PKCα/β I Inhibitor Go6976 Induces Dephosphorylation of Constitutively Hyperphosphorylated Rb and G_1 Arrest in T24 Cells

V. AALTONEN[1,2] and J. PELTONEN[2]

Departments of [1]Ophthalmology and [2]Anatomy, University of Turku, 20520 Turku, Finland

Abstract. *Background: Rb functions as a key controller of the G_1-S transition of the cell cycle, and its inactivation leads to a defective G_1 checkpoint. Bladder cancer frequently displays alterations in Rb such as constitutive hyperphosphorylation which results in inactive Rb and progression of cells to the S-phase. Several protein kinase C (PKC) inhibitors are currently undergoing clinical trials as anticancer drugs. Materials and Methods: T24 urinary bladder carcinoma cells, known to express hyperphosphorylated Rb, were treated with PKCα/βI inhibitor Go6976. The treated cells were subjected to cell cycle analysis, cell growth assay and Western blots for Rb and cdc2 phosphorylation. Results: The treatment resulted in Rb dephosphorylation at Ser 795 and Ser 807/811, and cdc2 dephosphorylation at Tyr15. Subsequent $G_{0/1}$ arrest and reduced proliferation rates were observed. Conclusion: The results show that Go6976 can be used to restore constantly hyperphosphorylated and therefore constantly inactive Rb function in cancer cells.*

Protein kinase C (PKC) family members are classified into three major groups: classical (α, βI, βII, and γ), novel (δ, ε, η and θ) and atypical (μ, ζ and ι). Activation of classical isoenzymes depends on calcium and phospholipids; novel enzymes are activated by phospholipids; and atypical enzyme activation occurs independently of calcium or phospholipids (1, 2). PKCα has been linked to cancer progression because it increases cell proliferation and migration, and inhibits apoptosis (3-7). PKC β has also been associated with an increased proliferation rate of cancer cells (8-10).

Previous studies have demonstrated that PKCα, -β, and -δ are the predominant isoenzymes in the normal epithelium of the urinary bladder. Furthermore, transitional cell carcinomas (TCCs) commonly display down-regulation of PKC β and δ, and increased expression of PKCα (11-13). Increased PKCα and βI expression and activity are associated with the high proliferation rate in TCC tumours (14).

Cancer cells may harbor defective cell cycle checkpoints resulting in uncontrolled growth and increased probability for new mutations. Cell cycle checkpoint defects are often caused by alterations in tumor suppressor genes such as Rb and p53. Mutations of p53 have been associated with ~50% of cancers, including urinary bladder carcinoma (15, 16). In addition, bladder cancers frequently display alterations in Rb, such as loss of expression, and interestingly, hyperphosphorylation, resulting in non-functional Rb (17).

Rb is regulated through phosphorylation by various signaling pathways, such as p53. Dephosphorylated Rb is bound to the S-phase-promoting E2F family transcription factors inhibiting progression to the S-phase (18). In contrast, the G_2 checkpoint is controlled by the mitosis-promoting factor (MPF), composed of activated cdc2/cyclin B1 complex. Activity of cdc2 is in part controlled by activating dephosphorylation on Tyr15 by active cdc25C. The PKCα/βI inhibitor Go6976 can induce MPF activation and mitosis in 5637 TCC cells. Furthermore, this feature can be exploited to overcome the resistance of cancer cells to paclitaxel (19).

Increasing evidence suggests that PKC inhibitors are effective chemotherapeutic agents. Non-specific and isoenzyme specific PKC inhibitors have been shown to exert various favorable effects in experimental cancer treatment, including growth arrest, apoptosis, and inhibition of invasion. UCN-01, a

Abbreviations: BSA, bovine serum albumin; cdc, cell division cycle; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; FACS, fluorescence activated cell sorting; FCS, fetal calf serum; HRP, horse radish peroxidase; MPF, mitosis promoting factor; PBS, phosphate-buffered saline; PKC, protein kinase C; PVDF, polyvinylidene difluoride; TCC, transitional cell carcinoma; Rb, retinoblastoma protein.

Correspondence to: Juha Peltonen, University of Turku, Institute of Biomedicine, Department of Anatomy, Kiinamyllynkatu 10, FI-20520 Turku, Finland. Tel. +35823337350, Fax. +35823337352, e-mail: juha.peltonen@utu.fi

Key Words: Go6976, protein kinase C, retinoblastoma, bladder, carcinoma.

wide range kinase inhibitor which inhibits PKC among others, has been shown to cause an accumulation of cells in the G_1 phase of the cell cycle and induction of apoptosis, but the molecular mechanism as an anticancer agent remains elusive (20). Recent evidence suggests that experimental cancer therapies with PKCα inhibitors are more effective than non-specific inhibitors. PKC isoenzyme specific inhibitors such as Go6976 (PKCα and -βI inhibitor), Safingol (PKCα inhibitor), and ISIS 3521 (PKCα antisense oligonucleotide) have proven to be effective anti-cancer drugs in cell cultures and animal models (21-27). PKC alpha and betaI inhibitor Go6976 induces a rapid translocation of various cell-cell and cell-matrix junction proteins and dramatically inhibits invasion and migration of urinary bladder carcinoma cells (28).

This study demonstrates that Go6976 induces Rb dephosphorylation, G_1 cell cycle arrest, growth retardation, and MPF activation in T24 cancer cells. The T24 cell line served as an excellent model of high grade TCC cells that are characterized by a *p53* mutation and inactivation of Rb due to chronic hyperphosphorylation (17).

Materials and Methods

Antibodies. Rabbit antibodies against human phospho-cdc2 (Tyr15), phospho-Rb (Ser795) (29), phospho-Rb (Ser 807/811) (30) and beta-actin (31) were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell culture. T24 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cell lines were maintained at DMEM supplemented with 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml) (DMEM plus 10% FCS). The experiments were performed without the antibiotics.

PKC inhibitor. PKCα and -βI isoenzyme inhibitor Go6976 was obtained from Calbiochem (La Jolla, CA, USA) and dissolved in DMSO (32, 33). All the control reactions were carried out with equal volumes of DMSO as in drug treatments.

FACS analysis. Flow cytometry using fluorescence-activated cell sorter (FACS) was used to study the effect of Go6976 on cell cycle. The cells were cultured in DMEM plus 10% FCS supplemented with 1 μM Go6976. The supplemented medium was changed at time points of 24 h, 48 h or 72 h. Non-treated samples served as controls and all analyses were performed in duplicate. In each time point, the cells were detached using trypsin-EDTA, combined with culture medium to collect floating cells, and subsequently centrifuged at 1000 rpm for 5 min. The pellet was washed with PBS and centrifuged twice. For propidiumiodide (PI) staining, the cells were fixed with 70% ethanol under continuous agitation, washed with PBS and centrifuged twice, finally resuspended at 10^6 cells/ml in PBS. The suspension was treated with 500 μg/ml RNAse, and incubated in 37˚C for 30 min. After incubation, the cell suspension was treated with 50 μg/ml PI for 10 min at room temperature, and immediately transferred to FACS analysis with FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). The results were analyzed and the graphs reproduced using Mod-Fit software (Venty Software House, Topsham, ME, USA).

Western transfer analysis. The cells were treated with 1 μM Go6976 or same amount of DMSO for 24 h, 48 h or 72 h. After treatment, the cells were rinsed once with PBS, lysed in boiling buffer containing 1% SDS, 10 mM Tris pH 7.4 and 1 mM sodium orthovanadate, and centrifuged at 16000 ×*g* for 5 min to remove the debris. Protein concentration was measured using DC Protein Assay (BioRad, Hercules, CA, USA) and equal amounts of protein were subjected to SDS-PAGE on 10-12% gel. The proteins were then electrophoretically transferred to a PVDF membrane and processed for immunoblotting. Membranes were first blocked with a solution containing 5% BSA/PBS and 0.05% Tween-20 and immunolabeled with anti-phospho-Rb antibodies. HRP conjugated anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA) was used as a secondary antibody and detected with ECL (Amersham Life Sciences, Little Chalfont, UK). Equal loading and transfer of each lane was evaluated with Ponceau-S staining of the membranes and by immunoblotting with beta-actin antibodies using the same samples.

Trypan blue exclusion. A total of 1×10^5 cells were plated on 6-well dishes and allowed to proliferate for 2 days. All samples were prepared in triplicate. The cells were treated with 1 μM Go6976 for 0 h, 24 h, 48 h, or 72 h, and detached with Trypsin-EDTA. Subsequently the cells were treated with DMEM + 10% FCS and 0.2% Trypan blue, incubated at 37˚C for 10 min, and viable cells were counted using a hemocytometer.

Results

PKCα and -βI inhibitor Go6976 induces G_1 arrest in T24 cells. Flow cytometry analysis of propidium iodide stained T24 cells were performed to test possible changes in the distribution of T24 cells in the cell cycle (Figure 1a and b). Treatment of the cells with 1 μM Go6976 induced a change in the cell cycle distribution within 24 h. During the first 24 h, a high proportion of cells arrested at $G_{0/1}$ phase of the cell cycle (Figure 1a). Specifically, the percentage of the cells in the $G_{0/1}$ phase increased from 34% to 38% (Figure 1b). The percentage of the cells in the S and $G_{2/M}$ phases decreased in the same proportion as a result from treatment with 1 μM Go6976. The accumulation of cells to $G_{0/1}$ phase continued in later time points (Figure 1a). The proportion of cells in $G_{0/1}$ phase at time points 48 h and 72 h was 45% and 60%, respectively (Figure 1b). The results show that the proportion of cells at S phase appeared to decrease at nearly all time points, from 44% to 24%. In contrast, the percentage of cells in the $G_{2/M}$ phase remained essentially the same, the percentage ranging from 16% to 24% at different time points.

PKCα and -βI inhibitor Go6976 induces MPF activation in T24 cells. To study changes in MPF activation in response to Go6976 treatment, Western transfer analysis using antibodies against phosphorylated cdc2 (Tyr15) was performed (Figure 2). The results showed that after treatment with 1 μM Go6976 for 24-72 h, cdc2 Tyr15 phosphorylation decreased. The results showed also that this activating dephosphorylation persisted up to the last time point, 72 h.

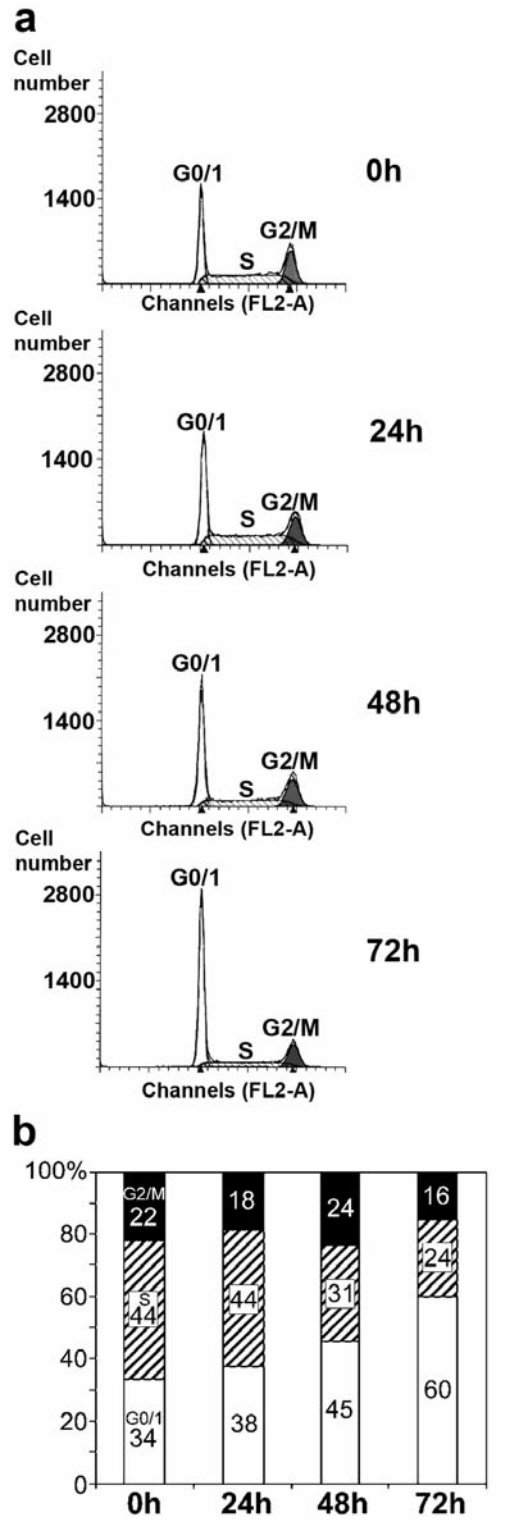

Figure 1. *Cell cycle distribution of Go6976-treated T24 cell culture. a: Cell cycle analysis of T24 transitional cell carcinoma cells labeled with propidium iodide after treatment with 1 μM Go6976 for 0, 24, 48 or 72 h. b: Cell cycle distribution is presented as percentages at each time point. The proportion of cells in the G_1 phase can be seen to increase with time.*

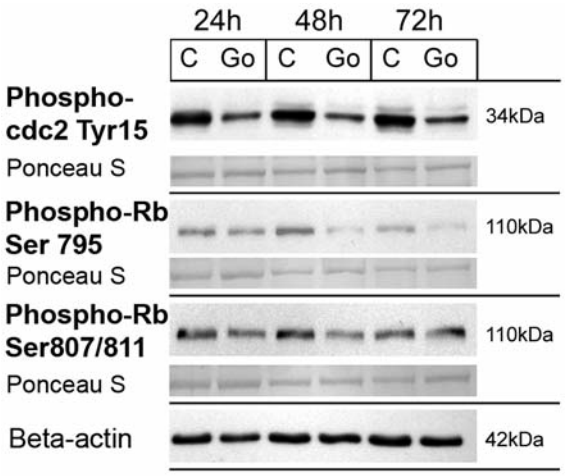

Figure 2. *Western transfer analysis of T24 cells treated with 1 μM Go6976. The levels of phosphorylated cdc2 (Tyr15), Rb (Ser 795) and Rb (Ser 807/811) were analyzed. Loading controls Ponceau-S stain of the membranes, and β-actin labeling show equal loading. Cdc2 (Tyr15), Rb (Ser 795) and Rb (Ser 807/811) are dephosphorylated at all time points.*

PKCα and -βI inhibitor Go6976 induces Rb dephosphorylation at Ser 795 and Ser 807/811. In order to study changes of the G_1 checkpoint control, Western transfer analysis using antibodies against phosphorylated Rb was performed. Two different antibodies were used: Phospho-Rb (Ser 795) and Phospho-Rb (Ser 807/811). The results showed that within 24 h both Ser 795 and Ser 807/811 were dephosphorylated in response to Go6976 treatment (Figure 2). Both sites remained in their dephosphorylated state at later time points 48 h and 72 h. The result was more prominent for the Ser 795 phosphorylation site.

PKCα and -βI inhibitor Go6976 induces growth retardation in T24 cells. Trypan blue exclusion experiments were carried out to find out whether the cell cycle arrest induced by Go6976 had an effect on cell growth. The results showed that 1 μM Go6976 treatment caused a growth retardation in T24 cells. Specifically, the absolute number of T24 cells increased within 72 h but was markedly lower (+443%) than that of non-treated (+966%). The results suggest that growth arrest occurred rather than cell death (Figure 3).

Discussion

Our results demonstrate that the function of a defective G_1 checkpoint due to Rb hyperphosphorylation in cancer cells can be activated using a pharmacological compound, Go6976, an inhibitor of PKCα and -βI isoenzymes. Go6976 induced a prominent G0/1 arrest in T24 cells and marked growth retardation. T24 urinary bladder TCC cells are known

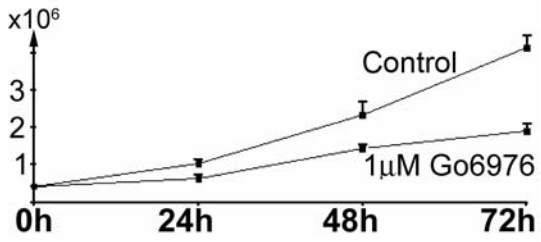

Figure 3. *Trypan blue exclusion of T24 cells treated with 1 μM Go6976. The X-axis shows the different time points 0 h-72 h and the Y-axis shows the viable cell number for each time point. Go6976 treatment at 1 μM results in prominent growth reduction when compared to controls.*

to have a defective G1 checkpoint which results from the expression of high levels of phosphorylated Rb (17). Thus, this cell line provided an excellent model to study the Rb phosphorylation status and its effect on the G_1 checkpoint and cell cycle distribution. In a previous study, T24 cells were transfected with p16 cDNA which resulted in a marked decrease in Rb phosphorylation and decreased cell proliferation (17). In the current study, we used a pharmacological compound, Go6976, which has previously been demonstrated to inhibit PKC isoenzymes α and -βI. However, this compound is not entirely PKCα and -βI isoenzyme-specific since it has been demonstrated to inhibit Chk1, JAK 2 and FLT3 kinases, and suggested to also inhibit C-TAK1, Chk2 and ATM which take part in the cell cycle checkpoint regulation (19, 34, 35). Thus, the biological changes reported in the current study must be considered as pharmacological effects of Go6976 which are not effects of PKCα and -βI inhibition alone.

The present study demonstrates that phosphorylated Rb, a key controller of the G_1 checkpoint, is dephosphorylated in response to Go6976. This is an important note since Rb hyperphosphorylation is a mechanism of cancer cells to inactivate Rb, which then causes the G_1 checkpoint to be defective. The current study shows that Go6976 may be useful in overcoming the inactivation of the G_1 checkpoint in the case of constant Rb hyperphosphorylation. Rb is phosphorylated, and thus its activity regulated by kinases associated with cyclins D and E. Cyclin D-cdk4/6 and cyclin E-cdk2 complexes are responsible for Rb phosphorylation (36-39). Thus, Go6976 may affect these kinases or kinases upstream of these.

In addition to our findings regarding the G_1 checkpoint and the phosphorylation of Rb, our results further confirm our previous findings in 5637 TCC cells. We demonstrated that Go6976 induces MPF activation and mitosis in non-synchronized, untreated 5637 cells (19). However, in contrast to T24 cells, 5637 cells are Rb null and thus incapable of G_1 arrest (40). The results of the current study suggest that MPF is also activated in T24 cells as a result of Go6976 treatment. Specifically, Go6976 induced an activating and critical dephosphorylation at cdc2. Activation of MPF (cdc2/cyclinB1 complex) induces activation of mitosis. We have previously demonstrated that non-synchronized, untreated 5637 TCC cells are forced into mitosis by Go6976. The results of the current study suggest that the cells entering mitosis as a result of the Go6976 treatment continue in the cell cycle since the percentage of the cells in the $G_{2/M}$ phase does not accumulate, while the proportion of them in S phase gradually decreases as a result of the Go6976 treatment.

In conclusion, we demonstrated that an Rb hyperphosphorylation-induced defective G_1 checkpoint can be reversed by using a PKCα and -βI isoenzyme inhibitor Go6976. Whether this phenomenon is due to the PKC-inhibiting action of this substance remains to be elucidated.

References

1 Nishizuka Y: Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science *258*: 607-614, 1992.

2 Nishizuka Y: Protein kinase C and lipid signaling for sustained cellular responses. FASEB J *9*: 484-496, 1995.

3 Aaltonen V and Peltonen J: Protein Kinase C Family. *In*: Encyclopedia of Cancer. Schwab M. (ed.). Berlin, Springer, pp. 2469-2472, 2009.

4 Mandil R, Ashkenazi E, Blass M, Kronfeld I, Kazimirsky G, Rosenthal G, Umansky F, Lorenzo PS, Blumberg PM and Brodie C: Protein kinase Cα and protein kinase Cδ play opposite roles in the proliferation and apoptosis of glioma cells. Cancer Res *61*: 4612-4619, 2001.

5 Masur K, Lang K, Niggemann B, Zanker KS and Entschladen F: High PKC alpha and low E-cadherin expression contribute to high migratory activity of colon carcinoma cells. Mol Biol Cell *12*: 1973-1982, 2001.

6 Yuspa SH, Długosz AA, Cheng CK, Denning MF, Tennenbaum T, Glick AB and Weinberg WC: Role of oncogenes and tumor suppressor genes in multistage carcinogenesis. J Invest Dermatol *103*: 90S-95S, 1994.

7 Yuspa SH: The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. J Dermatol Sci *17*: 1-7, 1998.

8 Sauma S, Yan Z, Ohno S and Friedman E: Protein kinase C beta 1 and protein kinase C beta 2 activate p57 mitogen-activated protein kinase and block differentiation in colon carcinoma cells. Cell Growth Differ *7*: 587-594, 1996.

9 Murray NR, Davidson LA, Chapkin RS, Clay Gustafson W, Schattenberg DG and Fields AP: Overexpression of protein kinase C betaII induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis. J Cell Biol *145*: 699-711, 1999.

10 Jiang XH, Tu SP, Cui JT, Lin MC, Xia HH, Wong WM, Chan AO, Yuen MF, Jiang SH, Lam SK, Kung HF, Soh JW, Weinstein IB and Wong BC: Antisense targeting protein kinase C alpha and beta1 inhibits gastric carcinogenesis. Cancer Res *64*: 5787-5794, 2004.

11 Koren R, Langzam L, Paz A, Livne PR, Gal R and Sampson SR: Protein kinase C (PKC) isoenzymes immunohistochemistry in lymph node revealing solution-fixed, paraffin-embedded bladder tumors. Appl Immunohistochem Mol Morphol *8*: 166-171, 2000.

12 Langzam L, Koren R, Gal R, Kugel V, Paz A, Farkas A and Sampson SR: Patterns of protein kinase C isoenzyme expression in transitional cell carcinoma of bladder. Relation to degree of malignancy. Am J Clin Pathol *116*: 377-385, 2001.
13 Varga A, Czifra G, Tállai B, Németh T, Kovács I, Kovács L and Bíró T: Tumor grade-dependent alterations in the protein kinase C isoform pattern in urinary bladder carcinomas. Eur Urol *46*: 462-465, 2004.
14 Aaltonen V, Koivunen J, Laato M and Peltonen J: Heterogeneity of cellular proliferation within transitional cell carcinoma: correlation of protein kinase C alpha/betaI expression and activity. J Histochem Cytochem *54*: 795-806, 2006.
15 Blanes A, Rubio J, Martinez A, Wolfe HJ and Diaz-Cano SJ: Kinetic profiles by topographic compartments in muscle-invasive transitional cell carcinomas of the bladder: role of *TP53* and NF1 genes. Am J Clin Pathol *118*: 93-100, 2002.
16 Sanchez-Carbayo M, Socci ND, Charytonowicz E, Lu M, Prystowsky M, Childs G and Cordon-Cardo C: Molecular profiling of bladder cancer using cDNA microarrays: defining histogenesis and biological phenotypes. Cancer Res *62*: 6973-6980, 2002.
17 Chatterjee SJ, George B, Goebell PJ, Alavi-Tafreshi M, Shi SR, Fung YK, Jones PA, Cordon-Cardo C, Datar RH and Cote RJ: Hyperphosphorylation of pRb: a mechanism for RB tumour suppressor pathway inactivation in bladder cancer. J Pathol *203*: 762-770, 2004.
18 Chen HZ, Tsai SY and Leone G: Emerging roles of E2Fs in cancer: an exit from cell cycle control. Nat Rev Cancer *11*: 785-797, 2009.
19 Aaltonen V, Koivunen J, Laato M and Peltonen J: PKC inhibitor Go6976 induces mitosis and enhances doxorubicin-paclitaxel cytotoxicity in urinary bladder carcinoma cells. Cancer Lett *253*: 97-107, 2007.
20 Akiyama T, Yoshida T, Tsujita T, Shimizu M, Mizukami T, Okabe M and Akinaga S: G1 phase accumulation induced by UCN-01 is associated with dephosphorylation of Rb and CDK2 proteins as well as induction of CDK inhibitor p21/Cip1/WAF1/Sdi1 in *p53*-mutated human epidermoid carcinoma A431 cells. Cancer Res *57*: 1495-1501, 1997.
21 Konopatskaya O and Poole AW: Protein kinase Calpha: disease regulator and therapeutic target. Trends Pharmacol Sci *31*: 8-14, 2010.
22 Roffey J, Rosse C, Linch M, Hibbert A, McDonald NQ and Parker PJ: Protein kinase C intervention: the state of play. Curr Opin Cell Biol *21*: 268-279, 2010.
23 Hofmann J: Modulation of protein kinase C in antitumor treatment. Rev Physiol Biochem Pharmacol *142*: 1-96, 2001.
24 Biswas DK, Cruz AP, Gansberger E and Pardee AB: Epidermal growth factor-induced nuclear factor kappa B activation: A major pathway of cell-cycle progression in estrogen receptor-negative breast cancer cells. Proc Natl Acad Sci USA *97*: 8542-8547, 2000.
25 Biswas DK, Dai SC, Cruz A, Weiser B, Graner E and Pardee AB: The nuclear factor kappa B (NF-kappa B): a potential therapeutic target for estrogen receptor negative breast cancers. Proc Natl Acad Sci USA *98*: 10386-10391, 2001.
26 Biswas DK, Martin KJ, McAlister C, Cruz AP, Graner E, Dai SC and Pardee AB: Apoptosis caused by chemotherapeutic inhibition of nuclear factor-kappaB activation. Cancer Res *63*: 290-295, 2003.
27 Tortora G and Ciardiello F: Antisense strategies targeting protein kinase C: preclinical and clinical development. Semin Oncol *30*: 26-31, 2003.
28 Koivunen J, Aaltonen V, Koskela S, Lehenkari P, Laato M and Peltonen J: Protein kinase C alpha/beta inhibitor Go6976 promotes formation of cell junctions and inhibits invasion of urinary bladder carcinoma cells. Cancer Research *64*: 5693-5701, 2004.
29 Kretschmer A, Moepert K, Dames S, Sternberger M, Kaufmann J and Klippel A: Differential regulation of TGF-beta signaling through Smad2, Smad3 and Smad4. Oncogene *22*: 6748-6763, 2003.
30 Joerges C, Kuntze I, Herzinger T and Herzinge T: Induction of a caffeine-sensitive S-phase cell cycle checkpoint by psoralen plus ultraviolet A radiation. Oncogene *22*: 6119-6128, 2003.
31 Nociari M, Ocheretina O, Schoggins JW and Falck-Pedersen E: Sensing infection by adenovirus: Toll-like receptor-independent viral DNA recognition signals activation of the interferon regulatory factor 3 master regulator. J Virol *81*: 4145-4157, 2007.
32 Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marmé D and Schächtele C: Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem *268*: 9194-9197, 1993.
33 Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ and Johannes FJ: Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase C isoenzymes. FEBS Lett *392*: 77-80, 1996.
34 Kohn EA, Ruth ND, Brown MK, Livingstone M and Eastman A: Abrogation of the S phase DNA damage checkpoint results in S phase progression or premature mitosis depending on the concentration of 7-hydroxystaurosporine and the kinetics of Cdc25C activation. J Biol Chem *277*: 26553-26564, 2002.
35 Kohn EA, Yoo CJ and Eastman A: The protein kinase C inhibitor Go6976 is a potent inhibitor of DNA damage-induced S and G_2 cell cycle checkpoints. Cancer Res *63*: 31-35, 2003.
36 Lundberg AS and Weinberg RA: Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol *18*: 753-761, 1998.
37 Harbour JW, Luo RX, Dei Santi A, Postigo AA and Dean DC: Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G_1. Cell *98*: 859-869, 1999.
38 Knudsen ES and Wang JY: Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. J Biol Chem *271*: 8313-8320, 1996.
39 Sherr CJ and Roberts JM: CDK inhibitors: positive and negative regulators of G_1 phase progression. Genes Dev *13*: 1501-1512, 1999.
40 Rieger KM, Little AF, Swart JM, Kastrinakis WV, Fitzgerald JM, Hess DT, Libertino JA and Summerhayes IC: Human bladder carcinoma cell lines as indicators of oncogenic change relevant to urothelial neoplastic progression. Br J Cancer *72*: 683-690, 1995.

Received May 10, 2010
Revised July 29, 2010
Accepted August 16, 2010