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The GPER1 agonist G-1 attenuates endothelial cell proliferation by inhibiting DNA synthesis and accumulating cells in the S and G2 phases of the cell cycle

by

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Abstract

GPR30 or G protein-coupled estrogen receptor 1 (GPER1) is expressed in the vasculature, but the importance of vascular GPER1 remains to be clarified. Here we investigate effects of the GPER1 agonist G-1 on endothelial cell proliferation using mouse microvascular endothelial bEnd.3 cells. The bEnd.3 cells express mRNA for GPER1. The bEnd.3 cells expressed both ERα and ERβ immunoreactivities. Treatment with G-1 reduced DNA synthesis and cell-number with IC_{50} values of about 2 μM. GPER1 siRNA prevented G-1-induced attenuation of DNA synthesis. G-1 accumulated cells in S and G2 phases of the cell cycle, suggesting that G-1 blocks transition between G2 and M. G-1 had no effect on DNA synthesis in COS-7 cells expressing GPER1 mRNA only weakly. 17β-estradiol had no effect on DNA synthesis in physiological concentrations (nM). The ER blocker ICI182780 reduced DNA synthesis with similar potency as G-1. Treatment with the ERK/MAP kinase inhibitor PD98059 had no effect on G-1-induced attenuation of DNA synthesis. G-1-induced anti-proliferation was observed not only in bEnd.3 cells but also in human HUVEC and HMEC-1 endothelial cells. We conclude that the GPER1 agonist G-1 attenuates endothelial cell proliferation via inhibition of DNA synthesis and by accumulation of cells in S and G2.

Key words: Cell cycle; DNA synthesis; Endothelial cells; Estrogen; Estrogen receptor; G-1; GPER1

Introduction

The female sex hormone estrogen possesses atheroprotective effects, observed in premenopausal women being less prone to cardiovascular disease than age-matched men [1, 2]. Several mechanisms have been suggested for the cardiovascular protective effect of estrogen, for example attenuation of vascular inflammation [3, 4], stimulation of NO formation [5] and prevention of vascular smooth muscle cell proliferation [6]. An intact endothelium is important for prevention of cardiovascular disease, and it is possible that the atheroprotective effects of estrogen are mediated through improved endothelial healing [7, 8].

Estrogen exerts its effect via two classical estrogen receptor subtypes named ERα and ERβ [9]. Expression of ERα and ERβ has been shown in endothelial and vascular smooth muscle cells on both mRNA and protein levels [10, 11]. Estrogen has been shown to prevent vascular smooth muscle cell proliferation after vascular injury via ERα [6]. Studies in knockout mice have shown that ERα is critical for estrogen-induced improvement of re-endothelialisation, while ERβ is not [7]. Also estrogen-induced anti-inflammatory effects have been reported to be mediated via ERα [12]. Estrogen has been reported to induce eNOS and increase NO production within minutes and this effect was attributed to a sub-population of ERα (ERα46) residing in the plasma membrane [13, 14]. Thus, a lot of evidence suggests that ERα mediates estrogen-induced vasculoprotection.

G protein-coupled receptor 30 (GPR30), now named G protein-coupled estrogen receptor 1 (GPER1), has been reported to bind 17β-estradiol with high affinity [15, 16]. GPER1 was shown to mediate proliferative effects of 17β-estradiol in breast cancer cells lacking ERα and ERβ [17]. Studies in GPER1 knockout mice have revealed that these mice show reduced

amount of T-lymphocytes expressing CD62L, hyperglycaemia, reduced body growth, reduced serum IGF-I levels and increased blood pressure [18-20]. Mesenteric arteries and endothelial cells have been shown to express GPER1, suggesting a functional role for GPER1 in the vascular system [18, 19].

Haas et al. [21] reported recently that the GPER1 agonist G-1, identified and characterized by Bologa et al. [22], reduces vascular smooth muscle cell proliferation when administered in μM concentrations, suggesting that G-1 might be used as a blocker of unwanted vascular smooth muscle cell proliferation in atherosclerosis. In order to obtain a more complete picture of how G-1 affects vascular function not only the effects of G-1 on vascular smooth muscle cells, but also its effects on endothelial cells, have to be considered. Therefore, we investigated the effects of G-1 on endothelial cell proliferation and disclose that G-1, in the μM concentration range, exerts an anti-proliferative effect on microvascular endothelial cells by inhibiting DNA synthesis and accumulating cells in the S and G2 phases of the cell cycle.

Material and methods

Cell culture

The mouse brain microvascular endothelial cell line (bEnd.3) was purchased from American Type Tissue Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum (FCS) and antibiotics (penicillin100 U/ml, streptomycin 100 μg/ml). The human dermal microvascular endothelial cell line HMEC-1 was cultured in GIBCO MCDB 131 medium supplemented with 10% FCS, 10 ng/ml epidermal growth factor and antibiotics. COS-7 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% FCS and antibiotics (penicillin100 U/ml, streptomycin 100 μg/ml). Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Lonza, Walkersville, MD, USA) and cultured in endothelial cell culture medium as recommended by Lonza. Cell culture dishes were placed in a water-jacketed cell incubator at 37˚C in 5% CO_2 in air. Experiments were performed when cells reached 80% confluence. Before experiments, normal culture medium was replaced with phenol red-free and FCS-free culture medium for 24 h, or 3 h for HUVEC, to make cells quiescent and to remove the estrogen-like activity of phenol red. The phenol red-free culture medium was used throughout the experiment. Experiments were normally performed under growth-arrested conditions (0% FCS), but in some experiments cells were growth-stimulated with (5%) dextran-coated charcoal stripped FCS. Dextran-coated charcoal was used to remove steroids from normal FCS.

Cell counting and cell viability

Number of cells was determined by cell counting in a Bürker chamber after trypsination (0.25%). Cell viability was assessed by trypan-blue exclusion test. After removing culture

medium cells were washed with 0.9% NaCl and incubated for 2 min with 0.4% trypan-blue (Sigma Chemicals, St Louis, MO, USA). Then the cells were washed three times and the number of cells containing trypan-blue was determined as a measure of dead/dying cells.

RT-PCR

The bEnd.3, COS-7 and RAW264.7 cells were washed carefully in PBS and then total RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was generated and mRNA for GPER1 determined as previously described by Mårtensson et al. [19]. Reverse transcriptase negative controls were included to exclude the presence of contaminating genomic DNA. The sequences of GPER1 (GPR30) primers employed are as follows: forward: GATCGTTAGATTAACAGAGCAG, reverse: CCTGGGAGCCTGTTAGTCTCAG. This primer-pair produces a GPR30 specific product [19]. One-step quantitative real-time PCR measurements were performed using QuantiFast SYBR Green RT-PCR kit (Qiagen) and QuantiTect primer assays (Qiagen) on a Step One Plus real-time thermal cycler from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in duplicate. GPER1 gene expression was calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene as described by Pfaffl [23]. The PCR primers (QuantiTect Primer Assays) for GPER1 (Mm_Gper_1_SG) and GAPDH (Mm_Gapdh_3_SG) were from Qiagen.

Immunocytochemistry

Cells grown on glass cover-slips were fixed in 4% formaldehyde in PBS for 2 h at 4 °C. After washing in PBS, the cells were incubated with polyclonal ERα (NeoMarkers, Fremont, CA, USA; code Ab-16) or ERβ (Affinity BioReagents, Golden, CO, USA; code PA1-311) antibodies raised in rabbits at a dilution of 1:100. ERα and ERβ antibodies were purchased

from Thermo Scientific, Waltham, MA, USA. For visualisation of ERα and ERβ immunoreactivity a fluorescein isothio-cyanate (FITC)-conjugated swine anti-rabbit antibody (Jackson Immuno Research Laboratories, West Grove, PA, USA) was used. For negative controls, the primary antibody was omitted.

DNA synthesis measurement

DNA synthesis was determined by measuring incorporation of [^{3}H] thymidine. The isotope was included for the last hour of the 24 h incubation. The cells were washed in PBS and then harvested using a rubber policeman. Cells were sonicated in 5M NaOH twice for 10 seconds. Aliquots of the sonicate was precipitated with 5% trichloroacetic acid and centrifuged (10621 g for 2 min at 4˚C). After washing with trichloroacetic acid the pellet was dissolved in soluene. Liquid scintilliation cocktail was added and radioactivity measured in a liquid scintilliation counter (Beckman, Fullerton, CA, USA). Protein concentration was determined by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) based on the Lowry method [24]. Radioactivity was expressed as D.P.M. and normalised to the concentration of total protein in each sample.

GPER1 siRNA

The bEnd.3 cells were treated with commercially available GPER1 siRNA (accession number S104924283) or scramble control (scramble, accession number S103650325) purchased from Qiagen. Free-floating, newly trypsinated, cells were treated with the GPER1 siRNA or scramble control and HiPerFect transfection reagent (Qiagen) for 3 h in serum-free and antibiotic-free culture medium. The final siRNA and scramble control concentration was 100 nM as recommended by the manual from Qiagen. The cells were allowed to attach and equilibrate for 48 h in normal culture medium before challenged with G-1 or DMSO vehicle.

A lower GPER1 mRNA level in siRNA vs. scramble control treated bEnd.3 cells, demonstrated by quantitative real-time PCR, confirmed down-regulation of GPER1 transcript by GPER1 siRNA.

Flow cytometry

Distribution of bEnd.3 cells in the cell cycle was determined by flow cytometry of propidium iodide stained nuclei as described by Odenlund et al. [25]. Briefly, flow cytometric DNA analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San José, CA, USA) equipped with Cell Quest PRO™ program for data acquisition. DNA contents were determined in about 13000 nuclei in each sample. Processor signals were digitised and sorted into frequency distribution, DNA histograms, with 256 units resolution. Cell cycle phase distribution was determined by using ModFit LT 3.1 (Verity Software House Inc., Topsham, ME, USA).

Drugs

G-1 was purchased from Cayman Chemical, Ann Arbor, MI, USA. E_2 was purchased from Sigma Chemicals. ICI182780 was a kind gift from Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK. PD98059 was purchased from Calbiochem, San Diego, CA, USA. G-1, E_2, ICI182780 and PD98059 were dissolved in DMSO. Controls received DMSO as vehicle.

Statistics

Summarized data are presented as means ± S.E.M. Statistical significance was calculated using ANOVA and Student's two-tailed t-test for unpaired comparison with Bonferroni correction for post hoc analysis as appropriate.

Results

Expression of GPER1, ERα and ERβ in bEnd.3 endothelial cells

In order to characterize ER expression in the bEnd.3 cells, we determined GPER1 mRNA and ERα and ERβ protein expression. The bEnd.3 cells expressed mRNA for GPER1 as demonstrated in figure 1A. For positive control we used mouse macrophage RAW264.7 cell cDNA (Fig. 1A). The RAW264.7 cells are well known to express GPER1 [26]. In COS-7 cells a weak GPER1 band was detected showing that these cells only weakly express GPER1 mRNA (Fig. 1A). This is in agreement with previous studies reporting that COS-7 cells lack GPER1 expression or only show weak GPER mRNA expression [27, 28]. Since there is no reliable antibody available for mouse GPER1, we could not confirm GPER1 transcript expression on the protein level. The bEnd.3 cells expressed ERα and ERβ proteins as demonstrated by immunocytochemistry (Fig. 1B). ERα and ERβ immunoreactivities were mainly observed in the nuclei, but some ERβ immunoreactivity was observed also in the cytoplasm.

Effects of G-1 on bEnd.3 cell DNA synthesis

Stimulation with a sub-maximal concentration of charcoal stripped FCS (5%) for 24 h increased DNA synthesis by about 3-fold compared to the basal level of DNA synthesis (0% FCS) as shown in figure 2. Treatment with G-1 at a low concentration (0.03 μM) had no effect on FCS-stimulated DNA synthesis, while a higher concentration (3 μM) fully prevented FCS-induced DNA synthesis (Fig. 2). An intermediate concentration of G-1 (0.9 μM) also reduced FCS-induced DNA synthesis but this effect was not statistically significant (Fig. 2). G-1 caused a concentration-dependent decrease in DNA synthesis under non-stimulated (0% FCS) basal conditions with an IC_{50} value of about 1.9 μM (Fig. 3). As seen in figure 4,

stimulation with G-1 (3 μM) for 24 h reduced basal DNA synthesis by about 80%. An equimolar concentration (3 μM) of the endogenous estrogen 17β-estradiol also reduced DNA synthesis (30%), but the effect of 17β-estradiol was smaller than that of G-1. Because 3 μM is not a physiological concentration of 17β-estradiol, we also tested lower concentrations (0.1-30 nM) of this estrogen. As shown in figure 4, treatment with 0.1-30 nM 17β-estradiol had no effect on DNA synthesis. Combined treatment with 3 μM 17β-estradiol and the ERα and ERβ antagonist ICI182780 (1 μM) reduced DNA synthesis by about 80% (Fig. 4). Treatment with 1 μM ICI182780 alone reduced DNA synthesis similar to the combination of 17β-estradiol and ICI182780 (Fig. 4). To provide direct evidence for the involvement of GPER1 we treated bEnd.3 cells with GPER1 siRNA and scramble control, respectively, and then monitored the effects of 24 h treatment with G-1 on FCS (5%) stimulated DNA synthesis. G-1 (3 μM) reduced DNA synthesis by 60% in scramble control, while it had no effect in GPER1 siRNA treated cells (Fig. 5A). Down-regulation of GPER1 mRNA level by siRNA was confirmed by quantitative real-time PCR (Fig. 5B).

We tested the effects of G-1 on DNA synthesis in a human microvascular endothelial cell line as well, i.e. human dermal microvascular endothelial HMEC-1 cells [29]. Treatment with G-1 (3 μM) for 24 h under non-stimulated (0% FCS) basal conditions reduced DNA synthesis by about 50% in HMEC-1 cells (Fig. 6), showing that G-1 reduces DNA synthesis in microvascular endothelial cells derived from a different origin than bEnd.3 cells as well. Treatment with 3 μM G-1 for 24 h (0% FCS) reduced DNA synthesis by about 80% in HUVEC (Fig. 6). We compared the effects of G-1 on DNA synthesis in GPER1 positive bEnd.3 cells with those in COS-7 cells only weakly expressing GPER1 [27, 28]. As demonstrated in figure 6, treatment with G-1 (3 μM) for 24 h (0% FCS) reduced DNA synthesis by about 80% in bEnd.3 cells, but had no effect in COS-7 cells.

Effects of G-1 on bEnd.3 cell number and cell cycle phase distribution

Treatment with G-1 (3 μM) for 5 days reduced the cell number by about 75% under conditions representing sub-maximal growth stimulation, i.e. 5% charcoal stripped FCS (Fig. 7A). Treatment with 3 μM G-1 for a shorter time, i.e. 2 days, reduced cell number by about 65% (70000±4100 cells/ml after G-1 treatment vs. 215000±45000 cells/ml in controls, $P<0.05$, n= 4 in each group). Trypan-blue exclusion test of cells cultured with 5% stripped FCS showed very few trypan-blue containing cells in both control cells and cells treated with 3 μM G-1, suggesting that G-1 has no effect on cell viability (data not shown). Treatment with G-1 (3 μM) for 24 h under the same culture conditions as used for measuring cell number (see above) accumulated cells in S and G2 phases of the cell cycle (Fig. 7B), suggesting that G-1 prevents the endothelial cells from entering the M phase.

Effects of ERK/MAP kinase inhibitor PD98059 on G-1-induced inhibition of DNA synthesis in bEnd.3 cells

The ERK/MAP kinase inhibitor PD98059 was included 2 h before G-1 and then present throughout the 24 h incubation (0% FCS) with G-1. As seen in figure 8, combined treatment with PD98059 (1 μM) and G-1 (3 μM) reduced DNA synthesis similar to G-1 alone, suggesting that G-1-induced reduction of DNA synthesis does not involve attenuation of ERK/MAP kinase activity. PD98059 at 1 μM had no effect on DNA synthesis by itself (Fig. 8). PD98059 is known to block ERK/MAP kinase activity already at 1 μM with an IC_{50} value of 2 μM [30]. Incubation with higher concentrations (10 and 50 μM) of PD98059 without G-1 reduced DNA synthesis in a concentration dependent manner (Fig. 8).

Discussion

Here we show that the GPER1 agonist G-1, in the μM concentration range, reduces endothelial cell proliferation by inhibiting DNA synthesis and accumulating cells in the S and G2 phases of the cell cycle. G-1-induced cell cycle arrest in S and G2 suggests that G-1 prevents the endothelial cells from entering the M phase. Haas et al. [21] reported recently that G-1 at 1 μM reduces vascular smooth muscle cell proliferation and proposed that G-1 might be used to attenuate unwanted vascular smooth muscle cell proliferation observed in atherosclerosis. Taken together, these findings suggest that G-1 reduces both endothelial cell and vascular smooth muscle cell proliferation. Unwanted endothelial cell proliferation occurs in e.g. angiogenesis in tumour growth, and G-1 may be used to prevent/reduce this unwanted formation of new blood vessels. Treatment with G-1 has been reported to reduce urothelial cell proliferation as well [28], but in this study growth-inhibition by G-1 was observed at a lower concentration (0.01-0.1 μM), suggesting that urothelial cells are more sensitive than endothelial and vascular smooth muscle cells to G-1. The difference in G-1 sensitivity of vascular and urothelial cells suggests that G-1 inhibits proliferation through different mechanisms depending on the cell type.

We show in the present study that G-1 reduces mouse brain microvascular bEnd.3 endothelial cell proliferation by inhibition of DNA synthesis and by causing cell cycle arrest in S and G2. G-1 induced attenuation of DNA synthesis was observed also in the widely used and well-characterized human dermal microvascular endothelial cell line HMEC-1 [29, 31-33] and in HUVEC, showing that G-1-induced reduction of DNA synthesis may be general to endothelial cells independent of origin. The bEnd.3 endothelial cell line is a well-established and widely used endothelial cell line [34, 35]. Furthermore, the bEnd.3 cells are representative

for native endothelial cells as they possess endothelial cell morphology and express the endothelial cell marker protein eNOS [36, 37]. In the present study we show that bEnd.3 cells express ERα, ERβ, and GPER1 confirming their expression patterns observed in endothelial cells in vivo [11, 18]. The bEnd.3 cells thus express several endothelial cell markers, suggesting that the G-1-induced anti-proliferative mechanism of action that we observe in bEnd.3 cells is representative of native endothelial cells in vivo.

G-1 has been shown to reduce blood pressure both in normotensive Sprague-Dawley rats and in ovariectomized hypertensive MREN2.LEWIS rats [21, 38]. This agent relaxes rat mesenteric arteries pre-contracted with either UTP or angiotensin II, human internal mammary arteries pre-contracted with prostaglandin $F_{2\alpha}$, and mouse carotid arteries pre-contracted with prostaglandin $F_{2\alpha}$, suggesting that the G-1-induced reduction of blood pressure is achieved by reducing vascular resistance [21, 38]. Acetylcholine-induced vascular relaxation was not affected by G-1, suggesting that G-1 does not act via stimulation of eNOS activity and increased NO production [38]. Relaxation of isolated vascular preparations is observed in response to 1 - 3 μM G-1, which is in the same concentration range as that reducing proliferation of endothelial and vascular muscle smooth cells. Thus, the GPER1 agonist G-1 affects, in μM concentrations, vascular function by reducing vascular resistance but also by attenuation of endothelial and vascular smooth muscle cell proliferation.

The reduction in DNA synthesis by G-1 was unaffected by the ERK/MAP kinase inhibitor PD98059, suggesting that G-1 reduces endothelial cell DNA synthesis via another mechanism than by reducing ERK/MAP kinase activity. We used a concentration of PD98059 known to reduce ERK/MAP kinase activity [30] without having any effects on endothelial cell proliferation, suggesting that G-1 acts via another pathway than ERK/MAP kinase, but still

we cannot completely rule out the possibility that G-1, at least in part, acts via ERK/MAP kinase inhibition. The bEnd.3 endothelial cells express a strong mRNA band for GPER1, suggesting that the anti-proliferative effect of G-1 is mediated via GPER1. It is very well documented that COS-7 cells express no GPER1 or only weakly express this receptor [27, 28]. Here we show that G-1 reduces DNA synthesis by about 80% in GPER1 positive bEnd.3 cells, while it has no effect in COS-7 cells only weakly expressing GPER1 mRNA, suggesting that G-1-induced endothelial cell anti-proliferation is mediated by GPER1. The low GPER1 expression level in COS-7 cells is probably not sufficient to evoke anti-proliferation upon stimulation by G-1. Another support for the involvement of GPER1 is that the ER antagonist ICI182780, reported to act as a GPER1 agonist [22, 39], reduces endothelial cell DNA synthesis with similar potency as G-1. GPER1 siRNA abolished the G-1-induced attenuation of DNA synthesis providing direct evidence for the involvement of GPER1. Taken together these data show that G-1-induced endothelial cell anti-proliferation is mediated by the GPER1.

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Figure legends

Fig. 1. (A) The bEnd.3 cells express a strong GPER1 mRNA band (GPR30, 290 bp) as demonstrated by RT-PCR. The most left panel shows the DNA ladder. No band was observed in reverse transcriptase negative (RT-) control. RAW264.7 cell cDNA (RAW) was used as positive control producing a very strong 290 bp GPER1 band. In COS-7 cells a very weak 290 bp GPER1 band was observed. **(B)** The bEnd.3 cells express immunoreactivity for ERα as well as ERβ as demonstrated by immunocytochemistry. No immunoreactivity was observed after omission of the primary ERα or ERβ antibodies. Bars represent 20 μm.

Fig. 2. G-1 (3 μM) prevents FCS-induced DNA synthesis in bEnd.3 cells growth-stimulated with 5% stripped FCS for 24 h. Cells were pre-treated with G-1 for 2 h before FCS was introduced. G-1 was then present throughout the 24 h incubation. DNA synthesis was determined by incubation with radiolabelled thymidine during the last hour of incubation ([^{3}H]-thymidine, 5 μCi). Radioactivity was normalized to protein concentration and presented as per cent of the radioactivity in parallel controls. Values are presented as means ±S.E.M. of 3-11 observations in each group. * and ** represent $P<0.05$ and $P<0.01$, respectively, when compared to cells stimulated with 5% FCS alone. N.S. = not significant compared to 5% FCS.

Fig. 3. Dose-response curve showing the effects of increasing concentrations of G-1 on DNA synthesis in bEnd.3 cells. DNA synthesis in cells treated with G-1 was normalized to that observed in untreated control cells. DNA synthesis in control cells was set to 100%. The cells were treated with G-1 for 24 h under non-stimulated (0% FCS) basal conditions. The IC_{50}

value was calculated to 1.9 μM. Values are presented as means ± S.E.M. of 5-9 observations in each group. ** represents P<0.01 compared to controls.

Fig. 4. Effects of treatment with 17β-estradiol, ICI182780 and G-1 for 24 h on DNA synthesis in bEnd.3 cells (0% FCS). ICI182780 was introduced 2 h before 17β-estradiol and then present throughout the 24 h incubation. Values are means ± S.E.M. of 4-9 observations in each group. ** and *** represent P<0.01 and P<0.001, respectively, when compared to controls. ### represents P<0.001 when compared to treatment with 3 μM E2 alone.

Fig. 5. (A) GPER1 siRNA abolishes G-1-induced attenuation of DNA synthesis in bEnd.3 cells. The cells were stimulated with G-1 (3 μM) for 24 h under growth-stimulated conditions (5% stripped FCS). **(B)** Down-regulation of GPER1 mRNA level by siRNA was confirmed by quantitative real-time PCR. Values are means ± S.E.M. of 5-6 observations in each group. * and *** represent P<0.05 and P<0.001, respectively. N.S. = not significant.

Fig. 6. Treatment with 3 μM G-1 for 24 h reduces DNA synthesis by about 80% in both bEnd.3 and HUVEC endothelial cells and by 50% in HMEC-1 endothelial cells. G-1 (3 μM) has no effect in COS-7 cells. The cells were treated with G-1 under non-stimulated (0% FCS) basal conditions. Values are means ± S.E.M. of 3-9 observations in each group. *, ** and *** represent P<0.05, P<0.01 and P<0.001, respectively. N.S. = not significant.

Fig. 7. (A) Treatment with 3 μM G-1 for 5 days in the presence of 5% stripped FCS, reduces bEnd.3 cell number by about 75%. **(B)** Stimulation with G-1 (3 μM) for 24 h in the presence of 5% stripped FCS accumulates cell in the S and G2 phases of the cell cycle. DNA was stained with propidium iodide and distribution of cells in G0/G1, S and G2 phases computed

based on DNA histograms and presented as percentage. Values are means ± S.E.M. of 4-6 observations in each group. * and *** represent $P<0.05$ and $P<0.001$, respectively.

Fig. 8. The ERK/MAP kinase inhibitor PD98059 (1 μM) has no effect on G-1-induced (3 μM) inhibition of DNA synthesis in bEnd.3 cells. PD98059 was introduced 2 h before G-1 and then present throughout the 24 h incubation (0% FCS). Values are means ± S.E.M. of 3-18 observations in each group. *** represents $P<0.001$ compared to controls. N.S. = not significant.

Fig. 1

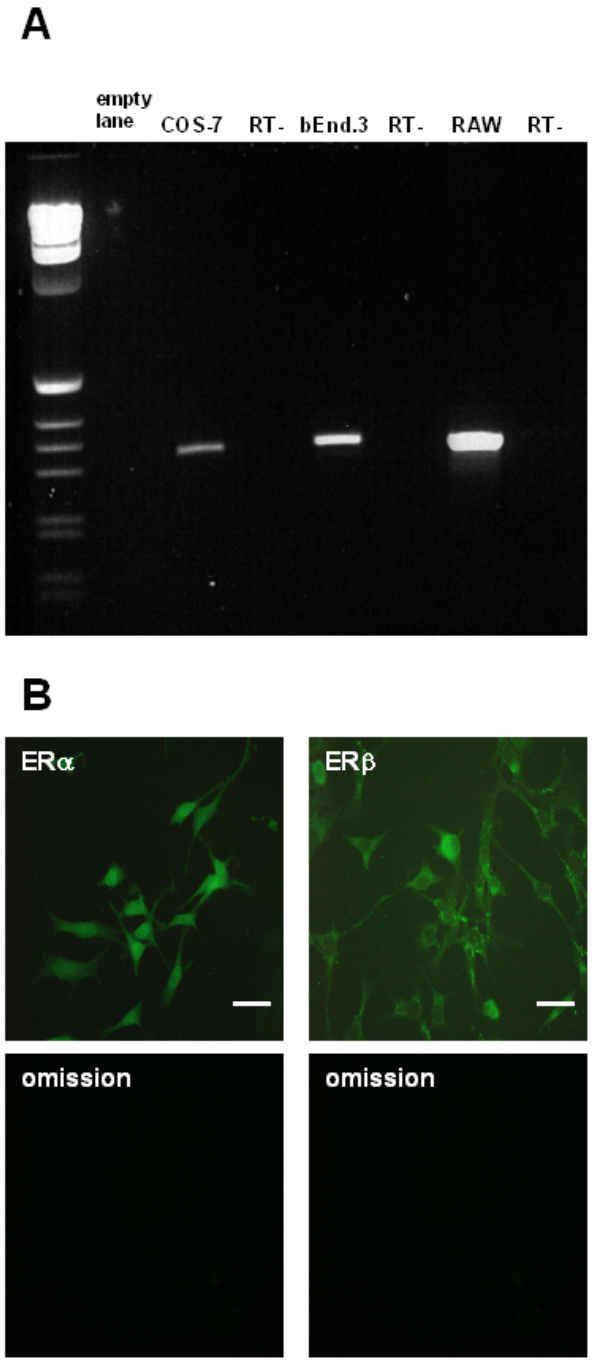

A
empty lane
COS-7
RT-
bEnd.3
RT-
RAW
RT-
B
ERα
ERβ
omission
omission

Fig. 2

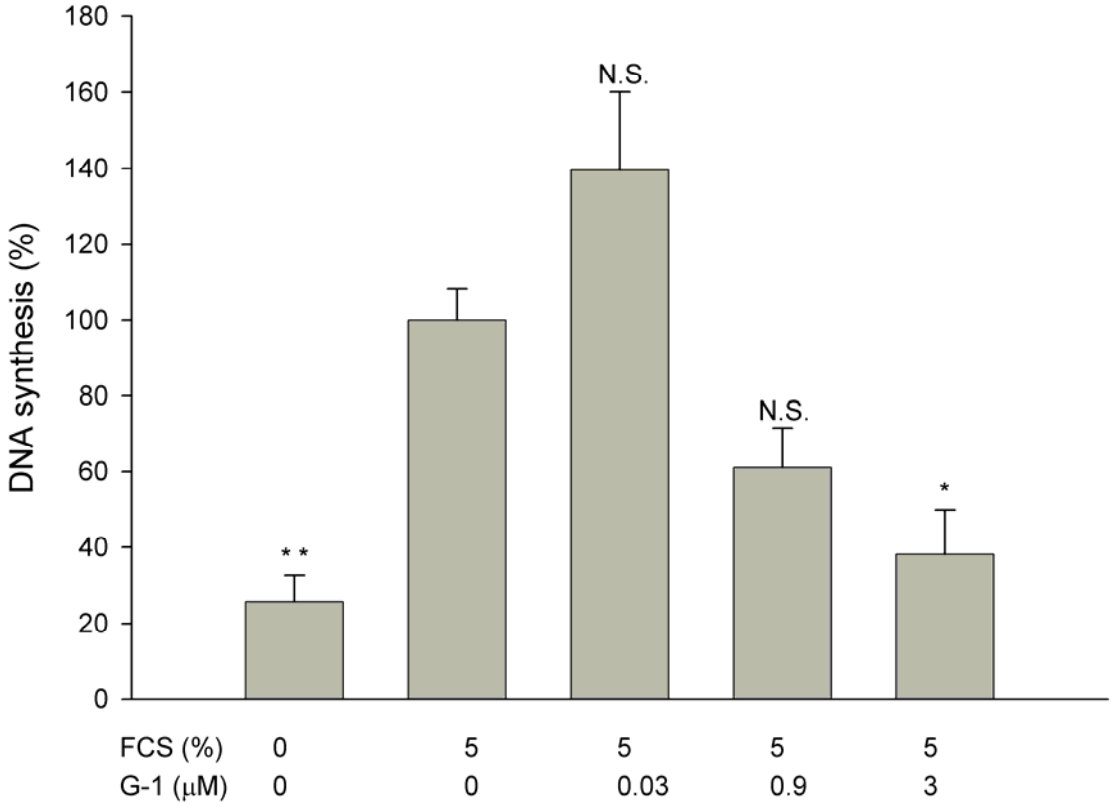

DNA synthesis (%)
180
160
140
120
100
80
60
40
20
0
N.S.
N.S.
* *
*
FCS (%) 0 5 5 5 5
G-1 (μM) 0 0 0.03 0.9 3

Fig. 3

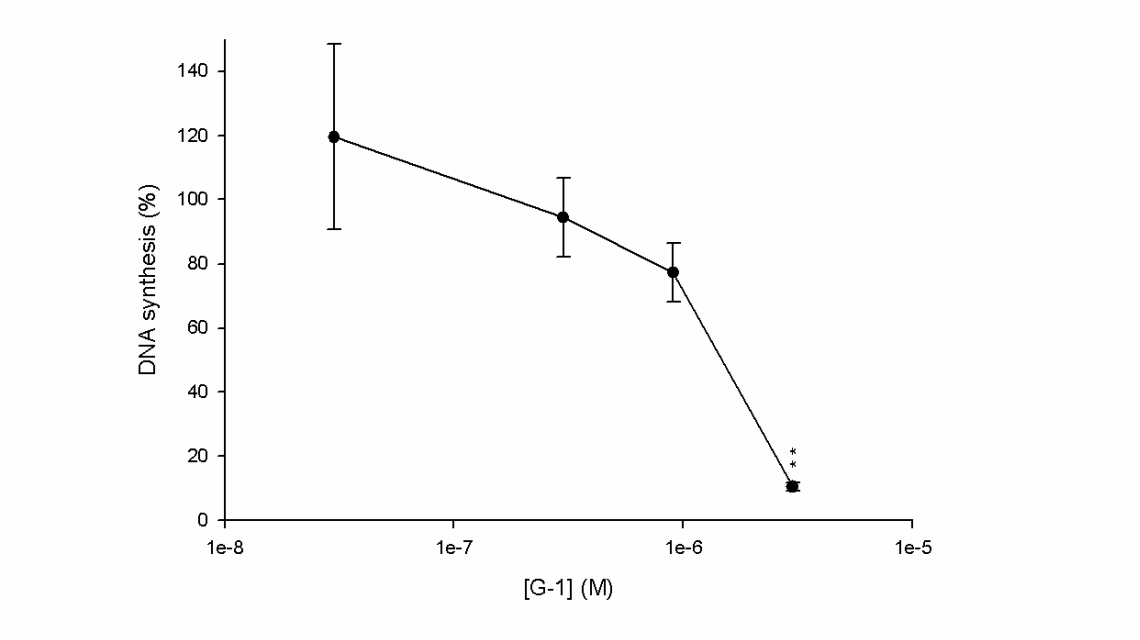


Fig. 4

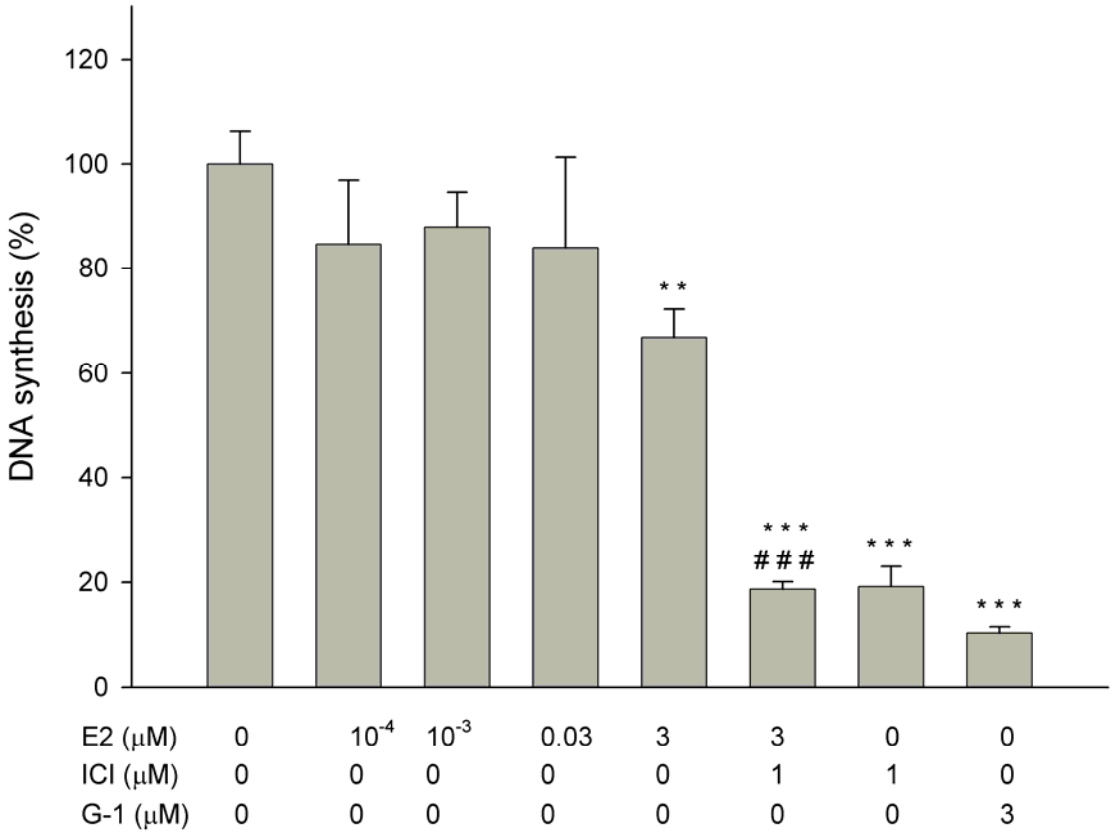

DNA synthesis (%)
120
100
80
60
40
20
0
**
***
###
***
***
E2 (μM) 0 10-4 10-3 0.03 3 3 0 0
ICI (μM) 0 0 0 0 0 1 1 0
G-1 (μM) 0 0 0 0 0 0 0 3

Fig. 5

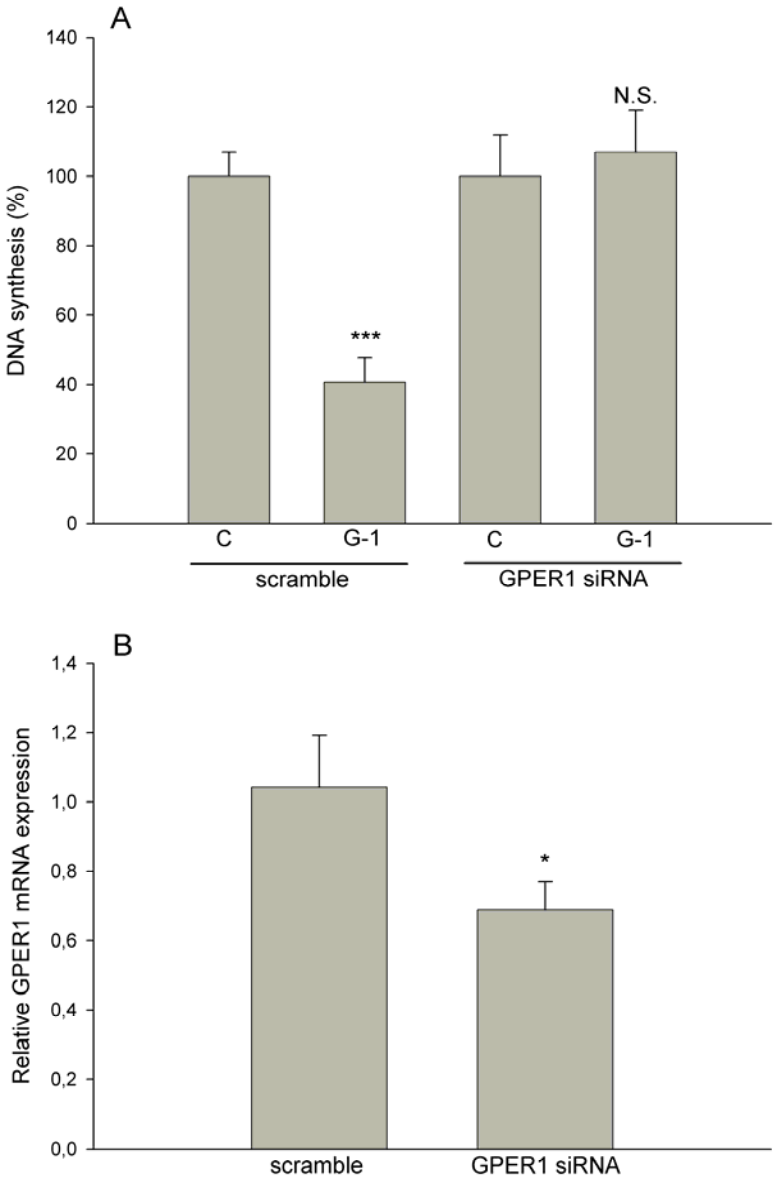

A
DNA synthesis (%)
140
120
100
80
60
40
20
0
***
N.S.
C
G-1
C
G-1
scramble
GPER1 siRNA
B
Relative GPER1 mRNA expression
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
scramble
GPER1 siRNA

Fig. 6

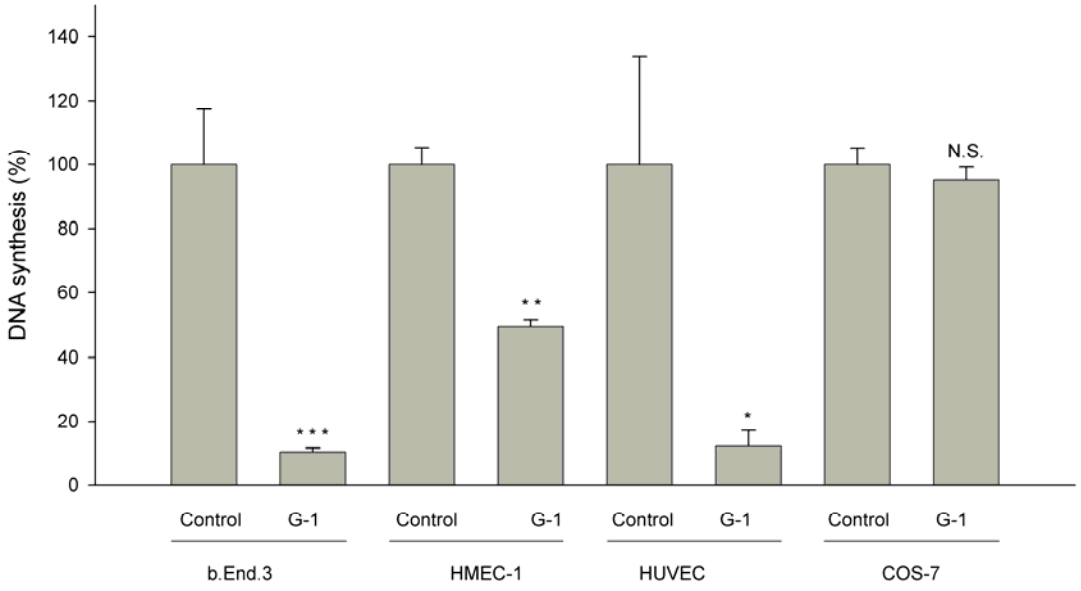

DNA synthesis (%)
140
120
100
80
60
40
20
0
***
**
*
N.S.
Control
G-1
Control
G-1
Control
G-1
Control
G-1
b.End.3
HMEC-1
HUVEC
COS-7

Fig. 7

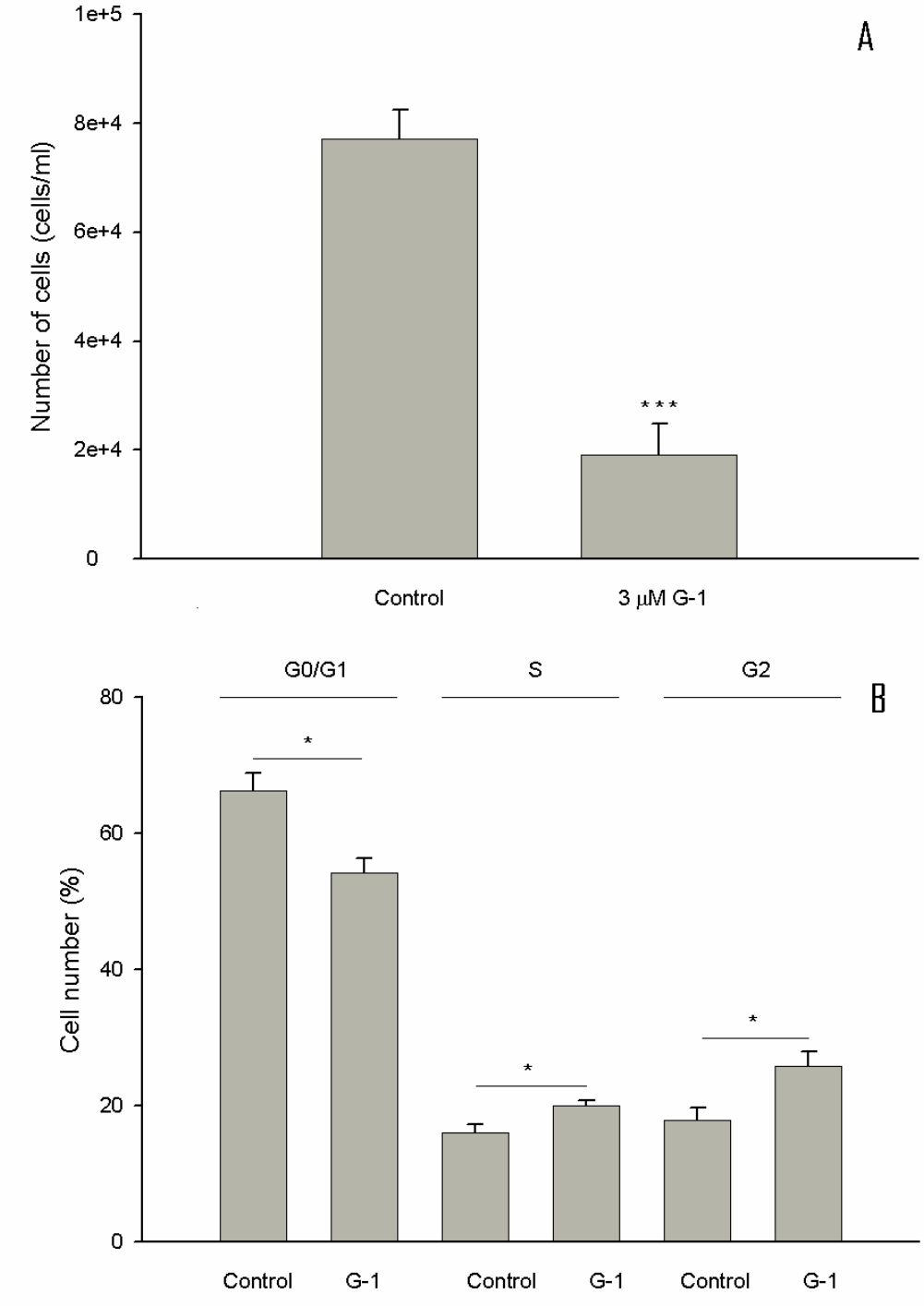
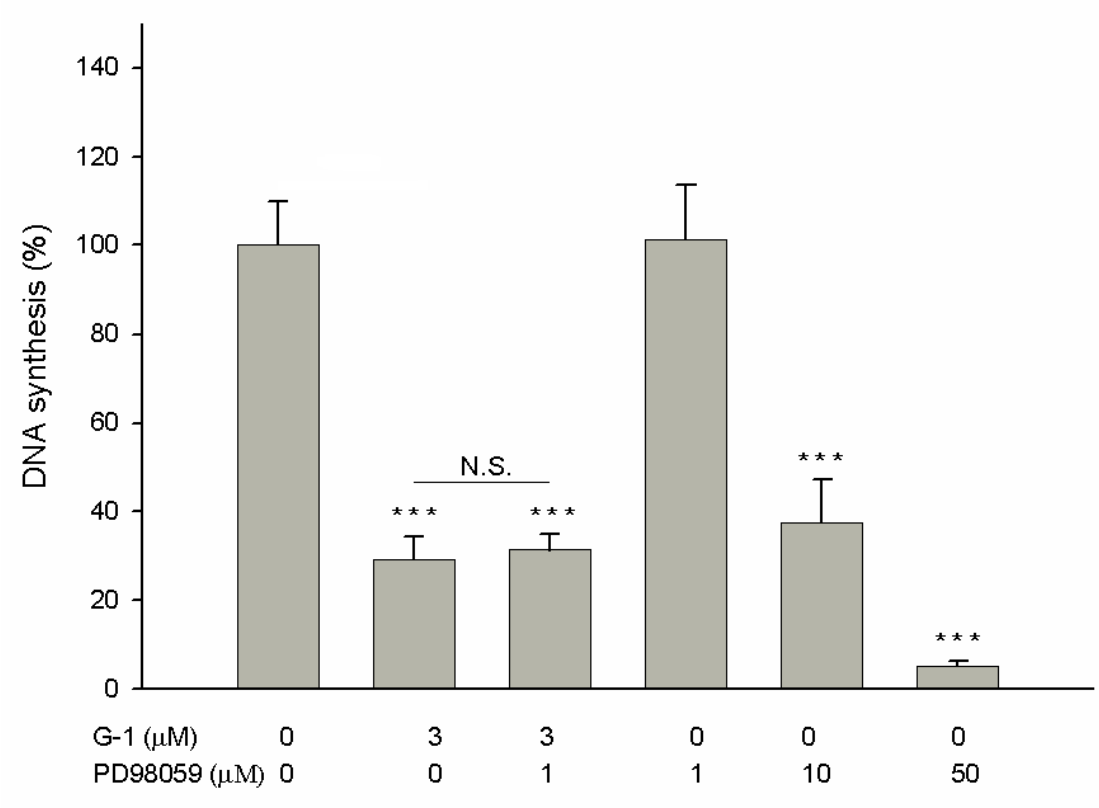

A
1e+5
8e+4
6e+4
4e+4
2e+4
0
Number of cells (cells/ml)
***
Control
3 μM G-1
B
G0/G1
S
G2
80
60
40
20
0
Cell number (%)
*
*
*
Control
G-1
Control
G-1
Control
G-1

Fig. 8


DNA synthesis (%)
140
120
100
80
60
40
20
0
N.S.
***
***
***
***
G-1 (μM) 0 3 3 0 0 0
PD98059 (μM) 0 0 1 1 10 50