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Role of soluble guanylyl cyclase-cyclic GMP signaling in tumor cell proliferation

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Abstract

Our previous studies demonstrate a differential expression of nitric oxide (NO) signaling components in ES cells and our recent study demonstrated an enhanced differentiation of ES cells into myocardial cells with NO donors and soluble guanylyl cyclase (sGC) activators. Since NO-cGMP pathway exhibits a diverse role in cancer, we were interested in evaluating the role of the NO receptor sGC and other components of the pathway in regulation of the tumor cell proliferation. Our results demonstrate a differential expression of the sGC subunits, NOS-1 and PKG mRNA and protein levels in various human cancer models. In contrast to $sGC\alpha_1$, robust levels of sGC β_1 were observed in OVCAR-3 (ovarian) and MDA-MB-468 (breast) cancer cells which correlated well with the sGC activity and a marked increase in cGMP levels upon exposure to the combination of a NO donor and a sGC activator. NOC-18 (DETA NONOate; NO donor), BAY41-2272 (3-(4-Amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine); sGC activator), NOC-18+BAY41-2272, IBMX (3-Isobutyl-1-methylxanthine; phosphodiesterase inhibitor) and 8-bromo-cGMP (cGMP analog) caused growth inhibition and apoptosis in various cancer cell lines. To elucidate the molecular mechanisms involved in growth inhibition, we evaluated the effect of activators/inhibitors on ERK phosphorylation. Our studies indicate that BAY41-2272 or the combination NOC18+BAY41-2272 caused inhibition of the basal ERK1/2 phosphorylation in OVCAR-3 (high sGC activity), SK-OV-3 and SK-Br-3 (low sGC activity) cell lines and in some cases the inhibition was rescued by the sGC inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one). These studies suggest that the effects of activators/inhibitors of NO-sGC-cGMP in tumor cell proliferation is mediated by both cGMP-dependent and independent mechanisms.

Keywords

Nitric Oxide; ovarian cancer; breast cancer; sGC; cyclic GMP

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Introduction

Nitric oxide (NO) and cyclic GMP (cGMP) are important signaling molecules that exhibit diverse physiological functions which range from smooth muscle relaxation, neurotransmission, inhibition of platelet aggregation, vasodilation and effects with host defense mechanisms. NO is synthesized by nitric oxide synthase(s) (such as nNOS (NOS-1), iNOS (NOS-2) and eNOS (NOS-3)) by the oxidation of L-arginine to L-citrulline with the release of the free radical NO (1-4).

NO has been shown to exhibit a paradoxical and diverse role in cancer. The effects of NO can be either cGMP-dependent, which involve the production of the second messenger, cGMP, following NO activation of soluble guanylyl cyclase (sGC), or cGMP-independent, which are mediated by reactive nitrogen species that are produced as a result of the interaction of NO with oxygen (O_2), oxygen reactive species or superoxide radicals ($O_2^{\bullet-}$). cGMP is synthesized by guanylyl cyclases (4-5) and there are two classes of guanylyl cyclase enzymes (particulate; pGC and soluble; sGC) which generate cGMP from intracellular GTP. sGC is a heme containing hetero-dimer composed of α (α_1 and α_2) and β (β_1 and β_2) subunits which make up the active enzyme. sGC can exert many physiological effects through production of cGMP which acts directly on the downstream effectors such as cGMP-dependent protein kinases (PKGs), cyclic nucleotide-gated channels (CNGs), and cGMP-regulated phosphodiesterases (PDEs) (4,6-8).

NO and other components of the signaling pathway such as nitric oxide synthases, sGC, pGC, PKG and PDE are known to regulate cell survival and cell death pathways in many cancer models (9-10). iNOS (NOS-2) enzyme is a poor prognostic marker in metastatic melanoma (11) and constitutive production of iNOS and NO may be involved in resistance to apoptosis in human melanoma (12). In addition, sGC and cGMP suppresses apoptosis in ovarian cancer cells by regulation of p53 tumor suppressor (13) and sGC in some cases mediates angiogenesis with endothelial cells (14). However, inhibition of sGC by antisense RNA dramatically decreases the tumor volume in glioma xenograft models (15). In contrast, some studies do point to the growth inhibitory and apoptotic functions of the NO-cGMP signaling pathway. For example, NO and ionizing radiation synergistically promote apoptosis and growth inhibition of colon cancer cells (16). Similarly, nitric oxide donating aspirin induces apoptosis of colon cancer cells due to induction of oxidative stress (17). Induction of protein kinase G is also involved in apoptosis and inhibition of cell migration in colon cancer cells (18).

Therefore, to further evaluate the significance of NO-cGMP in cancer cell proliferation, we focused on the expression, function and modulation of the NO receptor sGC and other components of the NO-cGMP pathway in various breast, ovarian and prostate cancer cells. Our studies demonstrate a differential expression of various subunits of the NO-receptor sGC, sGC activity and accumulation of cGMP in various cancer cell lines. In addition, our results show growth inhibition, apoptosis and inhibition of ERK phosphorylation in various cancer cell lines by using NO donors, sGC activators, combination of NOC-18+BAY41-2272, cGMP analog 8-bromo cGMP and PDE inhibitor IBMX.

Experimental Procedures

Reagents

RPMI1640 and DMEM-F12 media were purchased from Invitrogen; fetal bovine serum, atrial natriuretic peptide(ANP), and C-type natriuretic peptide (CNP) from Sigma Chemical Co. NOC-18 (DETA-NO, slow release NO donor), NOC-22 (Spermine-NO fast release NO donor), SNAP (S-nitroso-N-acetyl-penicillamine) L-NAME (N^G-nitro-L-arginine methyl ester), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), BAY 41-2272 (3-(4-Amino-5-

cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine), YC-1(3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole and IBMX (3-isobutyl-1-methyl xanthine) and cGMP analog (8-bromo-cGMP) were purchased from Calbiochem. Heat-stable entrotoxin (STa) was purchased from Bachem and (α-^{32}P) GTP from New England Nuclear.

Cell lines and antibodies

The A2780, OVCAR-3, SK-OV-3, C-2/HEY (ovarian cancer), MDA-MB-468, SK-Br-3 (breast cancer), LNCaP, PC-3, DU-145 (prostate cancer) and BE-2 human neuroblastoma cell lines were either purchased from American Type Tissue culture collection (Manassas,VA) or were gifts from Drs. Zahid Siddik (A2780) and Gordon Mills (C-2/HEY) both of M.D. Anderson Cancer Center, Houston, TX. All the cancer cell lines (except BE-2) were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1 mM glutamine and antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin). BE-2 cells were maintained in 1:1 mixture of DMEM/F-12K media supplemented with 10% FCS, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate and antibiotics (50 μg/ml of streptomycin and 50U/ml of penicillin) at 37° C.

Antibodies

Polyclonal anti-sGC α_1 and polyclonal anti-sGCβ_1 antibodies were raised against the peptides FTPRSREELPPNFP and SRKNTGTEETKQODDD respectively (19). Monoclonal β-actin (AC-15) antibody was obtained from Sigma Chemical Co. Phospho ERK1/2 (cat # sc-16982-R) and ERK1 (cat # 610030) antibodies were purchased from Santa Cruz Biotechnology and BD Transduction Labs respectively.

Real-time RT-PCR

Total RNA from cancer cells was isolated using ultraSpec total RNA isolation reagent from Biotecx (Houston, TX). cDNA was prepared using high capacity cDNA archive kit (Applied Biosystems, Foster City, CA), according to the manufacturer's suggestions. Real-time PCR assays for subunits of sGC (α_1 β_1 α_2 β_2), nitric oxide synthase(s) (NOS-1, 2, 3), PKGIα and GAPDH were purchased from Applied Bio systems and determined using the manufacturer's suggested protocol. All reactions were conducted using a 7900 HT Prizm Sequence Detector System for 40 cycles. The results were analyzed using the $_2$-$^{\Delta\Delta C}{}_T$ method (20).

Western Blot analysis

Human ovarian, breast, prostate and neuroblastoma cell lines were washed and collected in cell extraction buffer (Invitrogen) supplemented with 1mM PMSF and protease inhibitor cocktail for 30 mins on ice with vortexing at 10 min intervals. Equal protein aliquots (40 μg) were resolved on SDS-PAGE and transferred on to nitrocellulose membrane for 90 min at 4° C. The membranes were blocked and incubated with specific antibodies: anti-sGC α_1 polyclonal antibody at 1:2000 dilution, anti-sGC β_1 1:3000 or anti-β actin (1:5000) dilution for 2 h (most) or 30 min (β-actin) at room temperature. Proteins were detected with HRP-conjugated secondary antibodies (Goat-anti-rabbit or mouse HRP conjugate) and visualized by enhanced chemiluminescence.

sGC activity assay

sGC activity was measured by the formation of (α-^{32}P) cGMP from [^{32}P] GTP as described previously (21) The reaction volume (100 μl) contained 50 mM TEA (pH7.4) 1mM EDTA, 10 mM IBMX, 1 mg/ml BSA, 2 mM GTP (1-2×10^5 cpm), 5 mM phosphocreatine, 50 μg/ml phosphocreatine kinase, 3 mM $MgCl_2$ and 1 mM cGMP. BE-2 neuroblastoma cells were used as a positive control for sGC activity. In some cases, DEA-NO (200 μM) and BAY41-2272 (5 μM) were used to stimulate the activity. BAY41-2272 stocks were prepared in DMSO but the

final concentration of the DMSO in the reaction mixture did not exceed 0.1%. DEA-NO has a half life of ($t_{1/2}$) 2 mins and 16 mins at 37° C and 25° C respectively (22).

cGMP assay in human cancer cells

Cells grown in 12-well plate were washed 3 times with Dulbecco's PBS (DBPS) (0.5 ml/well) and exposed to 0.5 ml/well of DPBS containing 1 mM IBMX for 10 min. BAY 41-2272 (1μM) was added together with IBMX for 10 min preincubation. Activators of particulate GC (ANP, BNP, CNP, STa: 1 μM each) or soluble GC (NOC-22:100 μM) were then added and cGMP accumulation was determined as described (23). Data were expressed in pmol cGMP/mg protein. NO donor (NOC-22 or SPER/NO) has a half life of ($t_{1/2}$) of 39 mins at 37° C and 230 mins at 25°C. Based on our previous studies (23), 100 μM of SPER/NO were used to stimulate cGMP production.

MTT assay

The effects of NO donors, NOS inhibitor, sGC activators/inhibitors, PDE inhibitor and cGMP analog were determined by a modified MTT assay. Briefly, human breast (MDA-MB-468 and SK-Br-3), ovarian (A2780, OVCAR-3 and SK-OV-3) and prostate (LNCaP and DU-145) cancer cells were plated into 96 well plates for 24 h at 37° C. The stock solutions of NOC-18 (slow release NO donor), BAY41-2272 or YC-1, (sGC activators), ODQ (sGC inhibitor), IBMX (non specific phosphodiestrase inhibitor), 8 bromo-cGMP or other agents were diluted in media or DMSO and the cells were exposed to various drug concentrations for 48-72 h at 37° C in presence (IBMX) or absence (most agents) of serum. Then, 50 μl aliquots of MTT solution (3 mg/ml) were added to each well. The media was removed and replaced with 50 μl of 100% DMSO to dissolve the formazan crystals with agitation for 5-10 mins on a shaker. The % growth inhibition values were determined as described earlier (24). DETA/NO or NOC-18 has a half life of ($t_{1/2}$) 20 hours in aqueous solution (25).

Apoptosis Analysis

Oligonucleosome release into the cytoplasm was assayed using Cell Death detection ELISA^{PLUS} kit (Roche Diagnostics). Briefly, the cells were exposed to various concentrations of NOC-18, BAY41-2272 and the combination of the two for 48 h at 37° C in serum free media. The cell lysis and ELISA was conducted according to the manufacturer's suggested protocol.

Results

mRNA expression of soluble guanylyl cyclase subunits and other components of the NO pathway in various cancer cells

We initially examined the mRNA expression of different subunits of sGC, various isoforms of NOS and PKG in human ovarian, breast and prostate cancer cell lines. Our results demonstrate that the expression of sGC α_1 mRNA in PC-3 and LNCaP prostate cancer cells was 30 and 5000 fold greater compared to DU-145 prostate cancer cell line respectively (Figure 1A & 1B). DU-145 cell line which is known to exhibit low expression of both sGC subunits (26) was used as a negative control in this study and assigned an arbitratory Unit of 1. mRNA expression of sGC α_1 subunit ranged from 2 to 200 fold in (SK-OV-3 -108 fold, OVCAR-3-125 fold, C-2/HEY 2-fold and A2780-200 fold) in ovarian cancer cell lines. Similarly, MDA-MB-468 breast cancer cells exhibited ~ 2100 fold greater sGC α_1 mRNA levels compared to SK-Br-3 cells which showed very low expression of sGC α_1 subunit. Jurkat (T-cell leukemia) cells also demonstrated (540 fold) greater mRNA expression relative to DU-145 cells. Similarly, sGCβ_1 expression of OVCAR-3, SK-OV-3, MDA-MB-468 and Jurkat was 18, 50, 35 and 14- fold greater respectively. The expression of sGC α_2 ranged from 29-480 fold greater (OVCAR-3; 480 fold, SK-OV-3; 116 fold, LNCaP; 97 and A2780; 29 fold) and that of sGC

β_2 from 4.6-14 fold (SK-OV-3; 14 –fold, OVCAR-3 5-fold, PC-3; 5-fold and SK-Br-3; 6 fold) compared to DU-145 cells (Figure 1C and 1D).

Among the cell lines examined by us, only PC-3 (prostate), OVCAR-3 and SK-OV-3 (ovarian) cells demonstrated 700 and 7000-8000 fold greater NOS-1 mRNA expression compared to DU-145 cells. (Figure1E). All the breast cancer cell lines tested showed undetectable NOS-1 mRNA expression and similarly out of ovarian, breast and prostate cell lines tested, only A2780 a human ovarian cancer cell line showed PKG1 mRNA(1800-2000 fold) expression. NOS-2 and NOS-3 mRNA expression was undetectable in the tested cell lines (data not shown).

Western blot and sGC activity analyses in human breast and ovarian cancer cells

Compared to BE-2 cells (21) used as a positive control in this study, only OVCAR-3, HEY/C-2, A2780 and, MDA-MB-468 cancer cell lines (Figure 2A) showed varying levels of sGC α_1 protein. Similarly, OVCAR-3, MDA-MB-468, and BE-2 showed strong positive levels of sGC β_1 protein. HEY/C-2 and SK-Br-3 showed intermediate levels whereas ovarian cancer cell lines SK-OV-3, A2780 and prostate cancer cell line PC-3 and LNCaP showed low levels of sGC β_1 protein.

We then analyzed the sGC activity in various cancer cell lines by measuring the conversion of [α-^{32}P] GTP into [^{32}P] cGMP. Our results indicate that out of 9 cell lines tested (ovarian, breast and prostate) only three cell lines demonstrated detectable sGC activity. sGC activity was detected in positive control BE-2 as well as in human ovarian cell lines OVCAR-3, A2780 and human breast cancer cell line MDA-MB-468 (Figure 2B).

Basal cGMP levels and accumulation of cGMP in breast and ovarian cancer cells

To further explore the functional aspects of sGC and pGC, we plated the cancer cells in 12 well plates (at the confluence of 80-90%) and measured basal levels of cGMP production and its accumulation in response to NO donors and activators of sGC or particulate GC isoforms. Our results demonstrate that compared to SK-OV-3 (2 fold increase with NO donor, 6.7 fold increase with BAY41-2272 and ~ 13 fold increase with combination of NO donor + BAY 41 2272), OVCAR-3 human ovarian cancer cell line showed 5 fold stimulation with NO donor, 43 fold stimulation with allosteric sGC activator BAY41-2272. Combination of the two (NOC-22+BAY 41-2272) showed an additive effect (~ 50- fold) in stimulation of cGMP levels. Similarly, in contrast to SK-Br-3 (5.4 fold increase with CNP and 4.3 fold increase with NOC-22+BAY41-2272), MDA-MB-468 exhibited ~ 92 fold increase with NOC-22, ~ 8-fold increase with BAY41 2272 in cGMP levels. However, combination of NOC-22 and BAY41-2272 showed synergistic increase (1250 fold increase) in cGMP accumulation in MDA-MB-468 breast cancer cells ((Figure 3A-3D). These results demonstrate differential response of NO donors, sGC and pGC activators in stimulation of cGMP production in various cancer cell lines.

Growth inhibition and apoptosis with activators of NO-cGMP pathway in human breast, prostate and ovarian cancer cells

To study the effect of activators of the NO pathway (Figure 4A-4D) on growth of breast cancer cells, we exposed the cells (MDA-MB-468, SK-Br-3, OVCAR-3 and PC-3) to either slow release NO donor NOC-18, NO-independent and heme-dependent sGC activator BAY41-2272 or the combination of the two for 48 h at 37° C in serum free conditions.

Our results demonstrate that NOC-18 (25-100 μM) showed a dose-dependent growth inhibition in MDA-MB-468, SK-Br-3, OVCAR-3 and PC-3 cells respectively. sGC activator BAY41-2272 was slightly more effective in inhibiting the growth of most of the cancer cells. Combination of NOC-18 and BAY41-2272 showed modest increase over either NOC-18 or

BAY41 2272 alone (3-8% for SK-Br-3 and 0.5-5.5% for MDA-MB-468) in the growth inhibition (44%-65% for MDA-MB-468 and 61%-68% for SK-Br-3) of cancer cells. However, compared to BAY 41 alone, lower concentrations of NOC-18 and BAY 41(12.5+12.5 μM) showed 9-12% increase in mediating the growth inhibition of OVCAR-3 and SK-Br-3 cells. In contrast, NOC-18 alone (12.5 μM conc.) had no effect on the growth of these cells suggesting that the inhibition may be mediated by cGMP-independent manner (Figure 4A-4D). In addition, cGMP analog (8-bromo-cGMP) caused a dose-dependent growth (30-57% growth inhibition at 4 mM concentration) in various cancer cell lines tested with highest inhibition observed in SK-OV-3 and Sk-Br-3 (Figure 4E-4F) cells. Whereas, BAY41 2272 alone exhibited 10-20 % more growth inhibition in SK-OV-3 or DU-145 (low sGC expression) compared to MDA-MB-468 or OVCAR-3 (high sGC expression and activity) cells (Figure 5A).

Our results with non specific phosphodiesterase inhibitor IBMX demonstrates an IC_{50} of 2.5-2.8 mM for OVCAR-3, 1.6-2.2 mM for SK-OV-3 and 1.8-2.5 mM for BE-2 neuroblastoma cell line (positive control for sGC expression and activity). The IC_{50} for MDA-MB-468 was 1.2-1.4 mM and SK-Br-3 was in 1.4-1.8 mM range. These results demonstrate no significant difference in the IC_{50} of IBMX against high or low sGC expressing cancer cells (Figure 5B). However, the IC_{50} values of IBMX in various cell lines were slightly lower in serum free conditions.

In addition our results also indicate that compared to NOC-18 and BAY41-2272, various combinations of the two were able to induce modest but significant DNA fragmentation (4-8%) compared to DMSO in MDA-MB-468 (high sGC expression and activity) breast cancer cells (Figure 5C). In contrast, in SK-Br-3 (low sGC expression) cells both BAY41 and combination of NOC+BAY41 showed some DNA fragmentation (5D). Collectively, these results demonstrate that both cGMP-independent and -dependent pathways may be involved in growth inhibition and apoptosis mediated by these agents.

Effect of inhibitors/activators of NO signaling pathway on ERK phosphorylation

Previous studies have shown that low concentrations of NO (<50 nM) induce ERK phosphorylation which in turn can increase proliferation of some of the tumor cells. Therefore we examined the effect of NO donor, NO-independent allosteric sGC activator or sGC inhibitor on phosphorylation of ERK1/2. The cancer cell lines (MDA-468, SK-Br-3, OVCAR-3, SK-OV-3 in serum free media) were exposed to the slow release NO donor NOC-18 (50 μM), allosteric sGC activator BAY41-2272 (5 μM), sGC inhibitor ODQ (20 μM) or the combination of two or three for 18 hours at 37° C. The cells were stimulated with 100 ng/ml of EGF for 10 mins and the samples were resolved on SDS-PAGE for western analyses. The membranes were probed with phosho-specific ERK1/2 and total ERK antibodies and proteins were detected with HRP-conjugated secondary antibodies. Our results demonstrate that BAY41-2272 (sGC activator) or the combination of NOC18+BAY41-2272 inhibited the basal phosphorylation of ERK1/2 in OVCAR-3 (human ovarian cancer cells with high sGC expression and activity) cell line. However, ODQ (sGC inhibitor) was unable to rescue this inhibition. In contrast, in SK-OV-3 cells (undetectable-low sGC activity) modest inhibition of basal ERK phosphorylation was observed with combination of NOC-18+BAY412272 and ODQ, and ODQ rescued the NOC+BAY induced inhibition of ERK phosphorylation. We observed similar effect of various agents in SK-Br-3 (low sGC activity) cells, whereas MDA-MB-468 (high sGC activity) did not show any effect with NOC-18, BAY41-2272, combination of the two or ODQ on ERK phoshorylation (Figure 6A-6D). These results indicate that the drug induced inhibition of ERK phosphorylation may be mediated by a cGMP independent manner. Table 1 summarizes the results of the current manuscript.

Discussion

Previous studies have demonstrated that nanomolar concentrations of NO can increase the proliferation of tumor cells by activation of cancer promoting pathways such as phosphorylation of ERK via a sGC-dependent mechanism and by accumulation of hypoxia-inducible factor HIF. In contrast, intermediate levels (> 300 nM) and micromolar concentrations of NO can cause p53 phosphorylation which could also lead to apoptosis of tumor cells (10). NO has also been shown to inhibit cell motility and invasion by inducing tumor suppressor maspin in breast cancer cells (27). sGC which functions as a NO sensor has been shown to promote angiogenesis in endothelial cells (14) whereas, a nonspecific sGC inhibitor LY83583 inhibits tumor cell growth by induction of a cdk inhibitor p21 in a p53-independent manner in colorectal, breast and malignant melanoma cell lines (28). Similarly another sGC inhibitor ODQ has been shown to exert anti-tumor effect with prostate cancer cell lines in a cGMP independent manner (29). Additionally, sGC inhibitors NS2028 and ODQ and the PKG inhibitor KT 5823 have been reported to induce apoptosis of immortalized uterine epithelial cells thereby demonstrating the possible role of sGC and PKG in cell death. (30). Similarly Pervin et al (31) have shown that NO (depending on the concentration) can induce cell proliferation, cytostasis or apoptosis of breast cancer cell lines by modulating various cell cycle proteins, phosphatases (MKP-1) and kinases (ERK1/2). In addition, these investigators utilizing DETA- NONOate have demonstrated that low concentrations of NO induces proliferation in some human breast cancer cell lines, whereas higher concentrations lead to apoptosis and that both of these effects are cGMP- independent (25,32-33).

Therefore to further explore this dichotomous role of NO signaling components in proliferation/cell death of tumor cell lines, we evaluated sGC expression, activity and cGMP production and effect of various activators and inhibitors of the pathway on proliferation and apoptosis of various tumor cell lines. Our studies demonstrate differential expression of various sGC subunits, activity and sensitivity of cells to various activators and inhibitors of the pathway and inhibition of ERK phosphorylation independent of endogenous sGC status. Robust mRNA expression of NOS-1 gene was observed in SK-OV-3 (low sGC expression), OVCAR-3 (high sGC expression) human ovarian cancer and a PC-3 (low sGC expression) prostate cancer cell line. Similarly, out of 9 cell lines tested only A2780 human ovarian cancer cell line showed PKG1 mRNA and protein levels (data not shown). The significance of presence or absence of PKG I in tumor cell lines in our study is being further evaluated.

We propose that it is important to evaluate the expression of various components of NO-cGMP pathway to further study their role in regulation of tumor cell proliferation. Our previous studies with mouse (34) and human (35) ES cells demonstrate very low basal mRNA and protein levels of sGC α_1, α_2 and β_1, NOS-2 and NOS-3 in undifferentiated stem cells. However, there was a temporal induction of these components in differentiated stem cells. Based on the studies presented in this manuscript and a study conducted in our laboratory few decades ago (36) demonstrating the increased activity of particulate and decreased activity of soluble GC in regenerating and fetal liver and heptoma, we hypothesize that the cancer cells with low expression and activity of sGC may have higher fraction of cancer initiating cells compared to the cancer cells with high expression and activity of sGC. Therefore, we postulate that loss of NO receptor sGC can be used as a biomarker for aggressive tumors. Additional studies are ongoing to further validate this concept in various breast, ovarian and prostate cancer models.

Additionally, our recent studies with mouse and human embryonic stem cells indicate that NO donors and sGC activators combined induce enhanced differentiation of ES cells into myocardial cells than either of the two agents separately (37). Since stem cells and cancer cells share some common characteristics, such as self renewal and expression of stem cell markers such as Oct3/4, Sox-2, nanog (38), we were interested in examining the effect activators and

inhibitors of NO signaling pathway in various cancer cell models. In the current study, we demonstrate that sGC activator alone or combined with NO donor inhibits the tumor cell proliferation and ERK phosphorylation in some ovarian and breast cancer cell lines. In addition cGMP analog 8-bromo cGMP induced growth inhibition in some ovarian, breast and prostate cancer cell lines. A great number of previous studies have shown that another NO-independent and heme-dependent sGC activator YC-1, which is also hypoxia-inducible factor (HIF) inhibitor, is able to induce cell cycle arrest and apoptosis of many tumor cells *in vitro* and *in vivo in* human xenograft models. (39-40). However, the role of another potent sGC activator BAY41-2272 used in this study has not been explored in cancer therapy alone or in combination with other chemotherapeutic drugs. Therefore, we evaluated the efficacy of this inhibitor in our cancer cell studies and concluded that similar to YC-1, BAY41 2272 may have additional properties responsible for growth inhibition and apoptosis of cancer cells.

Although all the studies presented here were conducted in *in vitro* cell culture conditions, the role of tumor microenvironment *in vivo* in determining the tumor cell phenotype can not be ignored. Number of previous studies have show that the behavior of tumor cells *in vivo* is very different compared to the cells grown *in vitro* in tissue culture and one of the most important determinant of this phenomena is the tumor microenvironment (41-42). Tumor microenvironment is mainly comprised of stromal cells, activated fibroblasts and cells of immune system and it is the cross talk between different cell types that defines the tumor microenvironment (41-42). In epithelial tumors critical stromal elements include cancer activated fibroblasts which secrete number of growth factors and chemokines responsible for enhancing cell proliferation and invasion (43). It has also been shown that some cells of immune system such as tumor infiltrating lymphocytes and tumor associated macrophges are reprogrammed to inhibit physiological lymphocyte function through release of cytokines such as IL-10, prostaglandins and reactive oxygen species (ROS) (42). Similarly, myeloid suppressor cells in murine system have been shown to induce tumor progression by induction of iNOS and arginase 1, enzymes involved in L-arginine metabolism and NO production. Inflammatory cells present in the tumor microenvironment are largely thought to promote tumor progression by down regulating local and systemic antitumor activity by number of mechanisms. Additional factors in tumor milieu are hypoxic environment and induction of hypoxia responsive genes which lead to hyper production of ROS and induction of NFkB pathway. NFkB activation leads to the secretion of TNF-α and other proinflammatory cytokines which in turn induce tumor cell proliferation. Therefore, inhibition of NFkB signaling using TNF antibodies and targeting NFkB in tumor microenvironment has been proposed to represent strategy for arresting tumor growth. Our future studies will focus on evaluating the role of activators and inhibitors of NO signaling pathway in various nude or SCID breast and ovarian xenograft models to study molecular mechanisms and elucidate the efficacy of these cardiovascular drugs in cancer.

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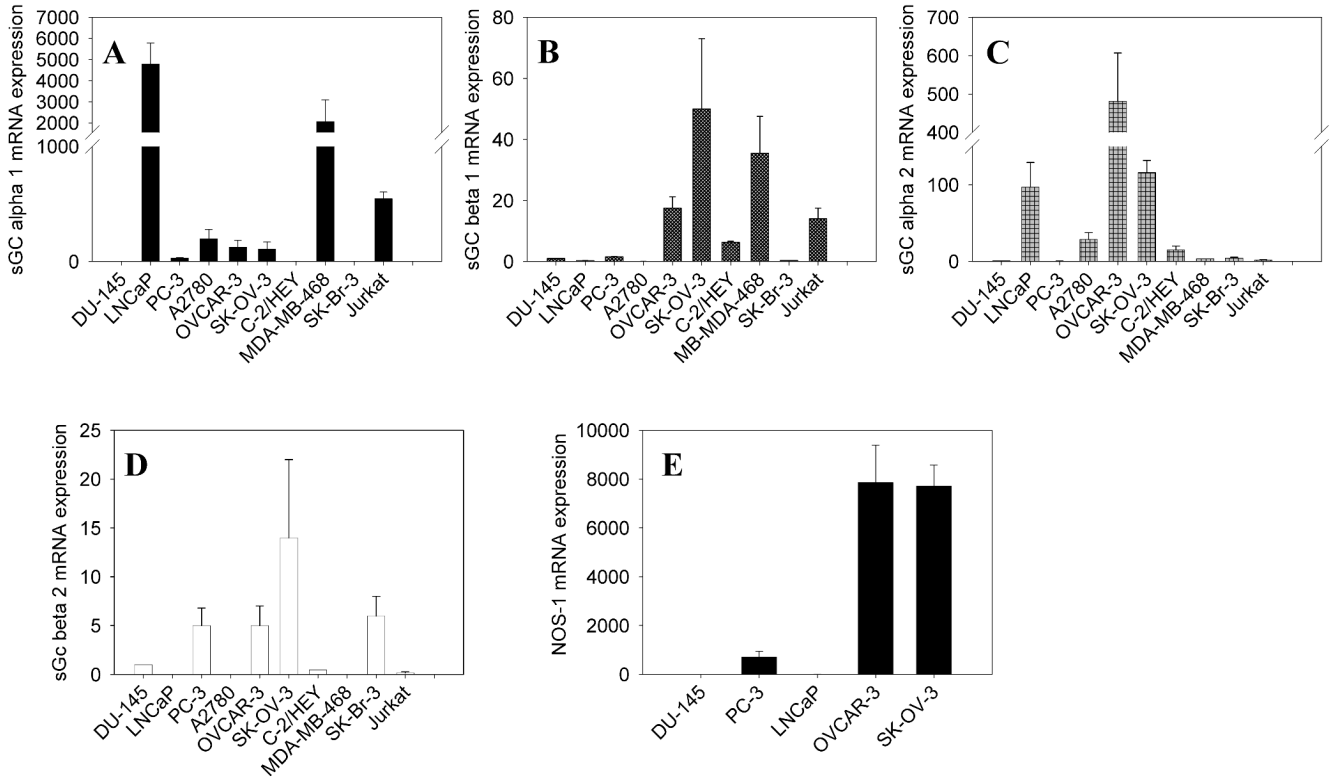

FIGURE 1. mRNA expression of sGC and NOS-1 in human cancer cell lines

mRNA levels for indicated genes (sGC α_1- A; sGC β_1- B; sGC α_2-C; sGC β_2-D and NOS-1-E) in various cancer cells were analyzed by real-time PCR and normalized using the housekeeping gene GAPDH and presented as fold expression compared to prostate cancer cell line DU-145. The data were analyzed using the $_2^{-\Delta\Delta C}{}_T$ method. Error bars indicate ± SEM. n= 6

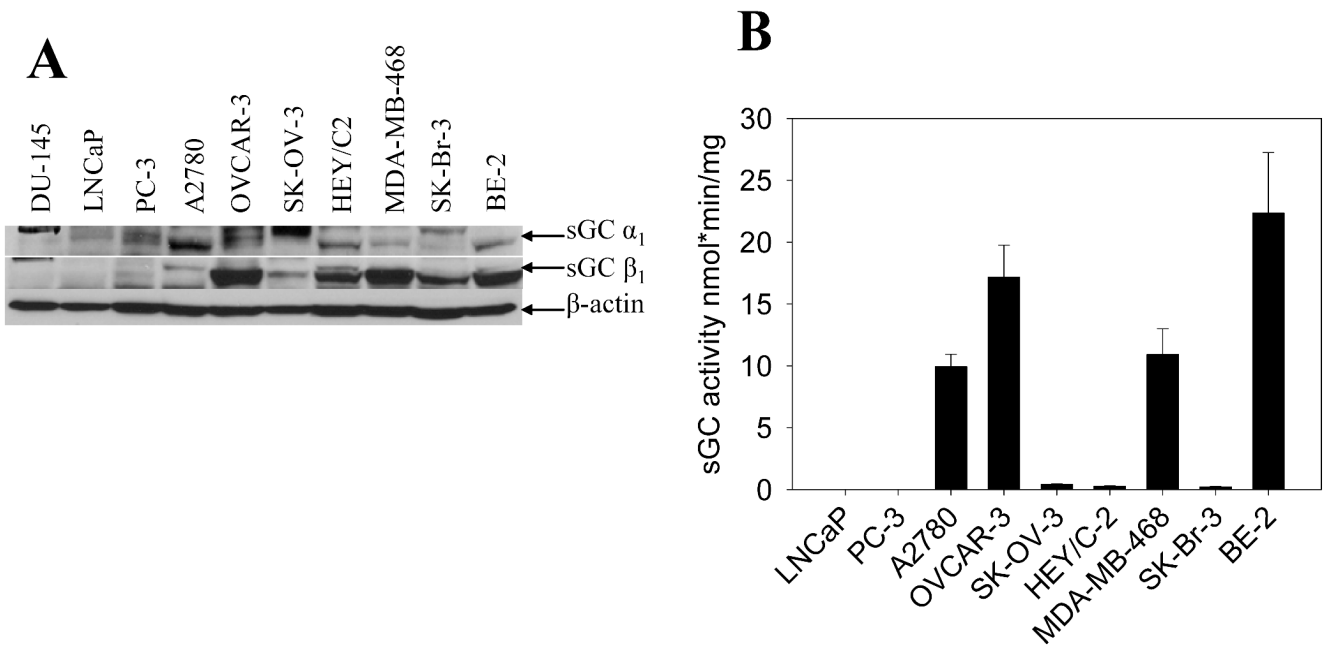

FIGURE 2. Protein levels and sGC activity in various human cancer cell lines

(A) Protein extracts from breast (SK-Br-3, MDA-MB-468), ovarian (SK-OV-3, OVCAR-3, C-2/HEY, A2780), prostate (LNCaP, DU-145, PC-3) and BE-2 (neuroblastoma) were tested with specific antibodies against sGC α_1, sGC β_1 and β-actin using western-ECL analysis. (B) sGC activity was determined in various DEA-NO treated lysates from cancer cell lines. Data are presented as mean ± SD of three independent experiments.

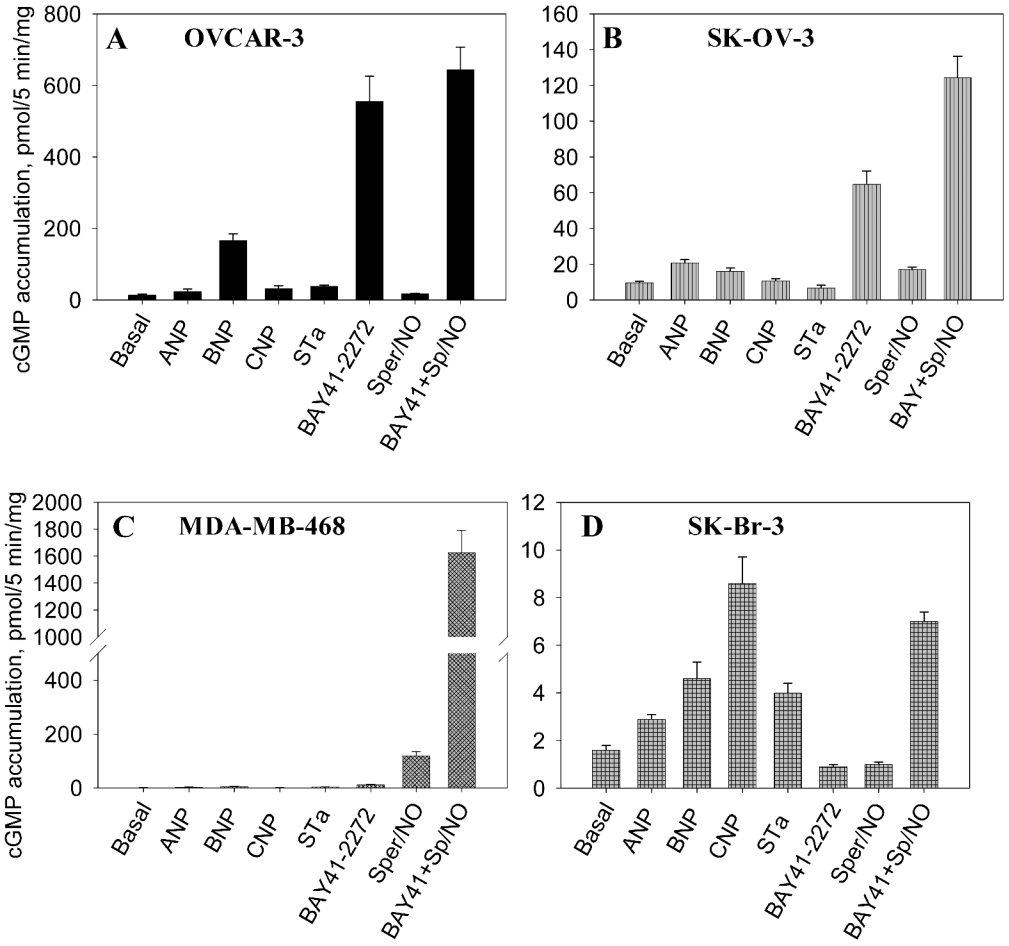

FIGURE 3. cGMP accumulation in cancer cells

Cancer cells were washed and exposed to 0.5 ml/well of DPBS containing 1 mM IBMX and BAY41-2272 (1μM) for 10 min followed by the addition of activators of particulate GC (ANP, BNP, CNP, STa-1μM) or soluble GC (NOC-22-100 μM). As described soluble GC is activated by NO and particulate are bunched together (GCA-ANP, BNP, GCB-CNP, GCC-STa). cGMP levels were assayed by ELISA and the data were expressed in pmol cGMP/mg protein. Data are presented as mean ± SEM of two independent experiments (n=6).

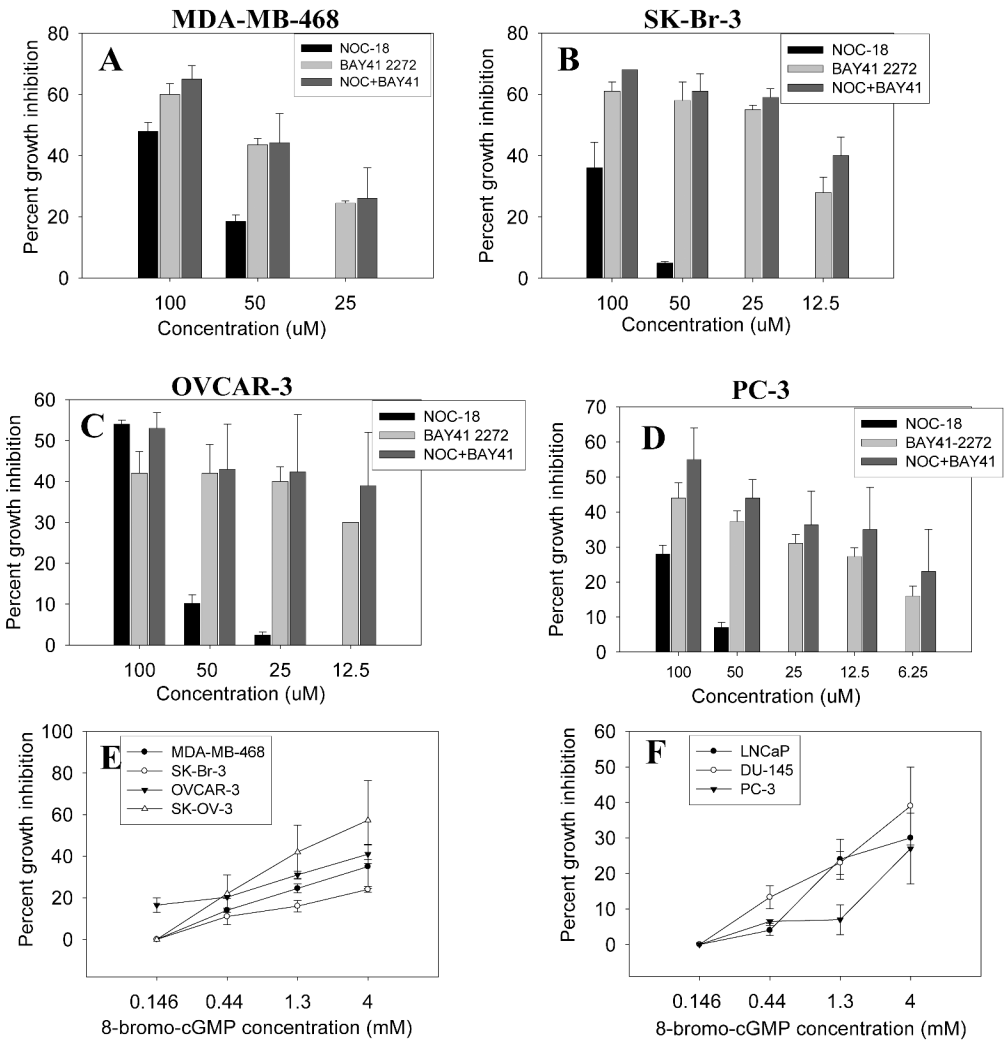

FIGURE 4. Growth inhibition with activators/Inhibitors of NO-cGMP

Human breast (MDA-MB-468 and SK-Br-3), ovarian (OVCAR-3 and SK-OV-3), prostate (LNCaP-3, PC-3 and DU-145) and neuroblastoma (BE-2) cells were exposed to different concentrations of NOC-18, BAY41-2272, NOC+BAY41, or 8-bromo-cGMP for 48 h in serum serum free media and growth inhibition was monitored using MTT assay. Error bars indicate ± SEM. n= 8-12 (A-F).

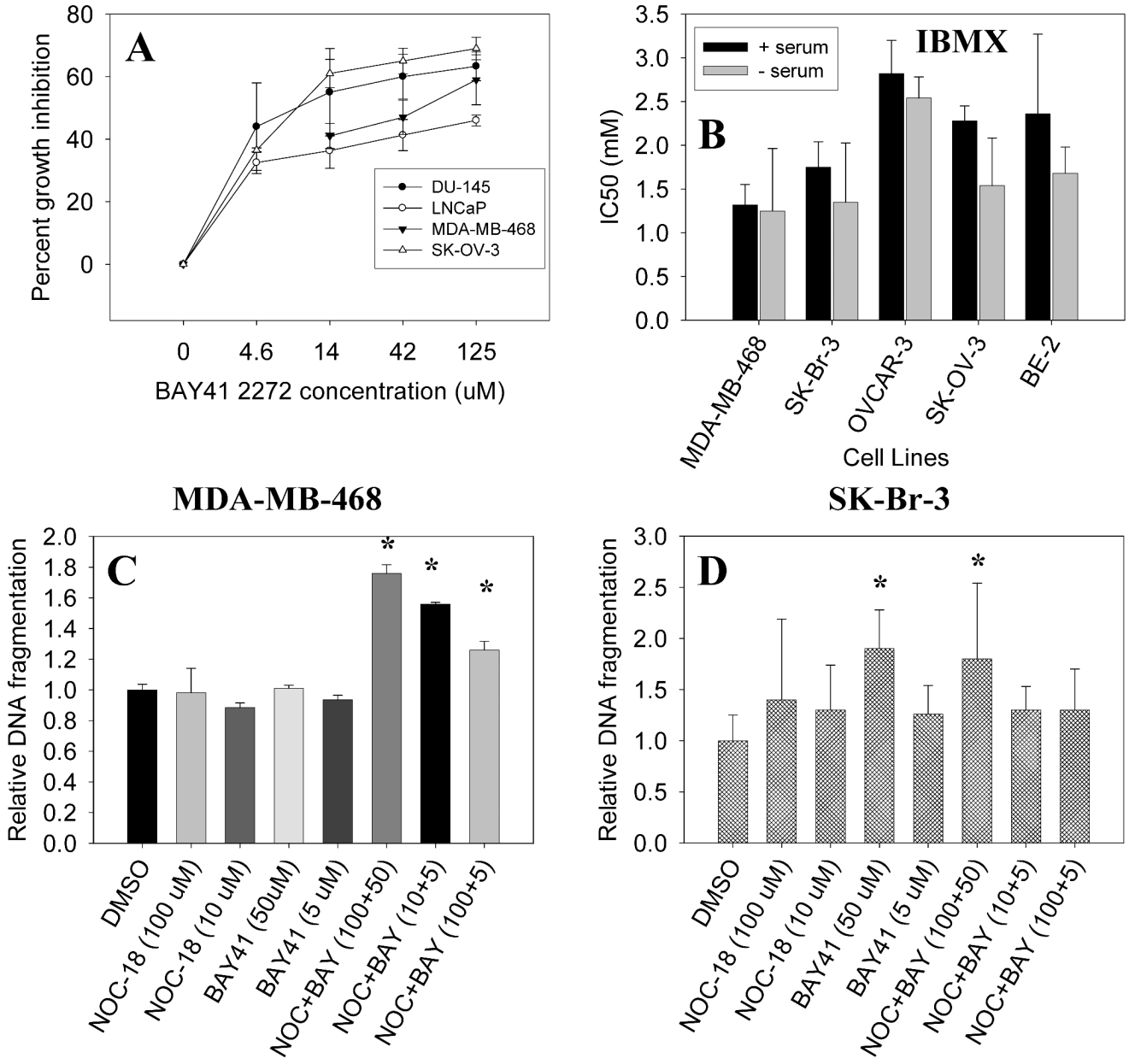

FIGURE 5. Growth inhibition and apoptosis with activators/Inhibitors of NO-cGMP

Human breast (MDA-MB-468 and SK-Br-3), ovarian (OVCAR-3 and SK-OV-3), prostate (LNCaP-3, PC-3 and DU-145) and neuroblastoma (BE-2) cells were exposed to different concentrations of BAY41-2272 or PDE inhibitor IBMX for 48 h in serum containing (dark bars) or serum free media (light bars) and growth inhibition was monitored using MTT assay. Data are presented as mean ± SEM. n=8-12 (A-B). Human breast cells (MDA-MB-468 and SK-Br-3) were exposed to different concentrations of NOC-18, BAY41-2272 or the combination of the two for 48 h in serum free media and oligonucleosome release in the cytoplasm was measured with Cell Death detection ELISA^{PLUS} kit (Roche Diagnostics). Error bars indicate ± SEM. n= 6. Significance using paired Students t-test is indicated: * $p < 0.05$ (C-D).

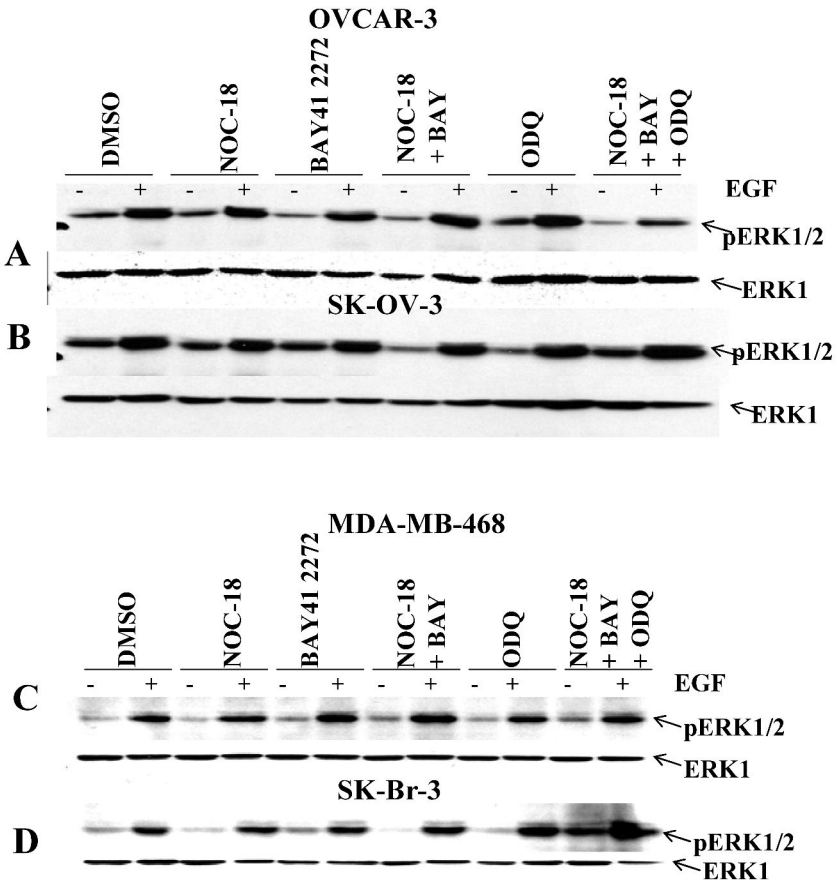

FIGURE 6. Effect of activators and inhibitors of NO-cGMP pathway on ERK phosphorylation

The cells (OVCAR, SK-OV-3, MDA-MB-468 and SK-Br-3) were exposed to the indicated compounds for 18 h at 37° C. Equal amount of protein (40 μg) was resolved on SDS-PAGE and membranes were probed with phospho-specific ERK and total ERK antibodies and proteins were visualized by ECL.

Table 1

Expression of various subunits of sGC and NOS and effect of activators/inhibitors NO-cGMP pathway In tumor cell proliferation

	RNA					Western		Activity	cGMP	Growth Inhib						
Cell Lines	**sGCα1**	**sGCβ1**	**sGCα2**	**sGCβ2**	**NOS-1**	**sGCα1**	**sGCβ1**	**sGC**	**Sp**	**BAY41**	**S+B**	**NO**	**BAY**	**N+B**	**8BG**	**IBMX**
DU-145	-	-	-	-	-	-	-		ND	ND	ND	ND	+	ND	+	ND
LNCaP	+	-	+	-	-	±	-	-	ND	ND	ND	ND	+	ND	+	ND
PC-3	-	-	-	+	+	±	+	-	ND	ND	ND	ND	ND	ND	+	ND
A2780	+	-	±	-	-	+	+	+	-	-	-	ND	ND	ND	ND	ND
OVCAR-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SK-OV-3	±	+	+	+	+	-	+	-	+	+	+	ND	+	ND	+	+
C-2/HEY	-	±	±	-	ND	+	+	-	ND	ND	ND	ND	ND	ND	ND	ND
MDA-MB-468	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
SK-BRr-3	-	-	-	+	-	-	+	-	±	±	±	+	+	+	+	+
Jurkat	+	+	-	-	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND
BE-2	ND	ND	ND	ND	ND	+	+	+	+	+	+	ND	ND	ND	ND	+

ND= not determined