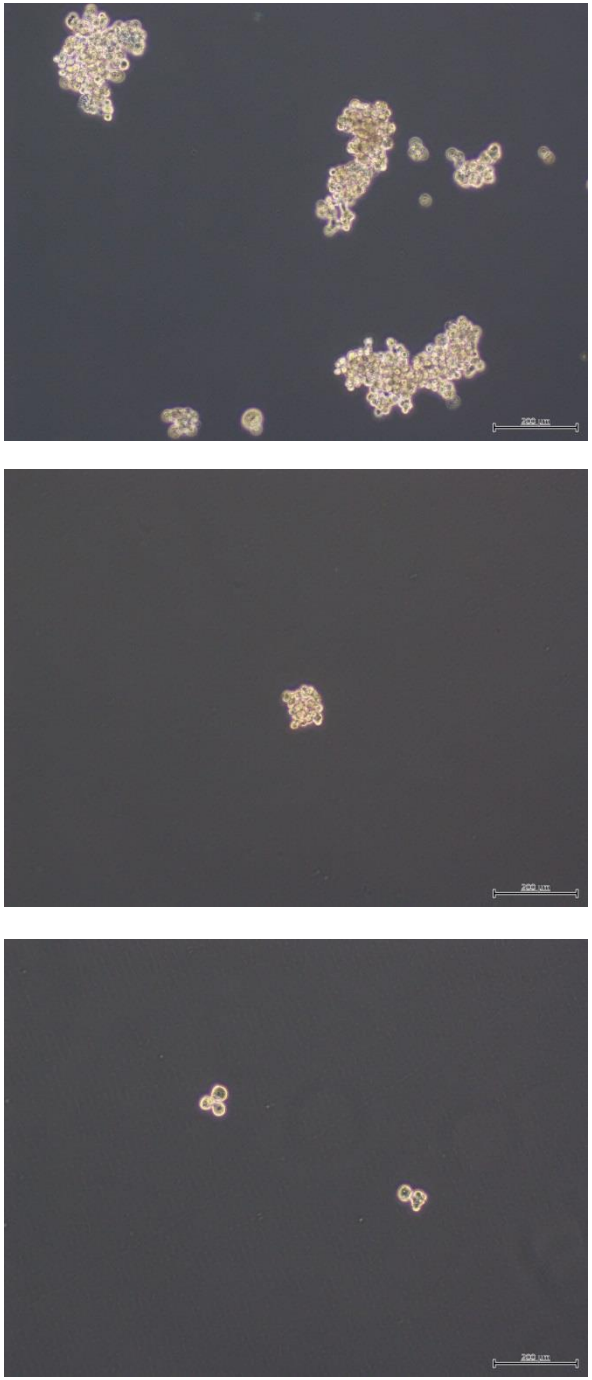

MDA-MB-231 cells
Number of mammospheres/
1000 cells seeded
40
30
20
10
0
*
*
**
0
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 5B



MDA-MB-231 cells

Control

Treated with 10 μM GANT61

Treated with 20 μM GANT61

Figure 5C

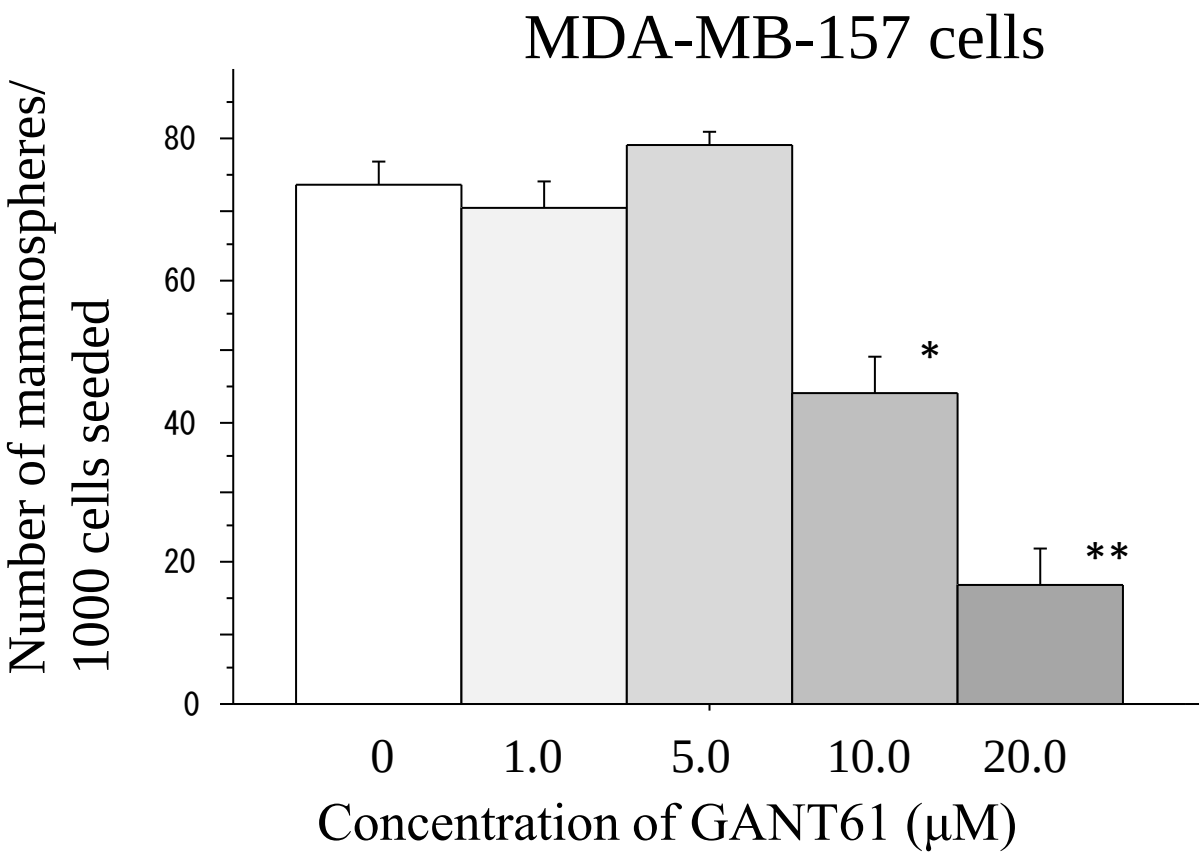
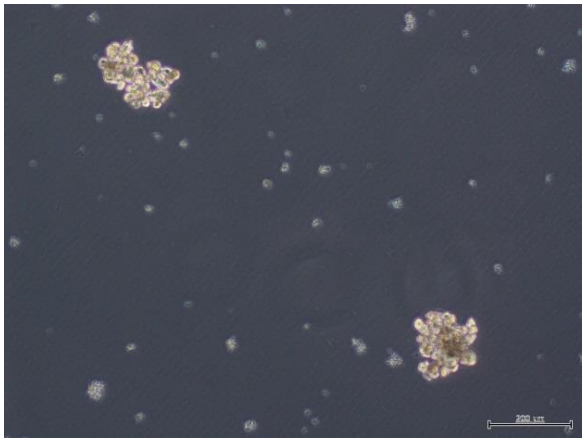
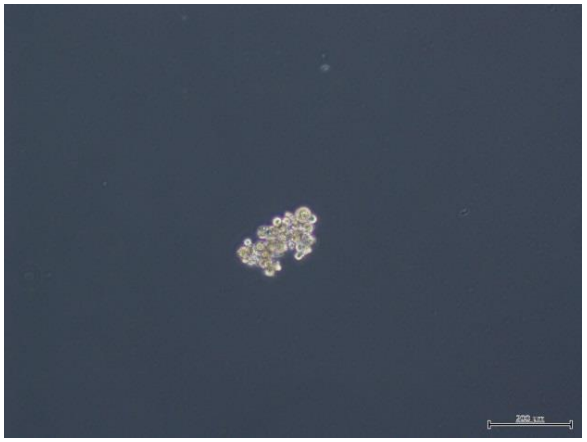

MDA-MB-157 cells
Number of mammospheres/
1000 cells seeded
80
60
40
20
0
*
**
0
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 5D



MDA-MB-157 cells

Control



Treated with 10 μM GANT61

Treated with 20 μM GANT61

Figure 5E

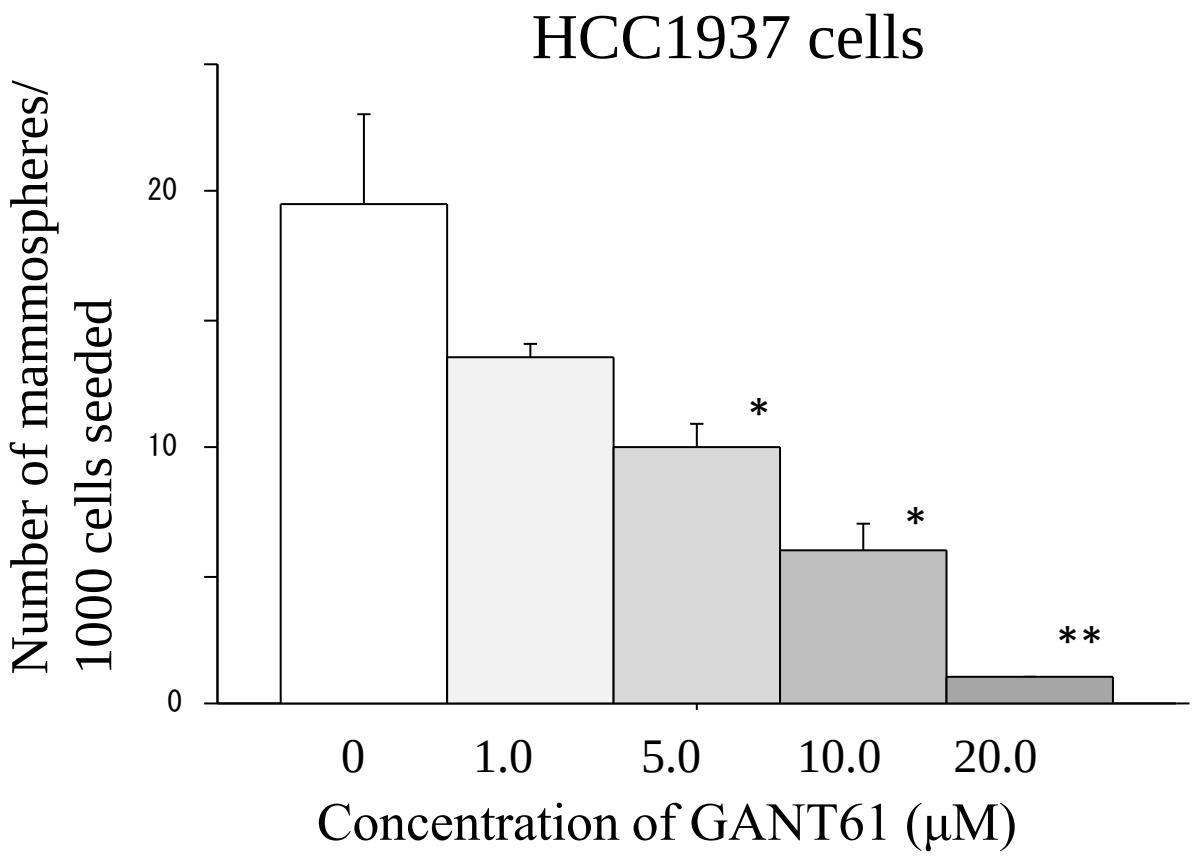
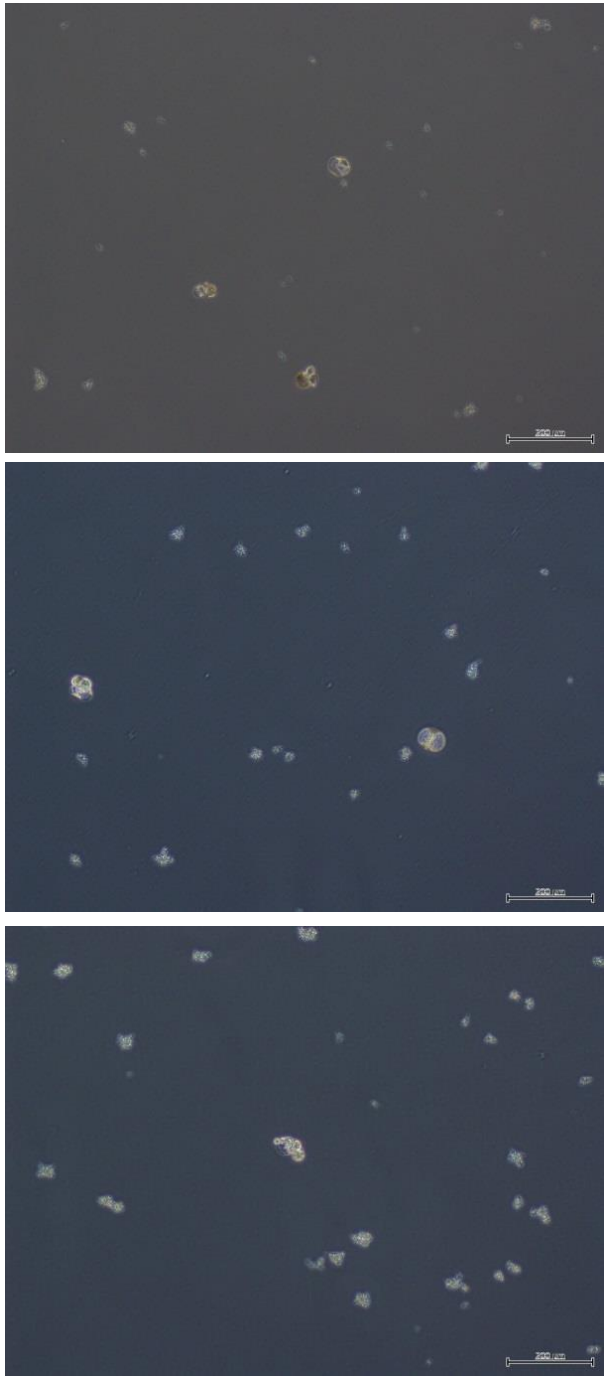

HCC1937 cells
Number of mammospheres/
1000 cells seeded
20
10
0
*
*
**
0
1.0
5.0
10.0
20.0
Concentration of GANT61 (μM)

Figure 5F



HCC1937 cells

Control

Treated with 10 μM GANT61

Treated with 20 μM GANT61

Figure 6A

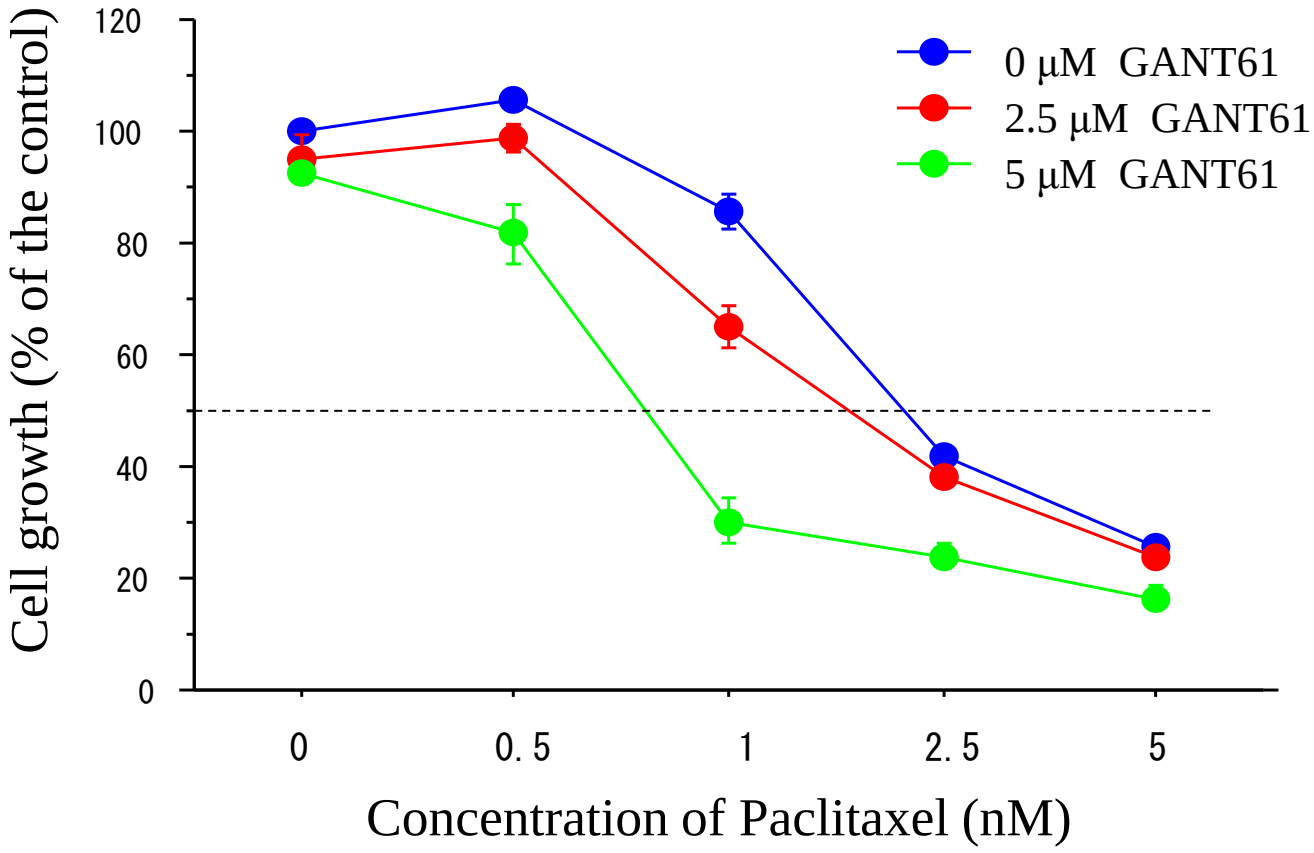

Cell growth (% of the control)
120
100
80
60
40
20
0
0
0.5
1
2.5
5
Concentration of Paclitaxel (nM)
0 μM GANT61
2.5 μM GANT61
5 μM GANT61

Figure 6B

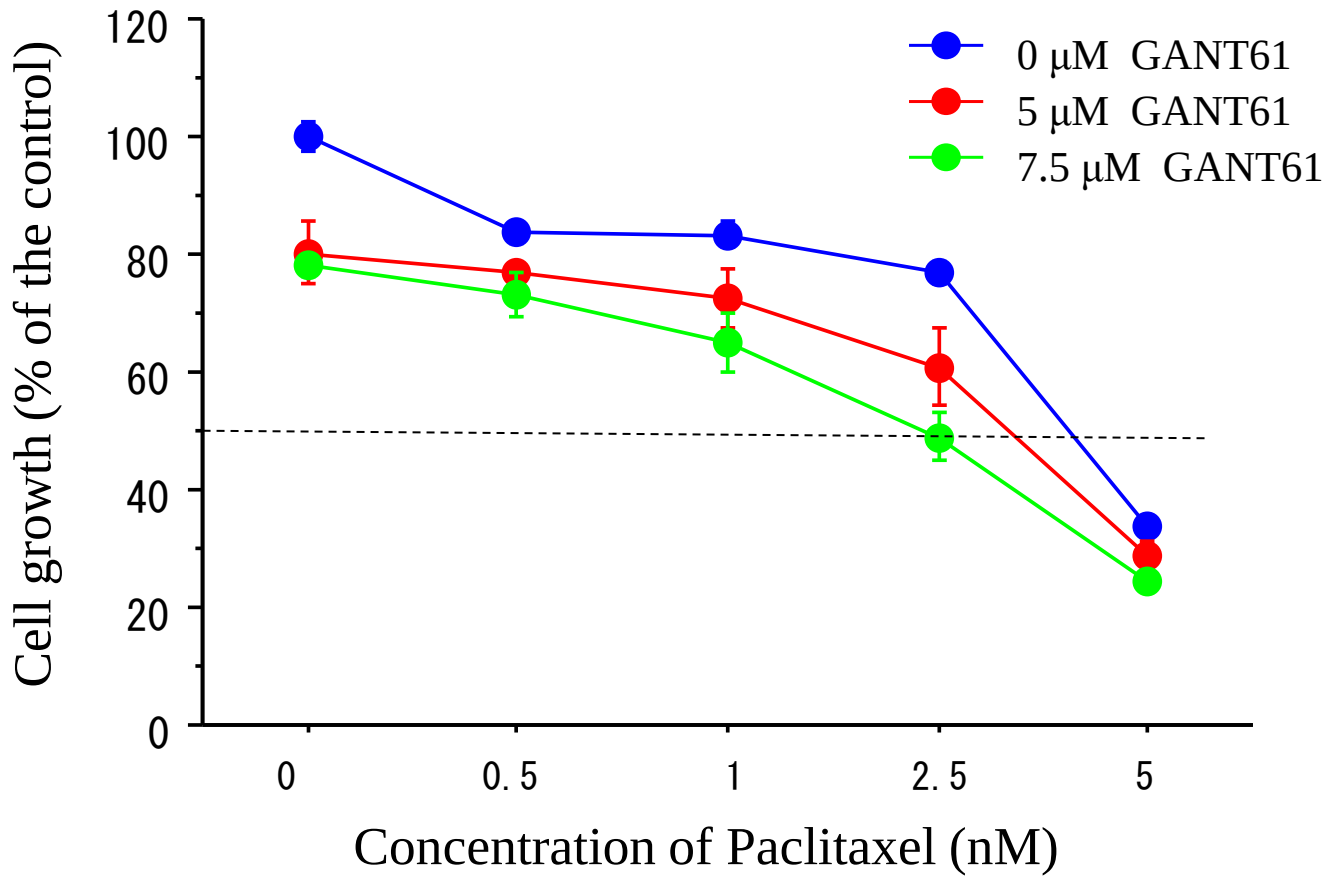

120
100
80
60
40
20
0
Cell growth (% of the control)
0
0.5
1
2.5
5
Concentration of Paclitaxel (nM)
0 μM GANT61
5 μM GANT61
7.5 μM GANT61

Figure 6C

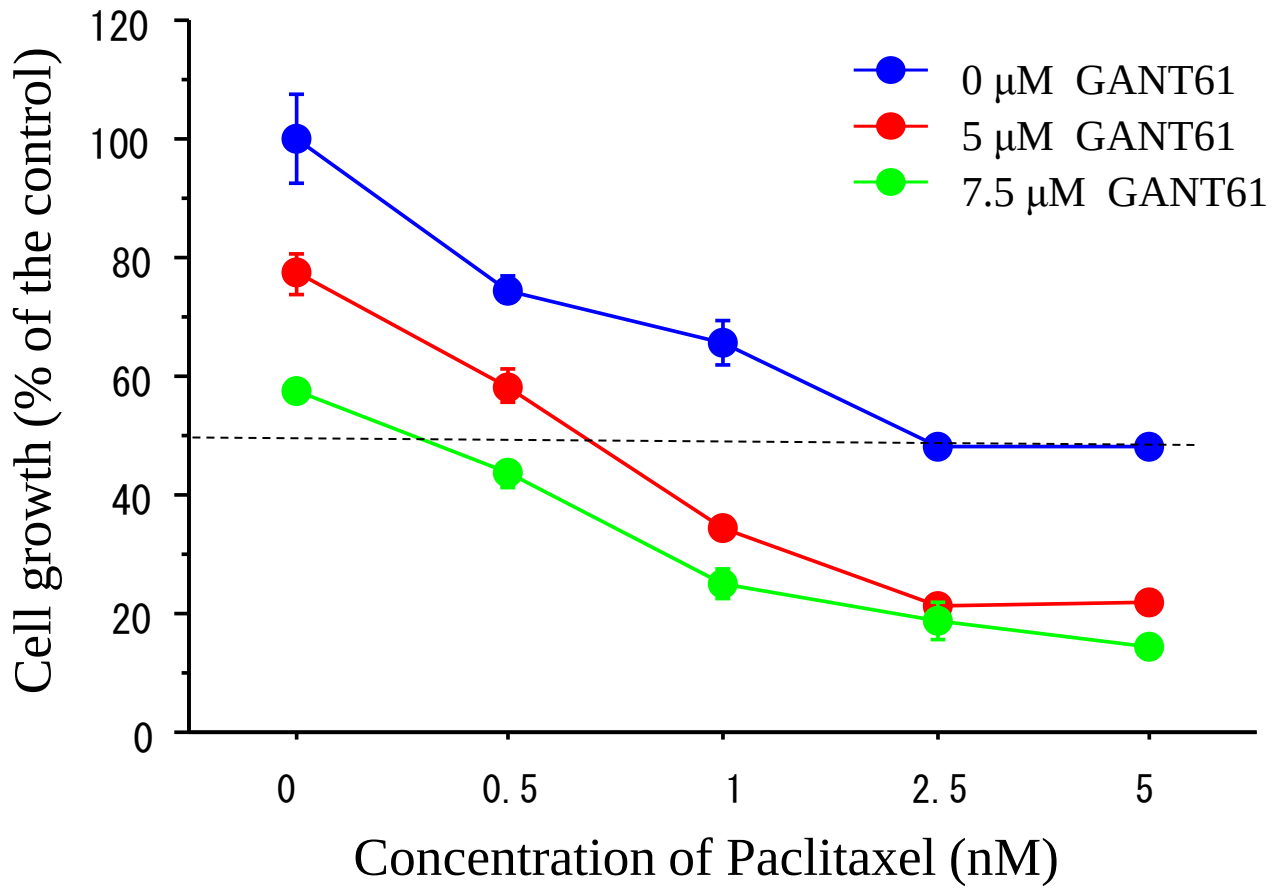

120
100
80
60
40
20
0
Cell growth (% of the control)
0
0.5
1
2.5
5
Concentration of Paclitaxel (nM)
0 μM GANT61
5 μM GANT61
7.5 μM GANT61

Figure 7A

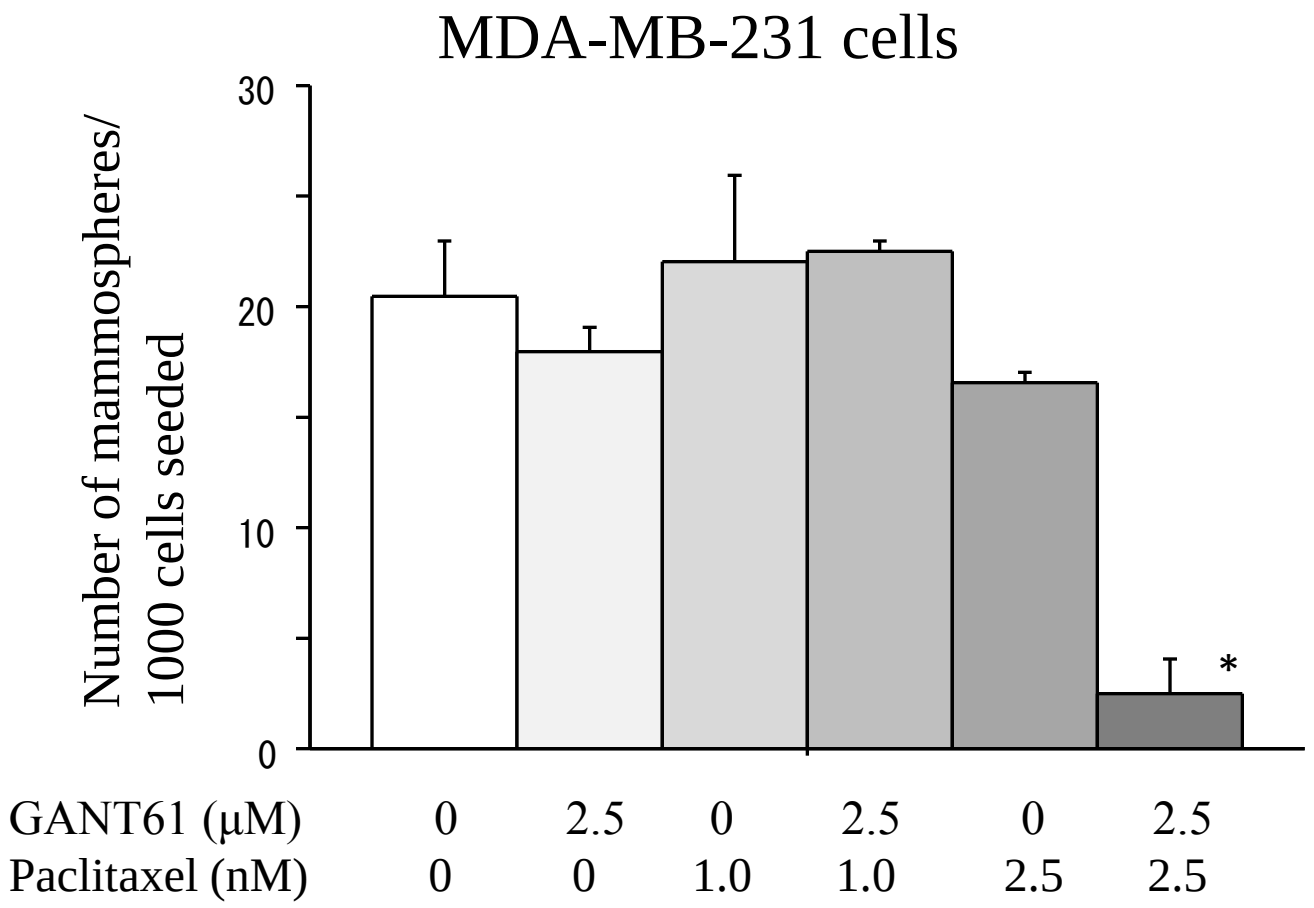

MDA-MB-231 cells
Number of mammospheres/
1000 cells seeded
30
20
10
0
*
GANT61 (μM) 0 2.5 0 2.5 0 2.5
Paclitaxel (nM) 0 0 1.0 1.0 2.5 2.5

Figure 7B

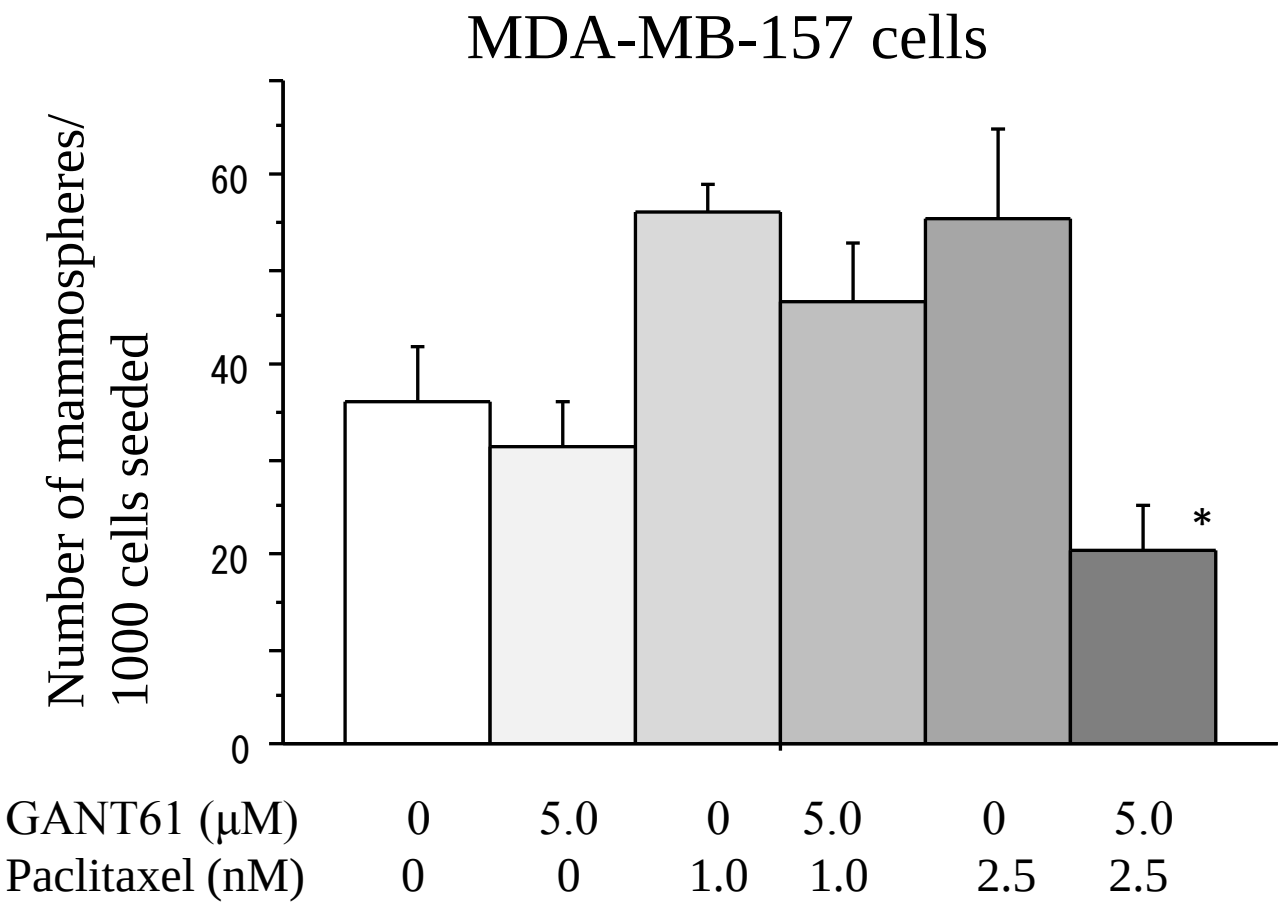
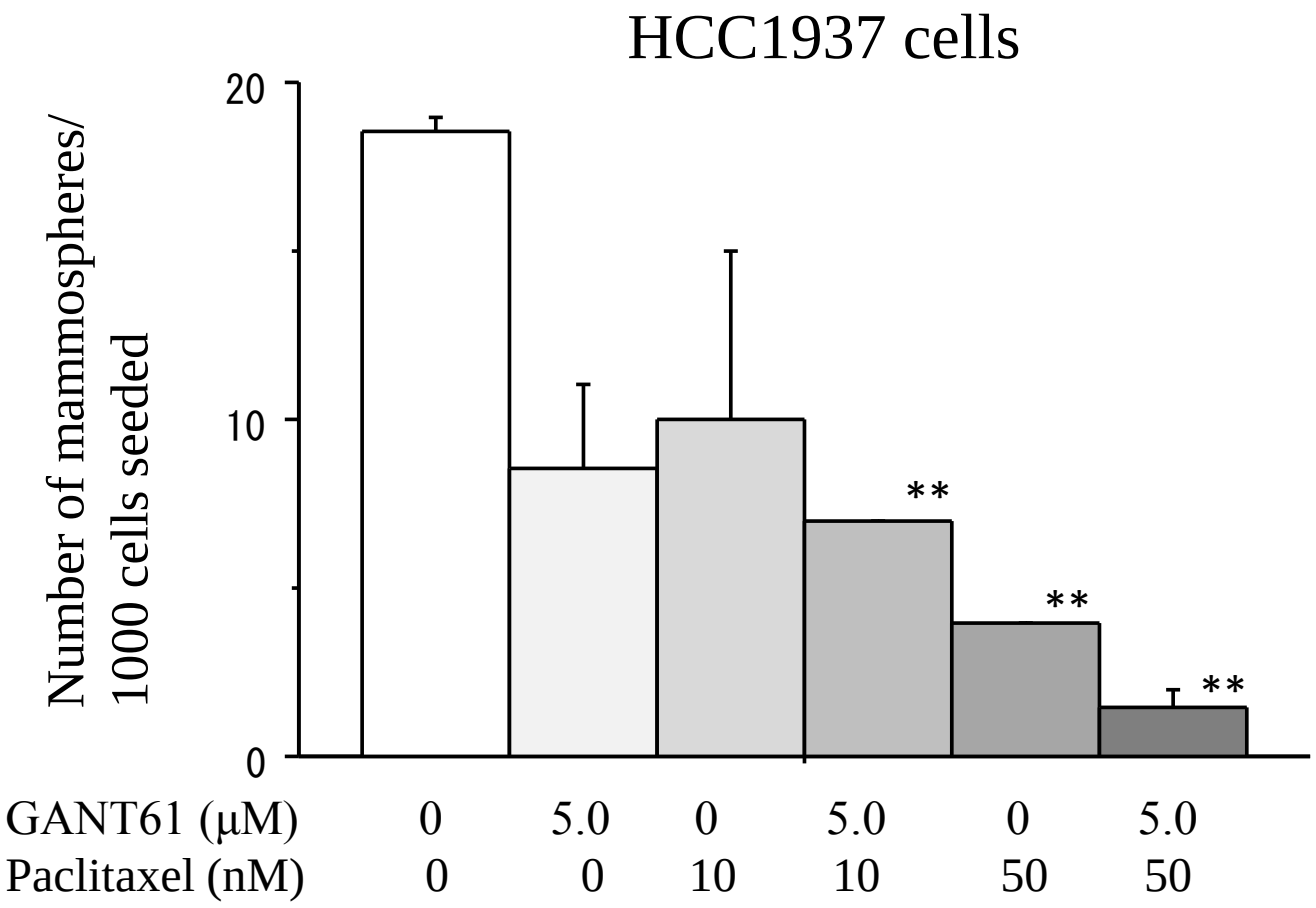

MDA-MB-157 cells
Number of mammospheres/
1000 cells seeded
60
40
20
0
*
GANT61 (μM) 0 5.0 0 5.0 0 5.0
Paclitaxel (nM) 0 0 1.0 1.0 2.5 2.5

Figure 7C


HCC1937 cells
Number of mammospheres/
1000 cells seeded
20
10
0
**
**
**
GANT61 (μM) 0 5.0 0 5.0 0 5.0
Paclitaxel (nM) 0 0 10 10 50 50