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# Penta-O-galloyl-β-D-glucose induces $G_1$ arrest and DNA replicative S-phase arrest independently of cyclin-dependent kinase inhibitor 1A, cyclin-dependent kinase inhibitor 1B and P53 in human breast cancer cells and is orally active against triple negative xenograft growth

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**ABSTRACT**

**Introduction:** Natural herbal compounds with novel actions different from existing breast cancer (BCa) treatment modalities are attractive for improving therapeutic efficacy and safety. We have recently shown that penta-1,2,3,4,6-O-galloyl-β-D-glucose (PGG) induced S-phase arrest in prostate cancer (PCa) cells through inhibiting DNA replicative synthesis and $G_1$ arrest, in addition to inducing cell death at higher levels of exposure. We and others have shown that PGG through intraperitoneal (i.p.) injection exerts a strong *in vivo* growth suppression of human PCa xenograft models in athymic nude mice. This study aims to test the hypothesis that the novel targeting actions of PGG are applicable to BCa cells, especially those lacking proven druggable targets.

**Methods:** Mono-layer cell culture models of p53-wild type estrogen receptor (ER)-dependent MCF-7 BCa cells and p53-mutant ER-/progesterone receptor (PR)- and Her2-regular (triple-negative) MDA-MB-231 BCa were exposed to PGG for a comprehensive investigation of cellular consequences and molecular targets/mediators. To test the *in vivo* efficacy, female athymic mice inoculated with MDA-MB-231 xenograft were treated with 20mg PGG/kg body weight by daily gavage starting 4 days after cancer cell inoculation.

**Results:** Exposure to PGG induced S-phase arrest in both cell lines as indicated by the lack of 5-bromo2'-deoxy-uridine (BrdU) incorporation into S-phase cells as well as $G_1$ arrest. Higher levels of PGG induced more caspase-mediated apoptosis in MCF-7, in strong association with induction of P53 $Ser^{15}$ phosphorylation, than in MDA-MB-231 cells. The cell cycle arrests were achieved without an induction of cyclin dependent

kinase (CDK) inhibitory proteins $P21^{Cip1}$ and $P27^{Kip1}$. PGG treatment led to decreased cyclin D1 in both cell lines and over-expressing cyclin D1 attenuated $G_1$ arrest and hastened S arrest. In serum-starvation synchronized MCF-7 cells, down-regulation of cyclin D1 was associated with de-phosphorylation of retinoblastoma (Rb) protein by PGG shortly before $G_1$-S transition. *In vivo*, oral administration of PGG led to a greater than 60% inhibition of MDA-MB231 xenograft growth without adverse effect on host body weight.

**Conclusions:** Our *in vitro* and *in vivo* data support PGG as a potential drug candidate for breast cancer with novel targeting actions, especially for a triple negative BCa xenograft model.

## Introduction

Breast cancer (BCa) is the major cause of cancer-related deaths for women of the US [1] and other Western countries. Approximately 60–70% of BCa cases express estrogen receptors (ER) and/or progesterone receptors (PR), and another ~ 20% of cases have amplified HER-2 proto-oncogene and express high levels of the HER-2 protein [2]. Approximately 15–20% of BCa cases are in the category of triple negative phenotype due to their lack of ER and PR and do not have amplification of HER-2 [2, 3]. These patients have a very poor prognosis because there is no clinically validated, molecularly targeted therapy as is the case for the other types of BCa. When surgical and radiation options are no longer applicable to these triple negative patients, treatment with available cytotoxic and genotoxic chemotherapy drugs produces limited efficacy and significant side effects. There remains a strong and urgent need for safer anti-cancer compounds for the treatment/management of the triple negative BCa and their metastasis. Novel agents with multiple-targeting ability distinct from the known drugable targets could be useful for circumventing the limitations of current treatment options.

Penta-1,2,3,4,6-O-galloyl-β-D-glucose (PGG) is a naturally occurring gallotannin polyphenolic compound in Oriental herbs such as *Galla Rhois,* the gallnut of *Rhus chinensis* Mill, and the root of peony *Paeonia suffruticosa* Andrews [4]. A couple of earlier papers have examined the *in vitro* effects of PGG using an $ER^+$ estrogen dependent and p53 wild type MCF-7 BCa cell culture model [5, 6]. Chen *et al* [5] reported that PGG induced $G_1$ arrest in association with up-regulated cyclin dependent kinase inhibitory (CDKI) proteins 1A ($p21^{Cip1}$) and 1B ($p27^{Kip1}$) abundance. Later, the same group showed that PGG decreased ERα and HER family of membrane tyrosine

kinase (EGFR, HER2, HER3) and PI3K/AKT signaling in MCF-7 cells [6]. A close inspection of the experimental designs of these studies revealed a lack of critical time-matched controls and therefore the conclusions and the validity of the mechansistic work reported are questionable.

In cell culture studies, we have recently shown that PGG induces caspase-mediated apoptosis in the human LNCaP prostate cancer (PCa) cells which express wild-type P53 [7]. The caspase-mediated apoptosis induction by PGG was in major part mediated by activation of P53 as established through siRNA knockdown and dominant negative mutant approaches [7]. More recently, we have shown the induction of cell death with autophagic features (autophagosome formation; addition of a phosphatidylethanolamine moiety to the microtubule-associated protein 1 light chain 3 (LC3) to a faster moving LC3-II form on electrophoresis) by PGG of p53-null, PTEN-null (high AKT) PC-3 PCa cells, which did not undergo caspase-mediated apoptosis after exposure to PGG [8]. We have also investigated the cell cycle effects of PGG in these and other PCa cells [9]. We showed, for the first time, that irrespective of the p53 and androgen dependence status of the PCa cell lines, PGG exerted a rapid (within 2 h) and potent inhibition ($IC_{50}$ ~ 6 μM) of BrdU incorporation into S phase cells. In isolated nuclei, PGG inhibited DNA replicative synthesis with superior efficacy than a known DNA polymerase-α inhibitor, aphidocolin. In addition to the S-arrest action, we have found a close association of down regulation of cyclin D1 with $G_1$ arrest induced by PGG. Taken together, our data with PCa cells indicate that PGG induced S-arrest, probably through DNA replicative blockage, and induced $G_1$-arrest via cyclin D1 down regulation, to contribute to its anti-cancer activity. These results sharply contrasted with the above-

mentioned, questionable, breast cancer cell culture studies [5, 6]. Therefore, whether the S- and $G_1$ cell cycle arrests and caspase-mediated or autophagic cell death actions of PGG are applicable to BCa cells needs to be experimentally tested.

In addition to PCa cells, PGG was shown to induce $G_1$ cell cycle arrest and apoptosis of leukemia [10, 11], to inhibit invasion-related molecules such as matrix metalloprotease (MMP)-9 in melanoma cells [12] and EGF-R signaling [13] and VEGFR2-signaling and angiogenesis *in vitro* and *in vivo* [14], supporting multiple targeting actions. A number of *in vivo* studies by us and others in Lewis lung cancer (LLC) allograft [14] and PCa xenograft models [7, 13] with a daily dose of 20 mg/kg or 25 mg/kg every other day have shown anti-cancer efficacy without adverse effect on body weight. These *in vivo* and *in vitro* studies suggest probable anticancer activity of PGG against BCa, especially triple negative BCa.

In this report, we evaluated the cell cycle and cell death actions of PGG against MDA-MB231 triple negative BCa cells as well as MCF-7 BCa cells; and we established, for the first time, an impressive oral efficacy of PGG against xenograft growth established from human MDA-MB-231 cells.

## Materials and Methods

### Chemicals and Reagents

Penta-1,2,3,4,6-O-galloyl-β-D-glucose (PGG) was prepared by methanolysis of tannic acid according to a published method [4, 15] in-house. The purity was ~99%. For treating cells in mono-layer culture, PGG was dissolved in DMSO as a stock solution.

The final DMSO added to cell culture medium was below 0.1%. Antibodies including anti-CDK4, anti-P21/CIP1, anti-P27/KIP1 and anti-ERα were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Additional antibodies specific for cleaved poly (ADP-ribose) polymerase (PARP; p89), cyclin D1, p53Ser$^{15}$P, pRbSer$^{795}$, pRbSer$^{807/811}$ and pAKT(Ser$^{473}$) were purchased from Cell Signaling Technology (Beverly, MA). Antibody for LC-3 was purchased from MBL International Inc (Watertown, MA).

**Cell Culture and Treatments**

MCF-7 and MDA-MB231 cell lines were purchased from the American Type Culture Collection, Manassas, VA. None was derived directly from human tumour tissue for the purposes of this study. MCF-7 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) without antibiotics in an incubator at 37$^{o}$C with 5% $CO_2$. MDA-MB231 cells were grown in L-15 medium supplemented with 10% FBS without antibiotics in an incubator at 37$^{o}$C with atomospheric $CO_2$. At 24 hours after plating, the medium was changed before starting the treatment with PGG or the other agents. To standardize all PGG/drug exposure conditions, cells were bathed in culture medium at a volume to surface area ratio of 0.2 mL per cm$^2$ (e.g., 15 mL for a T75 flask and 5 mL for a T25 flask).

**Cell growth assay by crystal violet staining**

For the evaluation of the overall inhibitory effect of PGG on cell number, the cells were treated with PGG daily (fresh medium) for 3 days. After treatment, the culture

medium was removed and the cells were fixed in 1% glutaraldehyde solution in phosphate-buffered saline (PBS) for 15 min. The fixed cells were stained with 0.02% aqueous solution of crystal violet for 30 min. After washing with PBS, the stained cells were solubilized with 70% ethanol. The absorbance at 570 nm with the reference filter 405 nm was evaluated using a microplate reader (Beckman Coulter, Inc., Fullerton, CA)

**BrdU incorporation and cell cycle measurement**

The protocol was based on our previous publications [9, 16]. After the desired experimental treatments, 10 µl of BrdU (9 mg/ml) solution was added to 5 ml of medium for 30 min before harvesting cells. The cells were collected by trypsinization, centrifuged at 1600 g for 6 min, fixed with 70% ethanol overnight, analyzed for cell cycle distribution by propidium iodide/BrdU bi-variate flow cytometry.

**Synchronic MCF-7 cell $G_0/G_1$ progression model**

MCF-7 cells were seeded in RPMI-1640 medium supplemented with 10% FBS without antibiotics in an incubator at 37$^{o}$C with 5% $CO_2$. Twenty-four hours later, the cells were washed with serum free phenol-red free RPMI-1640 medium, then incubated in serum free phenol-red free medium for another 24 hours. One flask of cells was reserved as 0 h baseline control. For the other flasks, serum free medium was replaced with complete medium (10% FBS) to release cells from $G_0$ arrest. At selected time points, cells were harvested for flow cytometry to analyze cell cycle distribution. For PGG treatment, cells were released into complete medium and treated simultaneously with serum stimulation or were exposed to PGG after different hours of $G_1$ progression

until 24 hours when the cells were collected for cell cycle analyses.

**Immunoblot Analyses**

The cell lysate was prepared in ice-cold lysis buffer as described previously [17]. Immunoblot analyses were essentially as described [17], except that the signals were detected by enhanced chemofluorescence with a Storm 840 scanner (Molecular Dynamics, Sunnyvale, CA).

**MDA-MB-231 xenograft model**

The animal use protocol was approved by the Kyunghee University Institutional Animal Care and Use Committee and carried out at the Cancer Preventive Material Development Research Center, College of Oriental Medicine, Kyunghee University, South Korea. One million MDA-MB-231 cells were mixed with 50% Matrigel (Becton Dickinson) and injected (in 100 µl) subcutaneously into the right flank of each 6-week-old female BALB/c athymic nude mouse (NARA Biotech, South Korea). Starting 4 days after inoculation, 10 mice per group were given a daily gavage treatment of 2% Tween-80 (vehicle) or 20 mg PGG per kg body weight. The dosage was based on our prostate cancer xenograft work [7] and lung cancer allograft work [14]. Tumors were measured twice per week with a caliper, and tumor volume was calculated using the following formula: 1/2 (w1 x w2 x w2), where w1 represents the larger tumor diameter, and w2 represents the smaller tumor diameter.

**Statistical analyses**

Numerical data were expressed as mean ± SE or SD (where noted). Statistical analyses were carried out with Graphpad Prism and Sigma Plot software, and $p<0.05$ was considered statistically significant. The data were analyzed by ANOVA followed by Dunnett's multiple comparison post tests or other appropriate tests.

## Results

### PGG inhibited MCF-7 and MDA-MB231 breast cancer cell growth and induced caspase-mediated as well as caspase-independent cell death

To evaluate the growth inhibitory effect on estrogen-dependent BCa cell line MCF-7 and the triple negative BCa cell line MDA-MB231, we exposed these cells to daily changes of fresh complete medium with increasing concentrations of PGG. After 3 days of daily exposure, PGG decreased the number of MCF-7 and MDA-MB231 cells in a dose-dependent manner, with the $IC_{50}$ of PGG for both cell lines lower than 12.5 μM (Fig 1A). The p53-wild type MCF-7 cells were more sensitive than the p53-mutant triple negative MDA-MB231 cells to the growth suppression action of PGG at each tested concentration of PGG.

The difference in sensitivity of the two BCa cell lines was in part associated with the propensity for MCF-7 cells to undergo caspase-mediated apoptosis preceded by P53 $Ser^{15}$ phosphorylation (P53-$Ser^{15}$P) (24 h) and cleavage of PARP (48 h) (Figure 1b). In the MDA-MB231 cells, whose mutant P53 phosphorylation was not responsive to PGG treatment, minimal cleavage of PARP (72 h) was preceded by autophagic features as indicated morphologically by cytosolic vacuolation (48 h), and biochemically by an early

(6 h) increase of phosphorylation of AMPK, a well-known autophagy signaling kinase in response to nutrient deprivation [18], and by increased phosphatidylethanolamine modification of the microtubule-associated protein 1 light chain 3 (LC3-I) to the faster moving LC-3II form (24 - 48h) (Figure 1b). These results therefore mirror our data on PGG induction of apoptosis and other types of cell death obtained with LNCaP [7] and PC-3 PCa cells [8], respectivley.

**PGG induced S and $G_1$ arrests in MCF-7 and MDA-MB231 cells**

Prompted by our findings of $G_1$ and S arrests in different PCa cell lines (LNCaP, DU-145 and PC-3) [8, 9], which contrasted with the reported $G_1$ arrest in MCF-7 cells [5], we measured the cell cycle distribution patterns of MCF-7 and MDA-MB231 cells after exposure in complete medium to different concentrations of PGG for 6, 24 and 48 h.

In both MCF-7 and MDA-MB231 cells, PGG exposure for 6 h led to a concentration-dependent increase of $G_1$-phase cells and was accompanied by a decrease of $G_2$ phase cells (Figure 2). The MB231 cells (Figure 2b) appearred to more readily achieve $G_1$ arrest than the MCF-7 cells (Figure 2a) in the presence of the lowest PGG concentration tested probably due to their faster growth. The % of S-phase cells remained relaltively steady in both cell lines. Inspection of the BrdU incorporation index (measured as we previously described for a 30 min pulse labeling before cell harvest [9]) showed a near complete blockage of DNA synthesis in the S-phase cells in MB231 cells at all 3 PGG exposure concentrations, whereas in the MCF-7 cells, a clear concentration-dependency on PGG was observed.

For both cell lines, as time progressed to 24 and 48 hours, the lowest concentration

of PGG (12.5 μM) was not able to hold the cells arrested in $G_1$, manifesting as the accumulation of S phase cells which remained incapable of incorporating BrdU. The higher concentrations of PGG (50 μM) kept cells arrested in $G_1$ phase and S phase throughout the 6 - 48 h period (Figure 2). The data therefore support both S-arrest and $G_1$ arrest by PGG in breast cancer cells, as in PCa cells [9].

**PGG did not alter $P21^{Cip1}$ and $P27^{Kip1}$ expression in breast cancer cells**

An earlier report by Chen [5] has claimed $G_1$ arrest and P21Cip1 and P27Kip1 induction by PGG in MCF-7 cells, without including critical time-matched controls. We therefore examined these proteins as possible molecular mediators for the $G_1$ and S arrests. Since we have reported the rapid P53-$Ser^{15}P$ by PGG treatment in LNCaP PCa cells [7] and have observed P53-$Ser^{15}P$ in PGG-exposed MCF-7 cells (Figure 1b), and since the P53-$P21^{Cip1}$ axis is best known for mediating $G_1$ arrest by genotoxic stress [19], we therefore focused on the relationship among these proteins in PGG-exposed MCF-7 cells.

We observed that PGG treatment activated P53$Ser^{15}P$ at 6 h with a clear concentration-dependency, but did not increase the protein abundance of either $P21^{Cip1}$ or $P27^{Kip1}$ (Figure 3a). Later, we also found the same pattern of dis-engaged P53/$P21^{Cip1}$ response (i.e., P53 $Ser^{15}P$, but not upregulated $P21^{Cip1}$) in the synchronic MCF-7 model (Figure 4). In MDA-MB231 cells, we did not observe any induction of these two CDKI proteins by PGG, either (Figure 3b). These results suggest that PGG induced $G_1$ arrest in the absence of detectable alterations of $P21^{Cip1}$ and P27 $^{Kip1}$ protein abundance and was independent of P53 function in these BCa cells.

**PGG decreased Cyclin D1 abundance in breast cancer cells**

In contrast to a lack of expression change of $P21^{Cip1}$ or $P27^{Kip1}$, PGG treatment significantly decreased the abundance of Cyclin D1 in MCF-7 and MDA-MB231 cells (Figure 3a and c). PGG treatment decreased Cyclin D1 expression as early as 6 h, and by 12 h, its expression decreased dramatically. From 24 h to 48 h, there was almost no detectable Cyclin D1 expression in MCF-7 and MDA-MB231 cells treated with PGG at a high dose (Figure 3a and c).

To test the contribution of Cyclin D1 down regulation to the $G_1$ arrest, we made stable transfectants of MCF-7 and MDA-MB231 cells with forced over-expression of Cyclin D1 (Figure 5a) (the expression plasmid was kindly provided by Prof. Joshua D Liao, Hormel Institute). Compared with vector transfectant cells, the Cyclin D1 over-expressing MCF-7 cells significantly attenuated PGG-induced $G_1$ arrest (Figure 5b). Similarly, MDA-MB231 cells over-expressing Cyclin D1 partially overcame PGG-induced $G_1$ arrest (Figure 5c). Instead, PGG exposure of the Cyclin D1 over-expressing cells hastened S arrest, without affecting $G_2$ phase decline. The data support $G_1$-arrest and S-arrest operating by independent mechanisms in BCa cells, as in PCa cells [9] and that cyclin D1 down-regulation by PGG was an important contributor (perhaps not sole mediator) to $G_1$ arrest.

**Defining $G_1$-targeting action of PGG in synchronic MCF-7 model**

To further probe the $G_1$-targeting mechanisms of action of PGG without the complication from S-arrest, we synchronized MCF-7 cells to $G_0$ by serum starvation for

24 h and released the cells into complete medium (this time point was referred to as 0 h). Cell cycle distribution patterns suggested that the $G_1$ phase cells started to transit into S phase between 20-22 h of FBS re-stimulation (Figure 6a).

In this synchronic MCF-7 cell model, inclusion of PGG at the time of serum stimulation (PGG@0 h) caused a complete block of $G_1$ to S transition, measured by flowcytometry at 24 h (Figure 6b). To determine whether the presence of PGG during the early stage $G_{0/1}$ progression was necessary for $G_1$ arrest and to pin-point the responsible molecular events, we delayed the starting time for PGG exposure in reference to serum stimulation. As shown in Figure 6b, delaying the starting exposure time to 14 h (i.e., PGG@14h) did not lessen the $G_1$ arrest action of PGG. Starting PGG treatment at 16-18 h was less able to prevent $G_1$-S transition. These data indicated that the crucial time window for PGG targeting during $G_1$/S progression was 16-18 h post serum stimulation.

**PGG decreased Cyclin D1 in synchrony with Rb de-phosphorylation in synchronic MCF-7 cells**

In the synchronic MCF-7 model, serum stimulation led to increased Cyclin D1 expression (4 h was the earliest point sampled), which persisted through 20 h ($G_1$/S transit) (Figure 4). Serum stimulation increased survival signaling, as indicated by AKT phosphorylation, in a similar temporal pattern as Cyclin D1 and suppressed background level apoptosis as indicated by the decreased cPARP. Serum stimulation decreased ERα, which declined progressively over time. A well-known downstream effector molecule of cyclin-CDK complexes for $G_1$ progression is the Retinoblastoma (Rb) protein [20]. Cyclin-CDK complexes phosphorylate Rb to decrease its binding to the

E2F transcriptional factor, releasing E2F to activate expression of its target genes for $G_1$/S transition. Indeed, we detected increased Rb phosphorylation at 12 h at the $Ser^{795}$ site and 16 h at $Ser^{807/811}$ sites, prior to the onset of $G_1$/S transition (20 h).

Exposure of synchronic MCF-7 cells to PGG at time of serum stimulation did not decrease Cyclin D1 until 16 h, coinciding with decreased Rb phosphorylation at $Ser^{795}$ and $Ser^{807/811}$ sites (Figure 4). Although P53-$Ser^{15}$P was detected by 8 h of PGG treatment, there was a clear absence of $P21^{Cip1}$ induction by PGG throughout 20 h. Increased cPARP was detected by 16 h, which was preceded by increased AKT($Ser^{473}$) phosphorylation by several hours. PGG treatment did not affect ERα until the 20 h time point. Considering that $P21^{Cip1}$ abundance was not upregulated throughout $G_1$ phase by PGG, the data suggest that the $G_1$ arrest was predominantly regulated by the Cyclin D-CDK-Rb axis, preventing the release of E2F to promote passage of the restriction point.

**Orally-administered PGG suppresses MDA-MB231 breast cancer xenograft growth**

The cell culture data presented above suggest probable *in vivo* anti-cancer efficacy of PGG against BCa growth. Because oral administration is the most practical and non-invasive way to deliver an anti-cancer agent, we evaluated the efficacy of PGG delivered by oral gavage against MDA-MB-231 cells injected subcutaneously into the right flank of each female athymic nude mice at the dosage of 20 mg/kg body weight, starting 4 days after cancer cell inoculation. This dosage of PGG did not exert any adverse effect on body weight of the host nude mice (Figure 7a). PGG treatment led to a significant inhibition of tumor growth rate over time (Figure 7b) and decreased the final tumor size by over 60% at necropsy.

**Discussion**

As pointed out in the Introduction, there is an urgent clinical need for safe and effective treatment and preventive agents for triple-negative BCa. Our results presented above provide *in vitro* and *in vivo* data that support the potential for PGG to be such a promising drug candidate with multiple targeting actions, distinct from known drugable BCa targets such as the ER (e.g., estrogen antagonist drug tamoxifen) and HER-2 (e.g., inactivating monoclonal antibody Herceptin). In cell culture, PGG treatment caused P53-$Ser^{15}$ phosphorylation (Figures 1, 3 and 4) and caspase-mediated apoptosis in MCF-7 BCa cells (Figures 1, 4). In p53-mutant MDA-MB231 triple negative BCa cells, PGG caused not only apoptosis, but also autophagic responses (Figure 1b). We showed that independently of P53 status or ERα status of the BCa cells, PGG induced S-arrest and $G_1$ arrest (Figure 2) without inducing $P21^{Cip1}$ and $P27^{Kip1}$ expression (Figures 3 and 4). Our data support Cyclin D1 down-regulation by PGG as an important mediating event for the $G_1$ arrest action (Figures 3, 4 and 5). The clear dis-engagement of P53 $Ser^{15}$ phosphorylation from the best known P53 transcriptional target $P21^{Cip1}$ in PGG-exposed MCF-7 cells remains an interesting question for further investigation.

Our findings are important in two aspects. First, they are consistent with recent published results for prostate cancer cells [7-9], suggestive of a treatment applicability of PGG for cancers of other organ sites. The documented ability in this study to generate high purity PGG in multi-gram quantities from tannic acid will enable us and others to explore the *in vivo* anti-cancer efficacy of PGG in relevant animal models of cancers of

other organ sites. Second, they point out the possibility that some published data are highly questionable concerning the action mechanisms of PGG in BCa cells. In contrast to the published data by others [5], our data did not detect a change of $P21^{Cip1}$ and $P27^{Kip1}$ expression to be associated with the $G_1$ arrest action of PGG (Figure 3). We also did not observe a dramatic impact of PGG on ERα abundance, nor a suppression of AKT phosphorylation (Figure 4), as were claimed [6]. Instead, PGG treatment increased AKT phosphorylation in MCF-7 cells (Figure 4), as we have reported for a similar increase of AKT phosphorylation in PC-3 cells by PGG [8]. Although many reasons could be cited for the discrepencies between our data and the previous reports [5, 6], their lack of time-matched controls could be the leading cause of confusion and misleading conclusions.

Our *in vivo* data demonstrated, for the first time, a growth inhibitory efficacy of PGG against triple negative breast cancer, and supported the oral bioavailability of PGG. The potency of PGG (20 mg per kg body weight) is remarkable, especially considering given by the oral route. Furthermore, just the fact that PGG is orally available and therefore can be self administered by patients will have a major impact on reducing the health care delivery cost, compared with injection-only drugs (such as paclitaxel) that have to be given by health care professionals. The data on efficacy and safety of PGG provide impetus for further studies about the therapeutic application of PGG and its *in vivo* molecular targets and mechanisms of action.

**Conclusions**

Our cell culture data showed that PGG could induce both $G_1$ and S arrests in BCa

cells, regardless of their ER or P53 functional status. Cyclin D1 down regulation by PGG was a mechanism for $G_1$ arrest in breast cancer cell lines and the data ruled out $P21^{Cip1}$ and $P27^{Kip1}$ for mediating $G_1$ arrest. We demonstrated for the first time that PGG given by oral administration was quite safe to the host nude mice and potent for suppressing a triple negative BCa xenograft model. The therapeutic and chemopreventive utility of PGG for breast cancer merits further study.

**Abbreviations**

AMPK, AMP activated protein kinase; BCa, breast cancer; BrdU, 5-bromo2'-deoxy-uridine; CDK, cyclin dependent kinase; CDKIs, cyclin dependent kinase inhibitors; cPARP, cleaved poly-ADP-ribose Polymerase; ER, estrogen receptor; IB, immunoblot or Western blot; i.p., intraperitoneal; LC3, microtubule-associated protein 1 light chain 3; PCa, prostate cancer; PGG, penta-O-galloyl-β-D-glucose; PR, progesterone receptor; P21Cip1, cyclin-dependent kinase inhibitor 1A; P27Kip1, cyclin-dependent kinase inhibitor 1B; siRNA, small interference RNA.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

JL and SHK conceived of, and coordinated the studies. JL, SHK, YC, HJL, JZ designed experiments. YC, KN and JZ did cell culture experiments and statistical analyses. HJL and SJJ carried out xenograft study. AAS and CX scaled up PGG preparation from tannic

acid and performed chemical characterization. JL, YC and HJL drafted the manuscript. All authors read, edited and approved the final manuscript.

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**Figure legends**

**Figure 1** Growth inhibitory and cell death actions of PGG in MCF-7 and MDA-MB231 cells. (**A**) Overall inhibitory effects of PGG on MCF-7 and MDA-MB231 cell growth after 3 days of daily treatment with PGG in fresh medium. Values are mean ± SEM, n= 3 wells of 12-well plate. **Statistical significance:** ***, ###, P<0.001; ****, ####, P<0.0001, vs untreated control. Results are representative of two independent experiments. (**B**). Immunoblot detection of apoptotic cleavage of PARP, P53 $Ser^{15}$ phosphorylation induced by PGG in MCF-7 or MDA-MB231 cells and autophagy responses (pAMPK, LC3-II) in MDA-MB231 cells. Phase-contrast photomicrograph shows vaculolation typical of autophagy. The medium was not changed for PGG exposure longer than 24 h.

**Figure 2.** The effect of PGG on cell cycle distribution of MCF-7 **(a)** and MDA-MB231 cells **(b)** detected by propidium iodide (PI)/BrdU-bivariate flow cytometric analyses. Cells were exposed to increasing concentrations of PGG for 6 h, 24 h and 48 h. BrdU was added for the last 30 min to label S-phase cells active in DNA replication. Values are mean ± SEM, n= 4. Results are from two independent experiments with duplicate values at each concentration. The medium was not changed for PGG exposure longer than 24 h. Statistical significance: (a) BrdU incorporation at all 3 time points, One-way ANOVA P<0.0001, with Dunnett's multiple comparison post test p value <0.01 for 0 vs. 12.5, 25 and 50 µM PGG. For $G_1$ , 6h p<0.05 for 0 vs. 25 and 50 µM PGG; 24h p<0.01 for 0 vs. 12.5 or 50 µM PGG; 48h, p<0.01 for 0 vs. 12.5 µM PGG and p<0.05 for 0 vs 50

μM PGG. For S, 24h/48h $p<0.01$ for 0 vs. 12.5 and 25 μM PGG. (b) BrdU incorporation at all 3 timepoints, One-way ANOVA $P<0.0001$, with Dunnett's multiple comparison post test p value <0.01 for 0 vs. 12.5, 25 and 50 μM PGG. For G1 , 6h $p<0.05$ for 0 vs. 12.5, 25 and 50 μM PGG; 24h $p<0.01$ for 0 vs. 25 and 50 μM PGG; 48h, $p<0.01$ for 0 vs. 50 μM PGG. For S, 24h/48h $p<0.01$ for 0 vs. 12.5 μM PGG.

**Figure 3.** Effect of PGG on Cyclin D1, $P21^{Cip1}$ and $P27^{Kip1}$ and other select cell cycle proteins in MCF-7 and MDA-MB231 cells detected by Western blot analyses. **(a)** Cyclin D1, CDK4, $P21^{Cip1}$ and $P27^{Kip1}$ expression and P53-$Ser^{15}P$ in MCF-7 cells. β-actin was reprobed as loading control. **(b)** $P21^{Cip1}$ and $P27^{Kip1}$ expression in MDA-MB-231 cells. **(c)** Time course of Cyclin D1 expression in MCF-7 and MDA-MB231 cells treated with PGG from 12 h to 48 h. Patterns are representative of two experiments. The medium was not changed for PGG exposure longer than 24 h.

**Figure 4**. Western blot analyses of the effect of PGG included at time of serum stimulation (as time 0) on cyclin D1 expression and phosphorylation of Rb, activation of P53-$Ser^{15}P$ and expressions of $P21^{Cip1}$ and estrogen receptor (ER) α in serum starvation synchronized MCF-7 cells.

**Figure 5.** Impact of overexpression of Cyclin D1 on PGG-induced $G_1$ arrest in MCF-7 and MDA-MB231 cells. **(a)** Western blot verification of stable over-expression of Cyclin D1 in MCF-7 and MDA-MB231 cells. **(b)** Cell cycle distribution of MCF-7 cells transfected with vector and cyclin D1 plasmid with or without PGG treatment for 24 h.

**(c)** Cell cycle distribution of MDA-MB231 cells transfected with vector and cyclin D1 plasmid with or without PGG treatment for 24 h. Each bar reflects the average of two T25 flasks. The patterns are representatstive of 2 experiments.

**Figure 6**. Effect of PGG on $G_{0/1}$-S progression in synchronized MCF-7 cells. **(a)** The temporal kinetics of serum-stimulated progression of starvation-synchronized MCF-7 cells. Each time point was average of duplicate flasks. *, #, P<0.05; **, ##, P<0.01; ***, ###, P<0.001 vs. 0 time. **(b)** Impact of delaying PGG treatment with reference to serum stimulation on $G_1$ arrest. Results are from two independent experiments with duplicate values at each time point. *, #P<0.05; **, ##, P<0.01; vs. SF or PGG@0h-14h.

**Figure 7.** PGG intake by oral gavage inhibits MDA-MB-231 tumor growth in female athymic nude mice. Starting 4 days after cell inoculation, PGG (20mg/kg) was gavaged with 2% Tween-80 as vehicle to these animals once a day. **(a)** Body weight, (**b**) tumor volume. Values are mean $\pm$ SD, n= 10 mice per group. Statistical significance: ANOVA PGG effect on tumor size, $p<0.0001$.

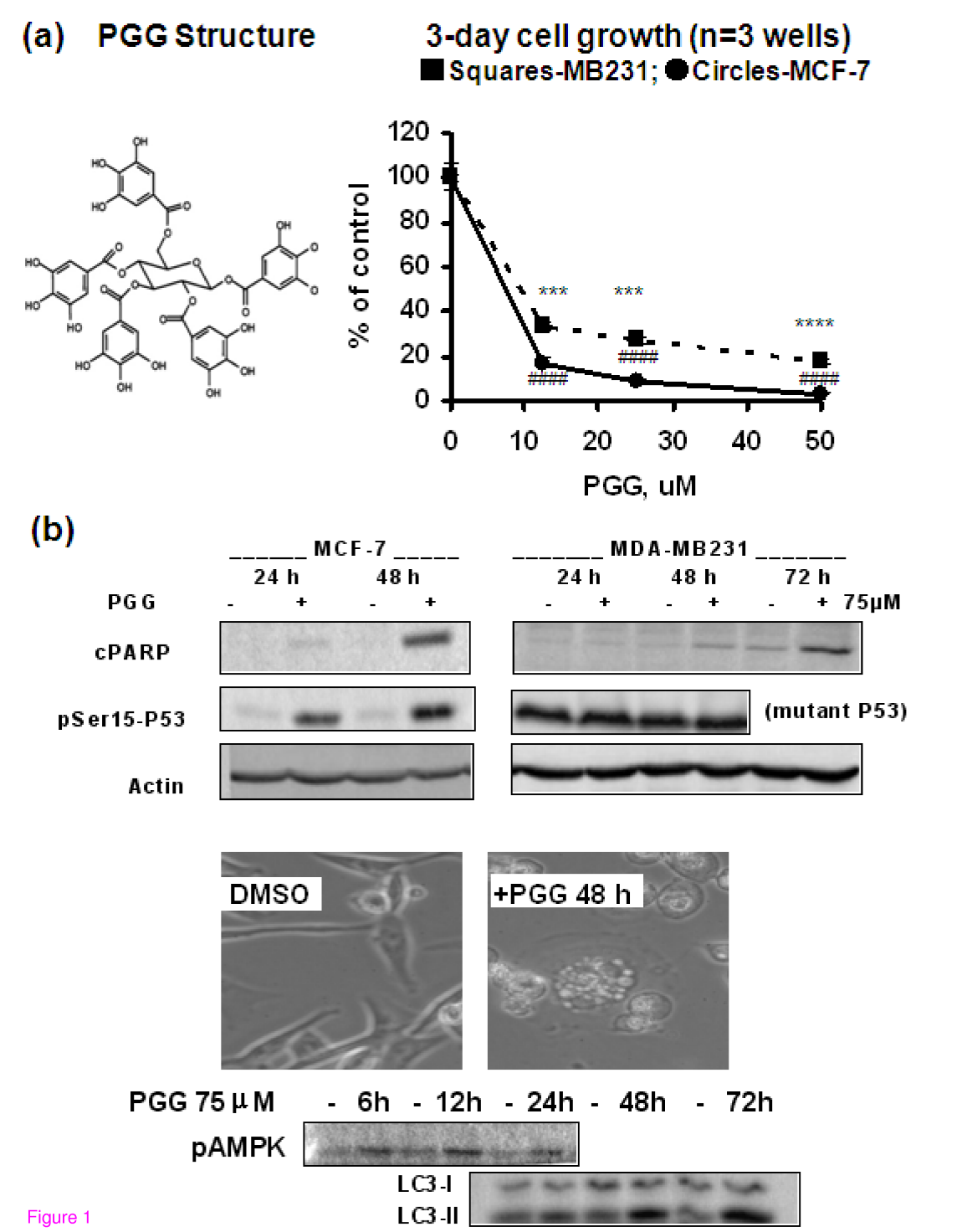
(a) PGG Structure
3-day cell growth (n=3 wells)
Squares-MB231; Circles-MCF-7
% of control
PGG, uM
(b)
MCF-7
MDA-MB231
24 h
48 h
72 h
PGG
75μM
cPARP
pSer15-P53
(mutant P53)
Actin
DMSO
+PGG 48 h
PGG 75 μM
6h
12h
24h
48h
72h
pAMPK
LC3-I
LC3-II

Figure 1

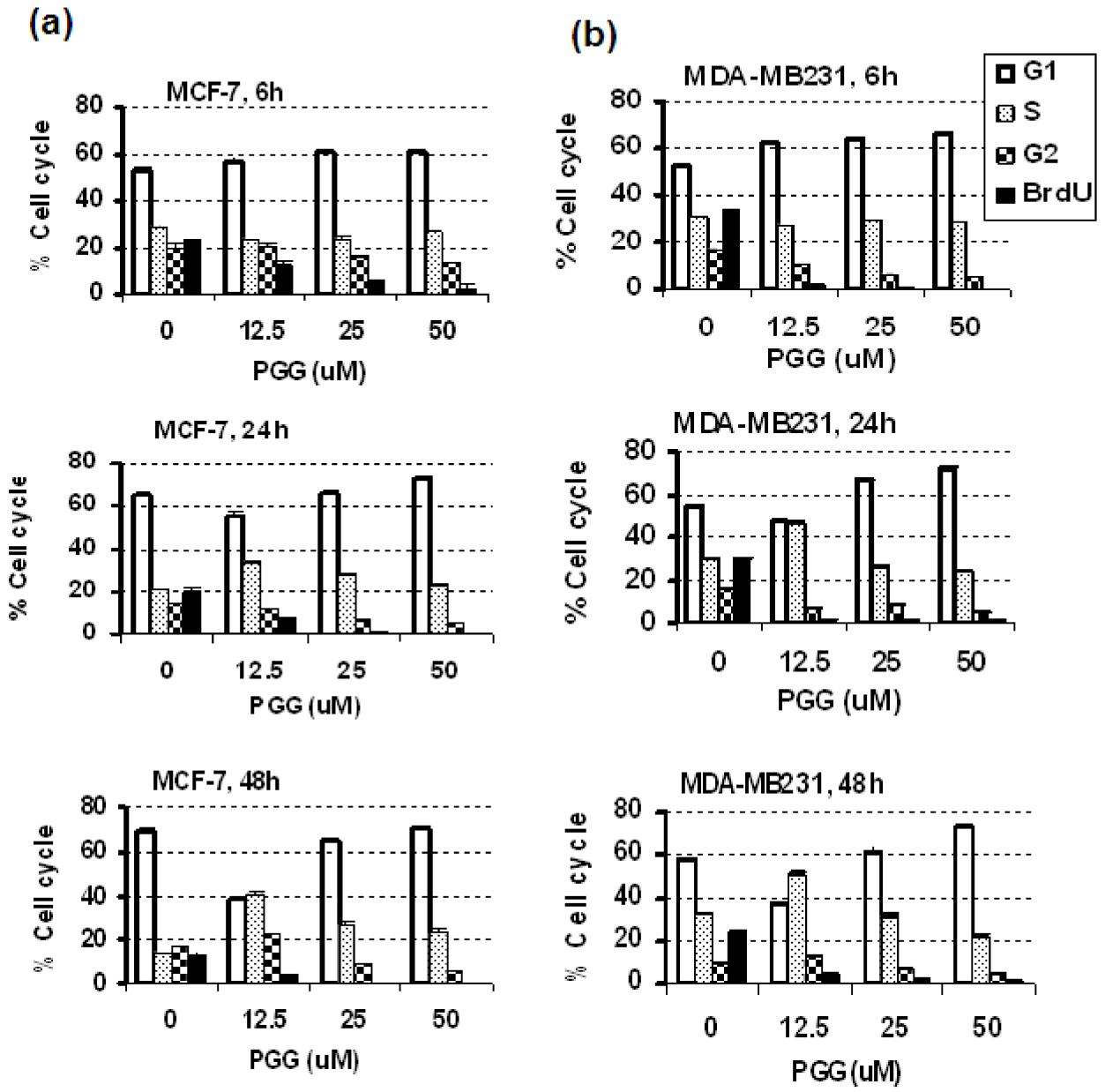
(a)
(b)
MCF-7, 6h
MDA-MB231, 6h
MCF-7, 24h
MDA-MB231, 24h
MCF-7, 48h
MDA-MB231, 48h
% Cell cycle
PGG (uM)
0
12.5
25
50
20
40
60
80
G1
S
G2
BrdU

Figure 2

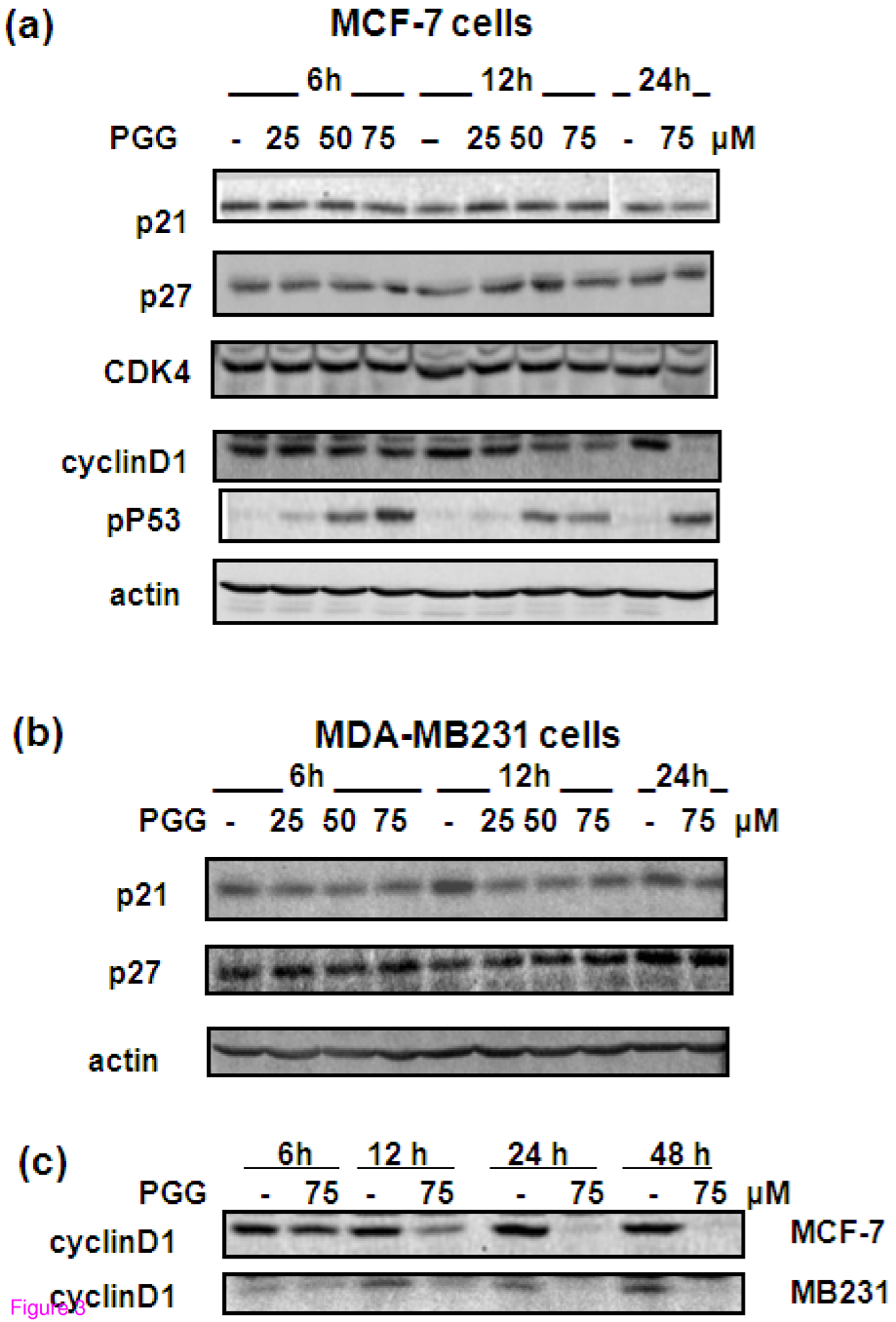
(a)
MCF-7 cells
6h
12h
24h
PGG - 25 50 75 – 25 50 75 - 75 μM
p21
p27
CDK4
cyclinD1
pP53
actin
(b)
MDA-MB231 cells
6h
12h
24h
PGG - 25 50 75 - 25 50 75 - 75 μM
p21
p27
actin
(c)
6h
12 h
24 h
48 h
PGG - 75 - 75 - 75 - 75 μM
cyclinD1
MCF-7
cyclinD1
MB231

Figure 3

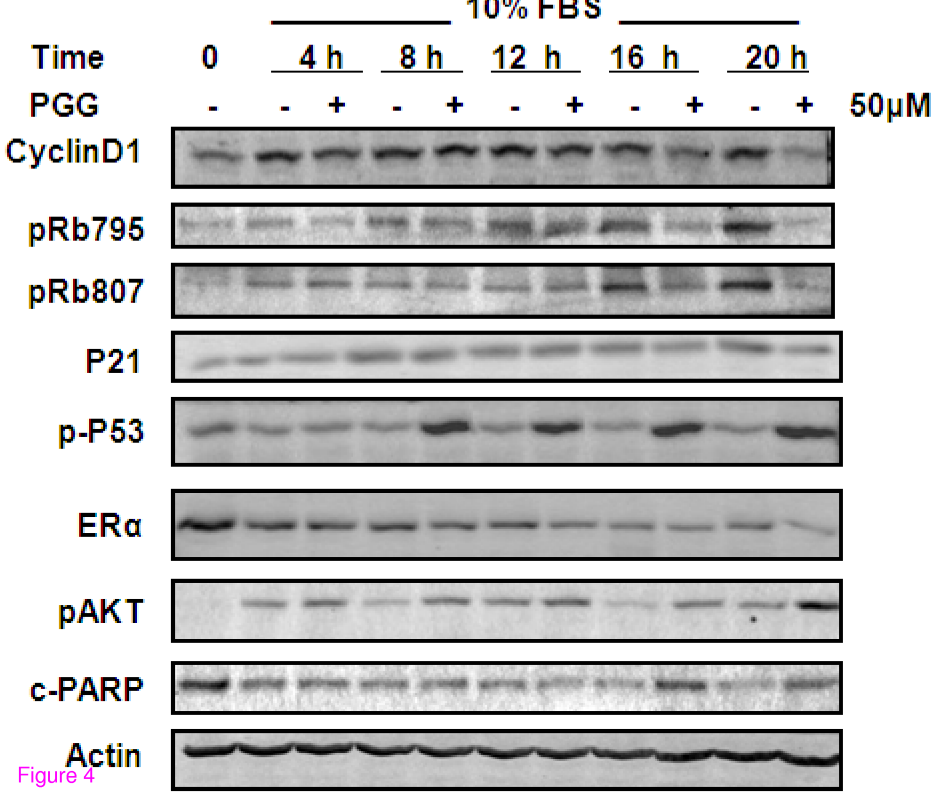
10% FBS
Time
0
4 h
8 h
12 h
16 h
20 h
PGG
- - + - + - + - + - +
50μM
CyclinD1
pRb795
pRb807
P21
p-P53
ERα
pAKT
c-PARP
Actin

Figure 4

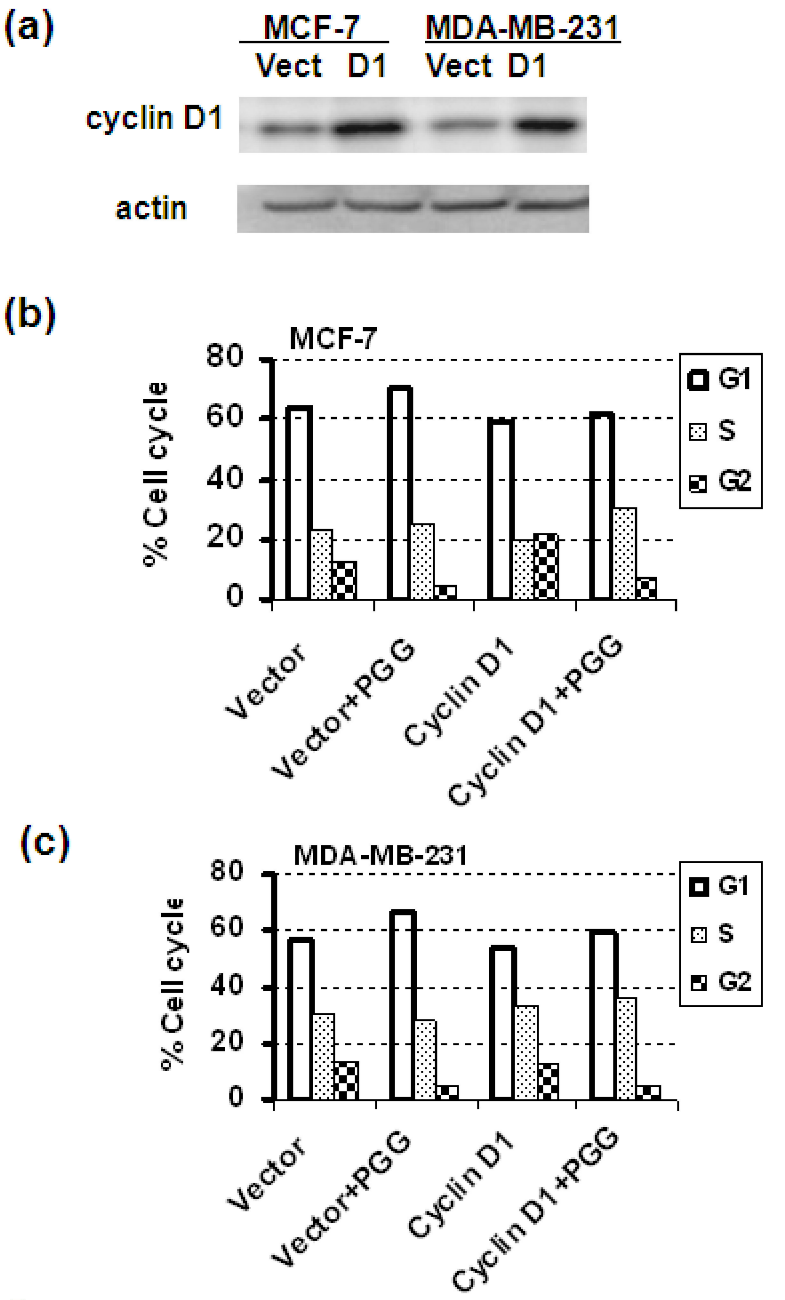
(a)
MCF-7
Vect D1
MDA-MB-231
Vect D1
cyclin D1
actin
(b)
MCF-7
% Cell cycle
80
60
40
20
0
G1
S
G2
Vector
Vector+PGG
Cyclin D1
Cyclin D1+PGG
(c)
MDA-MB-231
% Cell cycle
80
60
40
20
0
G1
S
G2
Vector
Vector+PGG
Cyclin D1
Cyclin D1+PGG

Figure 5

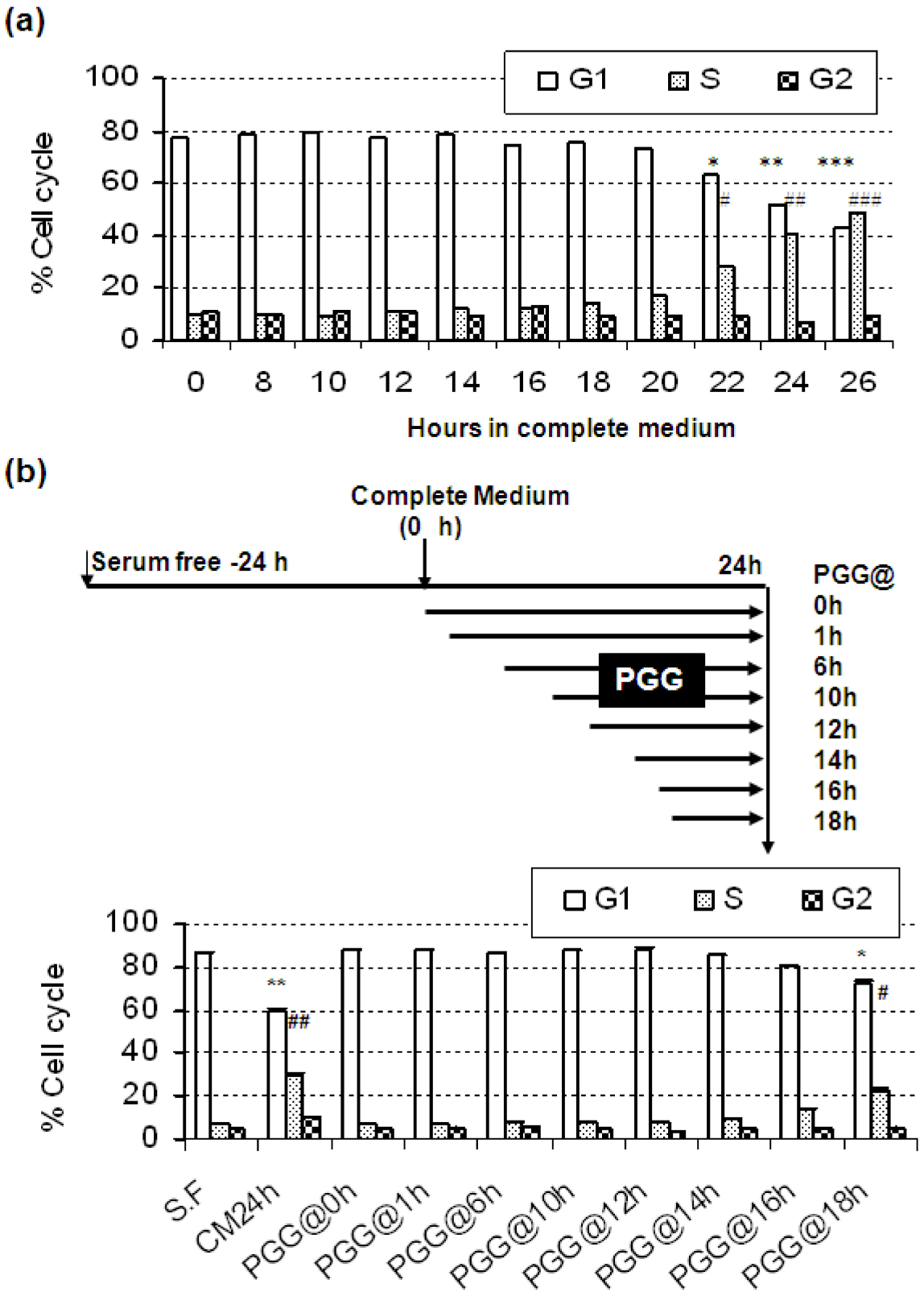
(a)
100
80
60
40
20
0
% Cell cycle
G1
S
G2
*
**
***
#
##
###
0
8
10
12
14
16
18
20
22
24
26
Hours in complete medium
(b)
Complete Medium
(0 h)
Serum free -24 h
24h
PGG@
0h
1h
6h
10h
12h
14h
16h
18h
PGG
G1
S
G2
100
80
60
40
20
0
% Cell cycle
**
##
*
#
S.F
CM24h
PGG@0h
PGG@1h
PGG@6h
PGG@10h
PGG@12h
PGG@14h
PGG@16h
PGG@18h

Figure 6

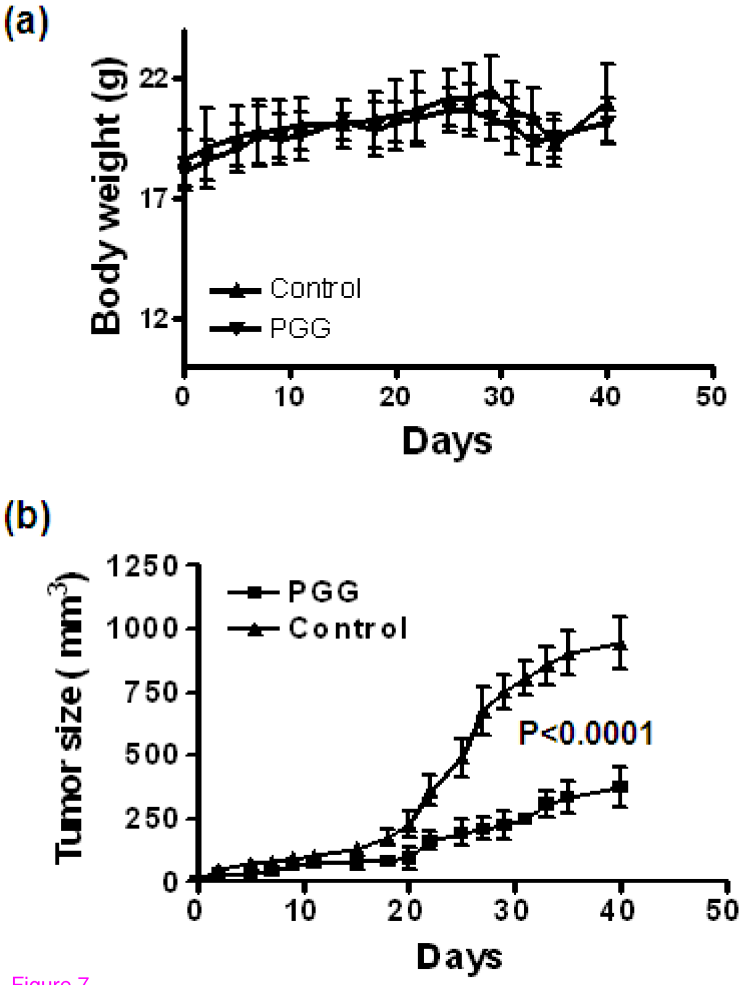
(a)
Body weight (g)
22
17
12
Control
PGG
0
10
20
30
40
50
Days
(b)
Tumor size ( mm3)
1250
1000
750
500
250
0
PGG
Control
P<0.0001
0
10
20
30
40
50
Days

Figure 7