

Protein Kinase C α/β Inhibitor Go6976 Promotes Formation of Cell Junctions and Inhibits Invasion of Urinary Bladder Carcinoma Cells

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ABSTRACT

Changes in activation balance of different protein kinase C (PKC) isoenzymes have been linked to cancer development. The current study investigated the effect of different PKC inhibitors on cellular contacts in cultured high-grade urinary bladder carcinoma cells (5637 and T24). Exposure of the cells to isoenzyme-specific PKC inhibitors yielded variable results: Go6976, an inhibitor of PKC α and PKC β isoenzymes, induced rapid clustering of cultured carcinoma cells and formation of an increased number of desmosomes and adherens junctions. Safingol, a PKC α inhibitor, had similar but less pronounced effects. In contrast, a PKC δ inhibitor, rottlerin, had an opposite effect on cell clustering and caused dissociation of cell junctions. A broad-spectrum PKC inhibitor bisindolylmaleimide I did not have any apparent effect on the morphology of the cultures or on the number of cell junctions. Additional studies with Go6976 demonstrated that inhibition of PKC α and β isoenzymes induced translocation of β 1-integrin from the cell-matrix junctions and that β 4-integrin was translocated to face the culture substratum. Go6976 was also highly effective in inhibiting migration of carcinoma cells and inhibited invasion through artificial basement membrane. Our results on urinary bladder carcinoma cells emphasize that Go6976 is a potential anticancer drug due to its effects on cell-cell and cell-matrix junctions, migration, and invasion. Furthermore, the results may be explained by changes in PKC activation balance promoted by inhibition of PKC α/β .

INTRODUCTION

Protein kinase C (PKC) family consists of serine-threonine kinases that act by phosphorylating their specific protein substrates. The PKC family members are classified into three major groups: classical (α , β , and γ); novel (δ , ϵ , η , and θ); and atypical (μ , ν , ξ). Activation of classical enzymes depends on calcium and phospholipids; novel enzymes are activated by phospholipids; and atypical enzyme activation occurs independently of calcium or phospholipids. PKCs are involved in various cellular processes such as regulation of gene expression, proliferation, cell junctions, apoptosis, and migration (1, 2).

PKC has been linked to cancer progression because most of the tumor promoters are PKC activators in two-stage carcinogenesis models (3). It has been suggested that different isoenzymes of PKC have opposite effects on cancer progression. Specifically, PKC α has been linked to cancer progression because it increases cell proliferation and migration and inhibits apoptosis (4–8). PKC δ is thought to have opposing effects on cancer progression by promoting apoptosis (5, 9–13). Thus, it has been suggested that inhibition PKC α and activation of PKC δ could be useful in cancer therapy. PKC isoenzyme-specific inhibitors such as Go6976 (PKC α/β inhibitor) and safingol (PKC α inhibitor) have proven to be effective anticancer

drugs in cell cultures and animal models (14–19). Furthermore, isoenzyme-specific PKC inhibitors seem to be more effective anticancer drugs than broad-spectrum inhibitors, suggesting the role of PKC activation balance in cancer (20).

Epithelial cells have abundant cell-cell junctions, which have a critical role in cell behavior and tissue morphogenesis. The most important anchoring structures between epithelial cells are adherens junctions and desmosomes. Adherens junctions are composed of transmembrane cadherin proteins; β -catenin, which attaches to cytoplasmic parts of cadherins; and α -catenin, a linker between β -catenin and actin cytoskeleton (21, 22). Desmosomes are formed by transmembrane desmosomal cadherins (desmocollins and desmogleins) and desmoplakin forming a link between cytoplasmic parts of desmosomal cadherins and intermediate filaments (23, 24).

Recent studies have enlightened the molecular mechanism of cell-cell junctions. It is thought that adhesional assembly starts with generation of filopodia, which penetrate and embed into adjacent cells. Adherens junctional proteins are clustered in the tip of the filopodia and generate a two-rowed adhesion zipper. Desmosomes clamp the opposing cell surfaces together and stabilize the junction. Finally, directed actin polymerization pushes the two-rowed adhesion zipper into a single row (25–27).

Cell-matrix junctions of epithelial cells are mainly formed by integrin receptors. Integrin receptors are heterodimers made up of different combinations of α - and β -chains. They act as receptors for various matrix proteins, including collagen, laminin, and fibronectin. Two major cell-matrix adhesion types in epithelial cells are focal adhesions and hemidesmosomes. Focal adhesions are characterized with β 1-chain expression and hemidesmosomes with α 6 β 4 expression (28, 29).

Increasing evidence suggests that changes in cellular junctions play an important role in development and progression of the malignant phenotype. The loss of cell-cell junctions is a crucial event in cancer progression and is commonly associated with increased aggressiveness of a tumor (30). Because loss of E-cadherin leads to aggressive tumors with high invasion rate, adherens junction proteins are often recognized as tumor suppressors (31). Furthermore, germ-line mutations in the *E-cadherin* gene have been described to cause a hereditary diffuse type gastric cancer syndrome and also predispose to other cancers (32–34). Down-regulation of desmosomal proteins has also been linked to aggressive cancers (35, 36). Furthermore, transfection of cancer cell lines with desmosomal components down-regulates invasion (37). Changes in integrin receptors have also been found in malignancies. β 1-Integrins are thought to contribute to invasive properties of cancer cells (38, 39). β 4-Integrins show often depolarization from their original localization in malignancies and may play a role in cancer progression (40, 41).

Urinary bladder transitional cell carcinoma (TCC) is one of the most common malignancies in western countries. The risk factors for bladder carcinogenesis are still largely undetermined, but tobacco smoke seems to be one among others. Nitrosamines, which are found in high concentrations in tobacco smoke, may be one factor contributing to bladder carcinogenesis, and interestingly, they are suggested to be PKC α/β activators and inactivators of PKC δ (42). Previous

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studies have demonstrated that PKC α , PKC β , and PKC δ are the predominant isoenzymes in the normal epithelium of urinary bladder, and TCCs often display down-regulation of PKC β and PKC δ (43, 44).

The present study investigates the effect of PKC inhibition on cell junctions and invasion of TCC cultures. The results suggest that PKC plays a central role in the formation of cell junctions and invasion and further point out the potential of PKC as a target for future chemotherapy of carcinomas.

MATERIALS AND METHODS

Transitional Cell Carcinoma Cell Culture. TCC cell lines used in the present study were 5637 (g 2–3) and T24 (g 3; American Type Culture Collection, Rockville, MD). The cells were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

Human Urothelial Cell Isolation and Cell Culture. Human urothelial tissue biopsies were obtained from ureteral tissue of a child undergoing open surgery for vesicoureteral reflux. After preliminary macroscopic preparation, the tissue specimen was placed in cell culture medium (Keratinocyte-Serum Free medium; Life Technologies, Inc., Gaithersburg, MD), 100 units/ml penicillin, 100 μ g/ml streptomycin, and 30 ng/ml cholera toxin (Sigma, St. Louis, MO) buffered with 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and processed within 15 min from removal. The urothelium was separated from the underlying stroma with a surgical knife and incubated in culture medium supplemented with 2.5% collagenase type II (Worthington Biochemicals, Lakewood, NJ) for 2 h. The specimens were subsequently homogenized with a plastic pipette and centrifuged at 1000 rpm for 5 min, resuspended in culture medium twice, and plated in Primaria cell culture flasks (Becton Dickinson, Cowley, United Kingdom). One-half of the medium was replaced twice a week. When confluent, the cells were rinsed in Ca²⁺ and Mg²⁺-free PBS and detached by incubation in trypsin-EDTA for 5 min. Cells were resuspended in medium containing soybean trypsin inhibitor, centrifuged, and seeded in 24-well plates for additional experiments.

PKC Inhibitors and Chemicals. Go6976, bisindolylmaleimide I, safinol, rottlerin, and cytochalasin D were obtained from Calbiochem (La Jolla, CA) and dissolved in DMSO. All of the control reactions were done with equal volumes of DMSO as in drug treatments.

Immunofluorescence. Cells intended for immunofluorescence were cultured on glass coverslips, rinsed once in PBS before fixation with methanol for 5 min at –20°C or for 10 min with 3% paraformaldehyde/PBS + 0.18% Triton X-100 at 20°C (for phalloidin staining). In Triton X-100 solubility studies, the cells were extracted with Triton X-100 buffer [1% Triton X-100, 10 mM Tris (pH 7.5), 5 mM EDTA, and 2 mM EGTA supplemented with Complete Mini EDTA-free protease inhibitors (Roche Biochemicals, Mannheim, Germany)] for 30 min at 4°C, rinsed with PBS, and fixed with methanol. Primary antibodies used in the present study were the following: mouse anti-pan-cadherin (Sigma); mouse anti-desmoplakin I/II (Roche Biochemicals); mouse anti- β 1-integrin (Life Technologies); and mouse anti- β 4-integrin (Life Technologies). Goat antimouse Alexa 488 (Molecular Probes, Eugene, OR) was used as a secondary antibody. For actin staining, samples were labeled with Alexa 568 phalloidin (Molecular Probes).

Electron Microscopy. Cells were rinsed with PBS and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 18 h at 4°C. Fixed cells were rinsed with dH₂O and further stained with 1% OsO₄ for 1 h at 20°C. The cells were rinsed with dH₂O and stained with 2% uranyl acetate for 30 min at 20°C. The cells were dehydrated and embedded in epon. Thin sections were cut on coated copper grids, and the samples were examined and photographed with Philips CM100 BioTwin electron microscope.

Western Transfer Analysis. Sequential extraction of proteins to Triton X-100 soluble and insoluble fractions was performed as described previously (45). After rinsing with PBS, the cells were extracted with a Triton X-100 buffer for 30 min at 4°C and scraped off from the bottom of the culture bottle with a rubber policeman. The lysate was centrifuged at 14,000 \times g at 4°C for 30 min to separate the soluble and insoluble proteins. Soluble pool was transferred to a new tube, and 3 \times SDS-PAGE loading buffer (46) was added to final concentration of 1 \times . Insoluble fraction was solubilized in SDS/urea buffer [1% SDS, 8 M urea, 10 mM Tris (pH 7.5), 5 mM EDTA, and 2 mM EGTA supplemented with Complete Mini EDTA-free protease inhibitors]. Triton

X-100 soluble samples were heated for 5 min at 95°C and centrifuged at 14,000 \times g at 4°C for 5 min. Equal volumes of soluble and insoluble protein lysates were subjected for SDS-PAGE on a 6% gel.

In PKC activation experiments, the cells were subjected to 4 h of treatment with inhibitors and subsequently to 30 min of treatment with phorbol 12-myristate 13-acetate (Sigma). After treatment, the cells were rinsed once with PBS and lysed in a buffer containing 1% SDS, 10 mM Tris (pH 7.4), and 1 mM sodium orthovanadate. Protein concentration was measured using detergent-compatible protein assay (Bio-Rad, Hercules, CA), and equal amounts of protein were subjected to SDS-PAGE on 10% gel.

The proteins were then transferred to polyvinylidene difluoride membrane and processed for immunoblotting. Membranes were first blocked with 5% BSA/PBS + 0.05% Tween-20 and immunolabeled with mouse anti-pan-cadherin or anti-desmoplakin I/II antibodies or in PKC inhibition studies with anti-phospho-serine PKC substrate antibody (Cell Signaling Technology, Beverly, MA) Goat antimouse horseradish peroxidase-conjugated antibody (Amersham Biosciences, Little Chalfont, United Kingdom) was used as a secondary antibody, and the signal was detected with ECL (Amersham Biosciences). Equal loading of each lane was evaluated with Coomassie Blue staining of the membrane after the immunolabeling or β -actin labeling of the same membranes.

Cell Migration Analysis. Chemically directed cell migration of TCC cells was performed using the Dunn chemotaxis chamber (Weber Scientific International Ltd., Teddington, United Kingdom). The chamber allows direct observation and thus time-lapse analysis of the cells under phase-contrast microscopy. The analysis was performed as described earlier, with minor modifications (47). TCC cells were seeded onto square coverslips and allowed to attach in DMEM supplemented with 10% FCS (DMEM + 10%FCS). The medium was changed to DMEM + 10%FCS supplemented with different concentrations of Go6976 or an equal volume of DMSO (DMEM + 10%FCS + Go/DMSO) for 16 h. Five h before chemotaxis analysis, the medium was changed to serum-free DMEM + Go/DMSO. After the 5-h starvation, the cells on coverslip were placed over the chamber, the outer and inner wells of which were filled with serum-free DMEM + Go/DMSO. The coverslip was sealed with molten 1:1 mixture of vaseline and paraffin around three edges to leave a slit for exchange of the medium in the outer well. To observe the chemotaxis, the medium in the outer well was changed to DMEM + 10%FCS + Go/DMSO, and all sides of the chamber were sealed. The chamber was set on a table of an inverted microscope equipped with an incubation hood at 37°C. A region of a bridge was viewed using a \times 20 or \times 40 objective and documented with a digital camera. The phase-contrast images were acquired every 10 min during 2.5 h of observation using MCID/M5+ system (Imaging Research Inc., Brock University, Ontario, Canada). A video of generated images was created using UTHSCSA Image tool (The University of Texas Health Science Center in San Antonio, San Antonio, TX). Thirty cells were chosen randomly over the image of the bridge. The angle and straight distance between the starting and end points was measured using the same software. The graphs were produced using Oriana 2.0 software (Kovach Computing Services, Pentraeth, Wales, United Kingdom) and statistical analyses with SPSS rel. 11.5.2.1 2003 (SPSS Inc., Chicago, IL) using univariate ANOVA.

Invasion Assay. Cell invasion was studied with Cell Invasion Assay kit (Chemicon, Temecula, CA) under the manufacturer's guidelines. In brief, 1 \times 10⁶ (T24) or 1.5 \times 10⁶ (5637) cells in DMEM without FCS, supplemented with Go6976, were plated into invasion chamber, which was placed on DMEM + 10% FCS with Go6976. Invasion was analyzed by counting the number of cells invaded through the invasion chamber 48 h after the plating.

RESULTS

Go6976 Induces Rapid Clustering of Cultured Transitional Cell Carcinoma Cells. Cultures of 5637 and T24 TCC cell lines displayed loosely organized morphology when analyzed by phase-contrast microscopy. Exposure to Go6976, a PKC α/β inhibitor, resulted in clustering of the cells (Fig. 1). Specifically, prominent intercellular contacts were formed, and the cells displayed a flattened phenotype. Clustering of the cells was seen as early as 2 h after application of Go6976 and development of clustering continued further for 24 h. The

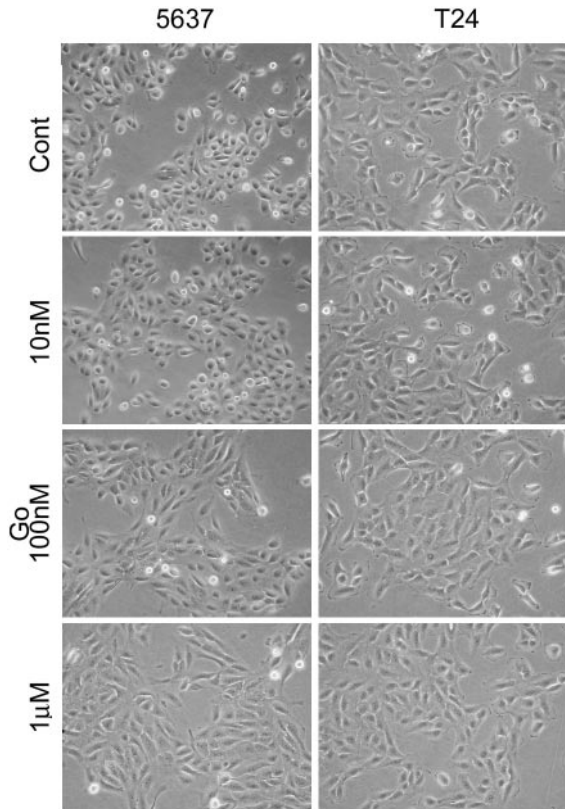


Fig. 1. Go6976 induces rapid clustering of cultured urinary bladder carcinoma cells. 5637 and T24 cell lines were incubated with varying concentrations of Go6976 (*Go*; 10 nM, 100 nM, and 1 μ M) for 4 h and photographed through a phase-contrast microscope. *Cont*, control.

effect of Go6976 was concentration dependent; the maximal effect was achieved with 1 μ M concentration (Fig. 1). Other PKC inhibitors were also tested for their ability to induce cell clustering. Bisindolylmaleimide I, a broad-spectrum PKC inhibitor, or safangol, a PKC α inhibitor, had no apparent effect on cell clustering in concentrations up to 10 μ M (not shown). Rottlerin, a PKC δ inhibitor, had an opposite effect on cell clustering causing dissociation of cells (not shown). Furthermore, rottlerin was able to inhibit morphological changes induced by Go6976 when these substances were applied together (not shown).

Go6976 Induces Formation of Desmosomes and Adherens Junctions. Because Go6976 was a potent inducer of cell clustering, the TCC cell lines were analyzed for the most important cell-cell junction proteins. Immunofluorescence studies with antibodies to cadherin (marker for adherens junctions) and to desmoplakin I/II (marker for desmosomes) demonstrated that untreated cells had abundant adherens junctions but only a low number of desmosomes, particularly in T24 cell line (Fig. 2A). Cells treated with 1 μ M Go6976 for 4 h displayed increased number of both adherens junctions and desmosomes. The number of desmosomes seemed to increase more than the number of adherens junctions (Fig. 2A). Electron microscopy showed that both cell lines had an extensive number of filopodia and adherens junctions in the tip of the filopodia. A low number desmosomes was detected in the 5637 cell line, but T24 cells were devoid of desmosomes (Fig. 2B). When the two cell lines were treated with 1 μ M Go6976 for 4 h, the cells moved closer together and formed abundant cell-cell junctions. Furthermore, both cell lines displayed an extensive number of desmosomes after treatment with Go6976 (Fig. 2B). Triton X-100 insolubility of adherens junctional and desmosomal proteins are known to increase when cells form stable cell junctions.

Consequently, Triton X-100 solubility of cadherin and desmoplakin was studied in Go6976-treated cells with two different methods: immunofluorescence labeling and Western analysis. The immunofluorescence studies on cells extracted with Triton X-100 containing buffer before fixation revealed an enhanced immunosignal for cadherin and desmoplakin at the cell-cell border zones after Go6976 treatment (Fig. 3A). It is feasible to assume that this finding may be explained by redistribution of proteins within the insoluble protein pool and by an increased level of this pool. This notion is also supported by Western transfer analyses, which demonstrated a moderate increase of the insoluble pool of cadherin and desmoplakin in 5637 cells and an increase of insoluble cadherin in T24 cells. In T24 cell line, desmoplakin was undetectable in both fractions, which is

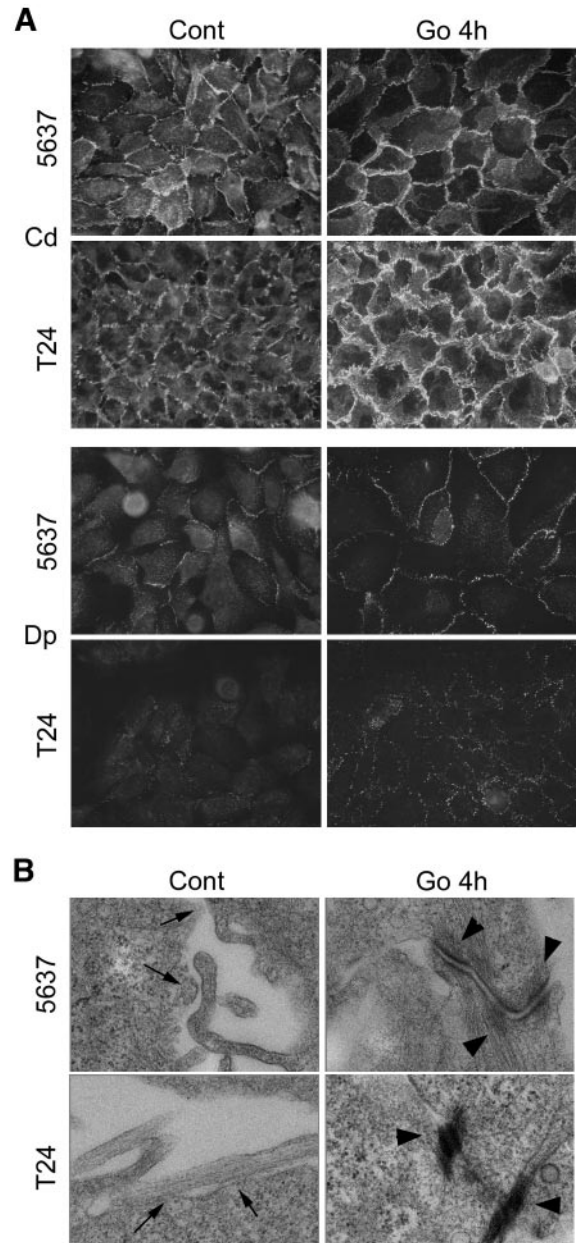


Fig. 2. Go6976 promotes formation of desmosomes and adherens junctions in TCC cultures. The cell lines 5637 and T24 were incubated with (*Go 4 h*) or without (*Cont*) 1 μ M Go6976 for 4 h and fixed. *A*, immunofluorescence labeling of cells with pan-cadherin (*Cd*) or desmoplakin (*Dp*) antibodies. *B*, electron micrograph of the cells treated with Go6976 as in *A*. Control cells of both cell lines display numerous adherens junctions (*arrows*) but only a few desmosomes. After treatment with Go6976 for 4 h, prominent desmosomes (*arrowheads*) are observed in both cell lines.

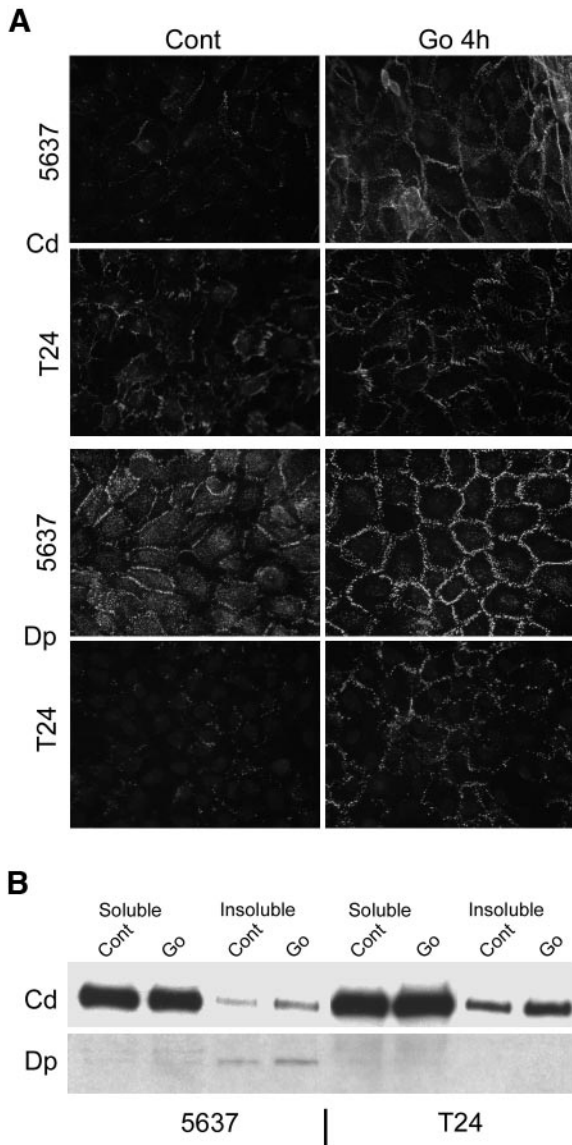


Fig. 3. The effect of Go6976 on Triton X-100 solubility of cell-cell junction proteins. The cell lines 5637 and T24 were incubated with (*Go 4 h*) or without (*Cont*) 1 μ M Go6976 for 4 h and treated with Triton X-100 buffer. *A*, immunofluorescence labeling with pan-cadherin (*Cd*) or desmoplakin (*Dp*) antibodies demonstrating Triton X-100 insoluble proteins. *B*, Western transfer analysis of Triton X-100 soluble and insoluble cadherin (*Cd*) and desmoplakin (*Dp*) proteins after treatment with 1 μ M Go6976 for 4 h.

likely to reflect low levels of desmoplakin expression in the cells (Fig. 3B).

Disrupting of Actin Cytoskeleton Does Not Affect the Formation of Cell-Cell Junctions Induced by Go6976. Immunofluorescence studies suggested that Go6976 had a particularly marked effect on the formation of desmosomes (Fig. 2A). Consequently, the actin cytoskeleton was disrupted with cytochalasin D, which is known to disturb the formation of adherens junctions (26). Adherens junctions attach to the actin cytoskeleton, and disruption of actin cytoskeleton is known to inhibit formation of stable adherens junctions. Desmosomes attach to intermediate filament network, and disruption of actin cytoskeleton should not have direct effect on desmosomal junctions. When the cells were treated with cytochalasin D for 4 h, they lost most of their cell-cell junctions (Fig. 4A). The effect was the most pronounced in T24 cell line, which was previously shown to be almost completely devoid of desmosomes (Fig. 2A). When cells were treated with combination of cytochalasin D and Go6976 for 4 h, the cells

retained their contacts, and increased desmoplakin-positive dots were observed at cell-cell junction sites (Fig. 4A). As expected, the number of adherens junctions between cells treated with cytochalasin D was the same as in cultures treated with cytochalasin D in combination with Go6976 (Fig. 4A). These findings strongly suggest that the effects of Go6976 are targeted on desmosomes. Complete disruption of actin cytoskeleton was confirmed by staining of actin cytoskeleton with fluorescence-labeled phalloidin (Fig. 4B).

The Effect of Different Protein Kinase C Inhibitors on Desmosomes. Because Go6976 proved to be a potent inducer of desmosome formation, the effects of other PKC inhibitors on desmosomes were

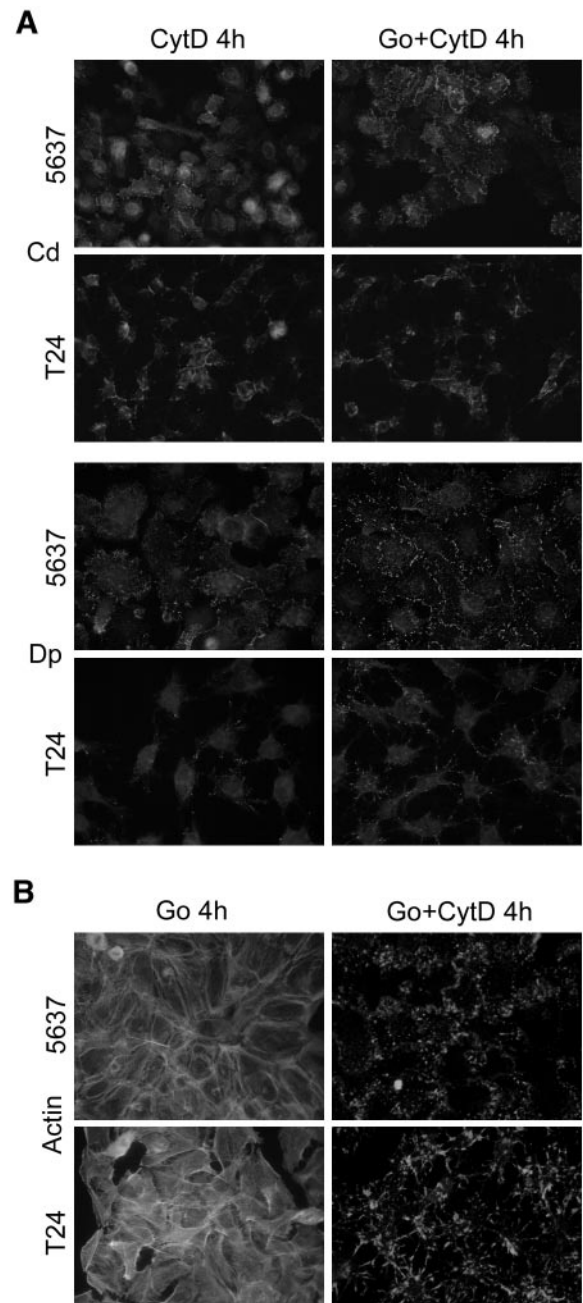


Fig. 4. Disrupting the actin cytoskeleton does not affect the Go6976-induced formation of desmosomes but decreases adherens junction formation. The cell lines 5637 and T24 were incubated with 2 μ M cytochalasin D (*CytD 4 h*) or with 2 μ M cytochalasin in combination with 1 μ M Go6976 (*Go+CytD 4 h*) for 4 h and fixed. *A*, immunofluorescence labeling for adherens junctions using pan-cadherin antibody (*Cd*) and for desmosomes using desmoplakin antibody (*Dp*). *B*, phalloidin staining for actin cytoskeleton of cells treated with or without cytochalasin in combination with Go6976.

studied. The cells were exposed to the inhibitors for a longer time period (24 h) to gain the maximal effect. Bisindolylmaleimide I (a broad-spectrum PKC inhibitor) did not have any apparent effect on the number of desmosomes as estimated by immunofluorescence labeling (Fig. 5). Safingol (PKC α inhibitor) increased the number of desmosomes, but the effect was less marked compared with Go6976 (Fig. 5). Treatment with rottlerin (PKC δ inhibitor) resulted in partial loss of desmosomes in both cell lines (Fig. 5). The effect of rottlerin was more pronounced in 5637 cells, which display more prominent desmosomes (Fig. 5). When Go6976 was applied to cells in combination with rottlerin, the effect of Go6976 was partially reversed, resulting in a decreased number of desmosomes compared with Go6976 treatment alone (Fig. 5).

Go6976 Induces Changes in Cell-Matrix Junctions. Because Go6976 proved to be a potent inducer of cell-cell junctions, the effect

of this PKC inhibitor on cell-matrix junctions was studied. Untreated cells of both TCC cell lines displayed an intense signal for β 1-integrin at focal adhesion sites, lamellopodia, filopodia, and retraction fibers and also at the cell-cell contact zone (Fig. 6A). Treatment of cells with Go6976 for 4 h had a marked effect on β 1-integrin localization. Specifically, β 1-integrin appeared to translocate away from the cell-matrix contact sites (Fig. 6A). Expression of β 4-integrin, a component of hemidesmosomes, was detected only in 5637 cell line. Untreated cells displayed β 4-integrin in filopodia and retraction fibers (Fig. 6B). Treatment with Go6976 resulted in pronounced translocation of the β 4-integrin to the bottom surface of the cells. Specifically, cells appeared to form β 4-integrin-positive cell-matrix junctions, which apparently represented hemidesmosomes (Fig. 6B). Calcium-induced differentiation of normal human urothelial cells resulted in markedly similar localization of β 1- and β 4-integrins as in TCC cells treated with Go6976. Specifically, increased calcium concentration resulted in transient translocation of β 1-integrin to cell-cell contact zone within 4 h, and subsequent fading of the immunosignal within 24 h. In addition, β 4-integrin translocated to the bottom surface of the cells within 24 h (Fig. 6C).

Go6976 Inhibits Cell Movement and Invasion. Invasive behavior of cancer cells is accompanied with increased cell movement. Furthermore, normal epithelial type cell junctions are known to inhibit invasive behavior of cancer cells. Because Go6976 seemed to promote the formation of cell contacts, the effect of the inhibitor was tested on TCC cell migration and invasion. Cell migration assay was performed using the Dunn chamber system, and the cell migration was followed for 2.5 h (Fig. 7). When the cells were not exposed to Go6976, the 5637 and T24 cells migrated rapidly toward FCS, which operated as a chemoattractant. When exposed to Go6976, 5637 and T24 cells markedly slowed their migration and tended to form clusters. Statistical analysis revealed a significant difference in the motility of the cells when different concentrations of Go6976 were used (control, 100 nM; 1 μ M Go6976) in 5637 and T24 cells ($P < 0.01$ and $P < 0.001$, respectively).

Furthermore, statistical analysis of three separate repeats revealed that the results were reproducible, with no significant difference ($P > 0.05$). Subsequently, *post hoc* tests between different concentration groups were performed. Treatment with 100 nM Go6976 caused a statistically significant decrease in T24 cell motility ($P < 0.01$), but not in 5637 cell motility ($P = 0.078$). A statistically significant decrease in the cell movement was observed when 5637 and T24 cells were treated with 1 μ M Go6976 ($P < 0.01$ and $P < 0.01$, respectively). In addition, no significant difference was found between three repeats in any of concentration groups ($P > 0.05$).

To examine the effect of Go6976 on the invasive phenotype of cells, the ability of TCC cells to invade through a reconstituted basement membrane was analyzed. The invasion assay showed that Go6976 inhibited cell invasion. In 5637 cells, invasion was almost completely blocked in 100 nM and 1 μ M concentrations (by $\sim 97\%$; Fig. 8, A and B). In T24 cells, cell invasion was also inhibited using Go6976 in 100 nM and 1 μ M concentrations (by $\sim 75\%$; Fig. 8, A and B).

Inhibition of Classical Protein Kinase C Isoenzymes by Go6976. To confirm the inactivation of PKC α/β by Go6976 and to show that rottlerin has no effect on PKC α/β inactivation, a Western analysis using anti-phospho-PKC substrate antibody was performed (Fig. 9). Anti-phospho-PKC substrate antibody detects phosphorylated serine residues, when surrounded by arginine or lysine at the -2 and $+2$ positions and a hydrophobic residue at the $+1$ position. Thus, it binds specifically to phosphorylated substrates of classical PKC isoenzymes (α , β , and γ) but not to those of novel isoenzymes, such as δ (48). Previous studies have shown that in urothelium, α , β , and δ are the

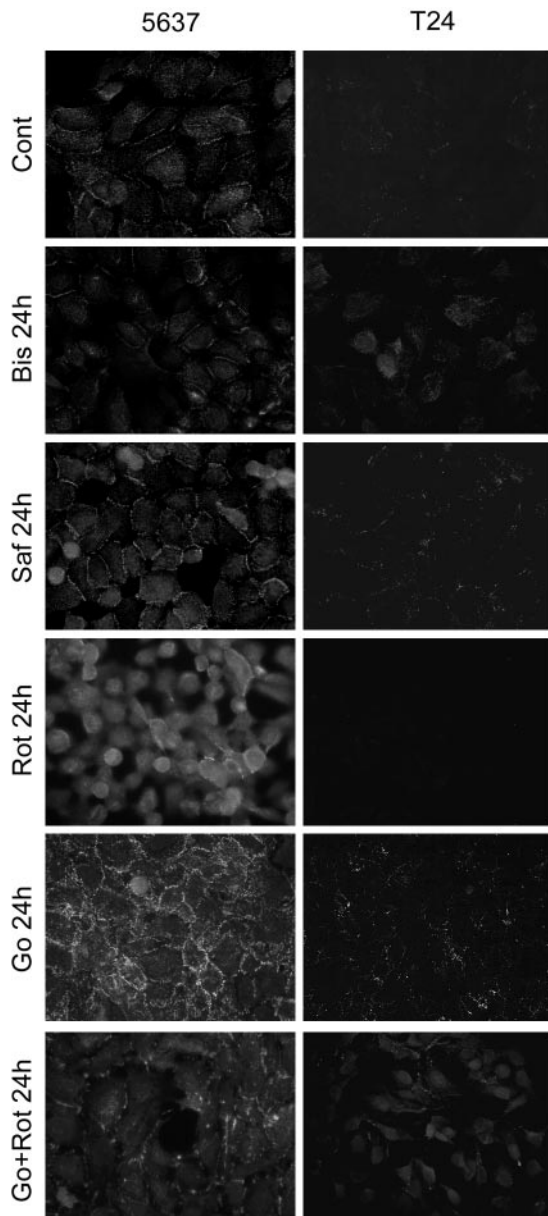


Fig. 5. The effect of different PKC inhibitors on the formation of desmosomes. The cell lines 5637 and T24 were treated with 10 μ M bisindolylmaleimide I (Bis 24 h), 10 μ M safingol (Saf 24 h), 10 μ M rottlerin (Rot 24 h), 1 μ M Go6976 (Go 24 h) or, 1 μ M Go6976 and 10 μ M rottlerin (Go+Rot 24 h) for 24 h. Desmosomes were visualized with immunolabeling for desmoplakin.

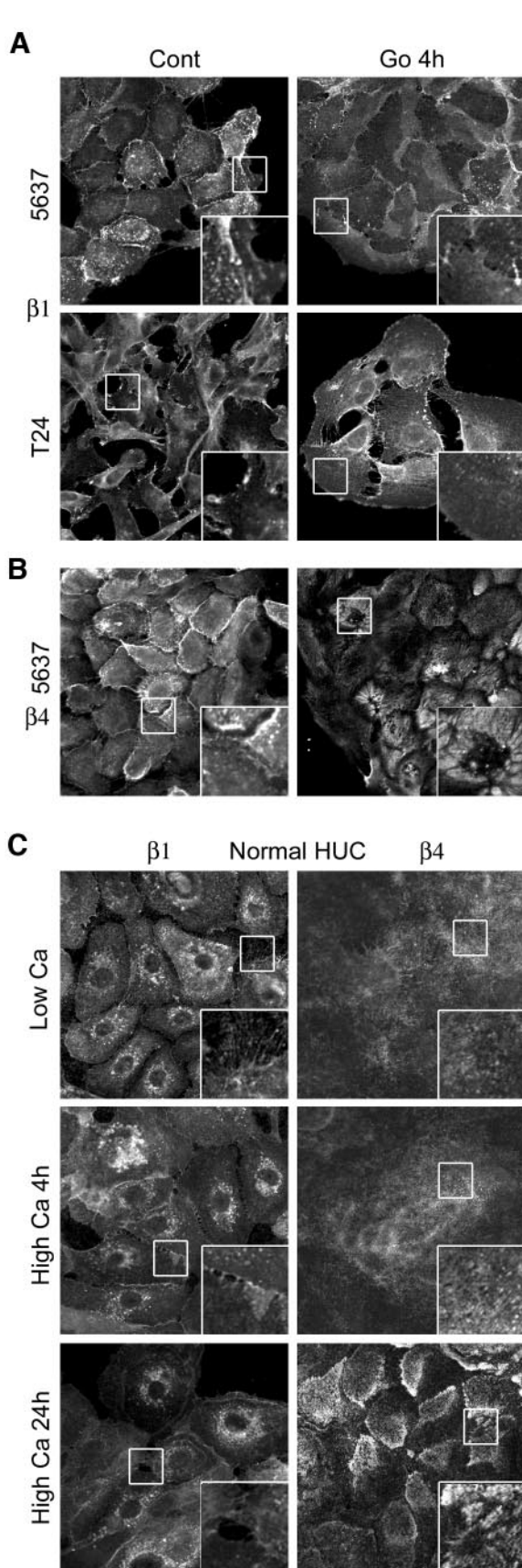


Fig. 6. Go6976 causes marked changes on cell-matrix junctions. Immunofluorescence labeling for $\beta 1$ - and $\beta 4$ -integrin were carried out to analyze the focal adhesions and hemidesmosomes. A, $\beta 1$ -integrin immunolabeling of 5637 and T24 cells treated with (Go 4 h) or without (Cont) Go6976 for 4 h. B, $\beta 4$ -integrin immunolabeling of 5637 cell line treated analogously to A. $\beta 4$ -integrin expression was not detected in T24 cell line. C, $\beta 1$ -

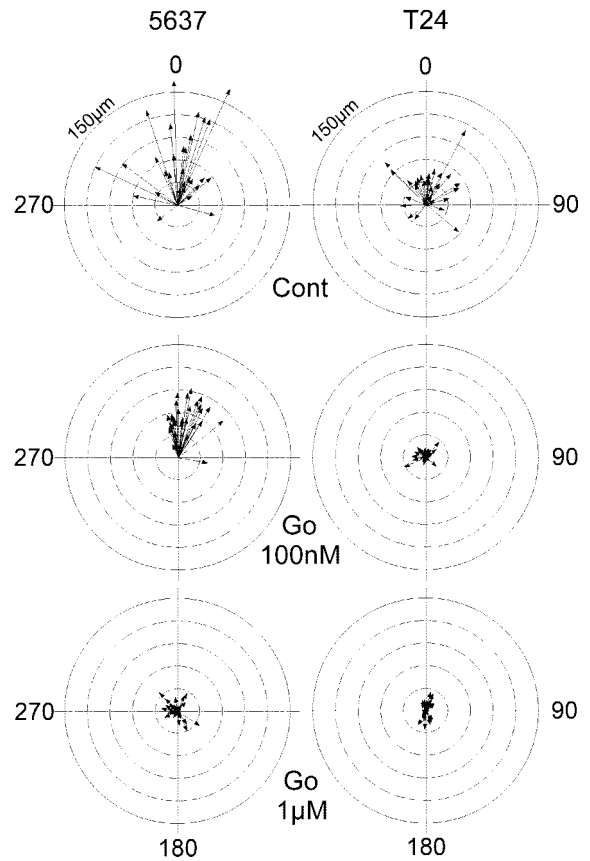


Fig. 7. Go6976 inhibits cell migration. Chemically directed migration of 5637 and T24 cells was analyzed using Dunn chamber and time-lapse analysis. Representative vector graphics demonstrating the distance (vector length, μm) and direction (degrees) of movement of individual cells ($n = 30$) from their original positions during the observation period of 2.5 h. The vertical axis points toward the outer well filled with chemoattractant, and the horizontal axis is directed parallel to the bridge of the chamber. Each interval between circles represents $30 \mu\text{m}$ distance, thus the outermost circle represents $150 \mu\text{m}$.

predominant PKC isoenzymes (43, 44). Our results showed that Go6976 inhibits phorbol 12-myristate 13-acetate-stimulated activity of classical PKC isoenzymes in a concentration-dependent manner (Fig. 9A). After treatment with $10 \mu\text{M}$ rottlerin, classical PKC activity was not decreased, suggesting that rottlerin does not inhibit classical isoenzymes in this concentration. In contrast, classical PKC activity showed a minor increase. Furthermore, when the cells were treated with a combination of $10 \mu\text{M}$ rottlerin and $1 \mu\text{M}$ Go6976, a slightly increased classical PKC activity was observed compared with $1 \mu\text{M}$ Go6976 treatment alone (Fig. 9B).

DISCUSSION

The present study investigated the link between PKC and cell junctions using cultured high-grade bladder carcinoma cells. The results showed that PKC α/β inhibitor Go6976 induced drastic changes in both cell-cell and cell-matrix contacts. Specifically, Go6976 induced formation of adherens and particularly desmosomal cell-cell junctions. Cell-matrix contacts underwent changes with a decreased number of $\beta 1$ -integrin-positive junctions and an increased number of $\beta 4$ -integrin-positive junctions. PKC δ inhibition with rot-

and $\beta 4$ -integrin immunolabeling of normal human urothelial cells (normal HUC), cultured either in medium containing 0.05 mM calcium (Low Ca) or in medium containing 1.8 mM calcium (High Ca) for 4 or 24 h. \square , areas are presented with higher magnification in lower right corner of each panel.

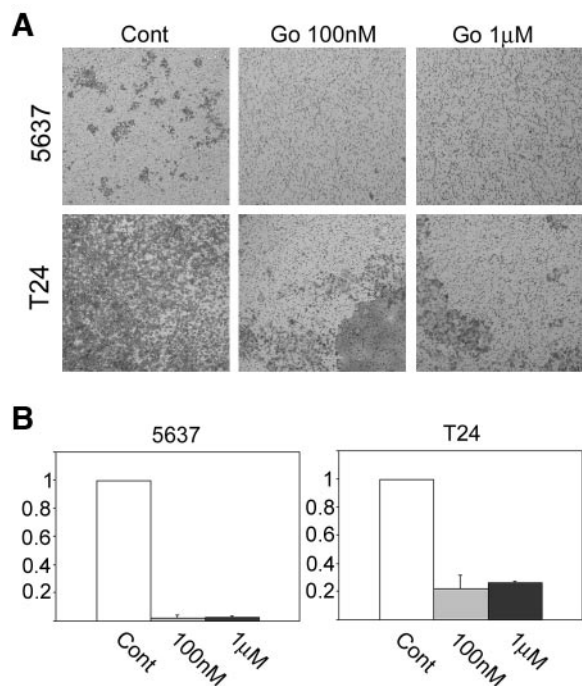


Fig. 8. Go6976 inhibits cell invasion. Cell invasion was analyzed using cell invasion assay in which the cells invade through reconstituted basement membrane. *A*, micrographs of the 5637 and T24 cells that have invaded through the basement membrane 48 h after plating following treatment with (*Go* 100 nM, *Go* 1 μ M) or without (*Cont*) Go6976. *B*, the relative invasion of the cells 48 h after plating when the cells were treated with (100 nM or 1 μ M) or without (*Cont*) Go6976. The means + SD are shown.

tlarin had an opposite effect to Go6976 with disruption of cell-cell junctions and partial reversion of the effect of Go6976. In addition to changes in cell junctions, Go6976 proved to be a very potent inhibitor of cell migration and invasion.

Changes in PKC balance have been linked to cancer development and progression. Activation of PKC α/β has a cancer-promoting effect, leading to increased cell proliferation and migration and inhibition of apoptosis (4–8). In opposite, PKC δ has been suggested to be a cancer suppressor because it promotes apoptosis (5, 9–12). High-grade carcinomas, including TCCs, display changes in cell adhesion. Specifically, disruption of E-cadherin-mediated junctions and desmosomes have been linked to invasive TCCs but not to superficial TCCs (49–51). In cell-matrix junctions, depolarization of β 4-integrin-dependent junctions (hemidesmosomes) is frequently seen in invasive TCCs (40), and β 1-integrins (focal adhesions) are commonly linked to invasive behavior of carcinomas (52, 53). The results of the present study showed that high-grade TCC cells display similar alterations in cell junctions *in vitro*. Interestingly, PKC α/β inhibitor Go6976 induced very rapid changes toward restoration of normal epithelial cell-cell and cell-matrix junctions. The changes occurred rapidly (<2 h), making it likely that they were caused by changes in protein phosphorylation rather than in gene expression. PKC δ inhibition disrupted cell-cell contacts and reversed the Go6976-induced phenotype. This suggests that increased proportional activity of PKC δ promoted by PKC α/β inhibition leads to stable cell adhesions. The results of the present study link together the two well-known events—a change in PKC balance and alterations in cell junctions—occurring during cancer progression.

As estimated by immunolabelings and electron microscopy, Go6976 seemed to have more pronounced effect on desmosomal cell-cell junctions than on adherens junctions. This was further enlightened by experiments in which actin cytoskeleton was disrupted.

The results showed that disruption of actin cytoskeleton could not reverse the effect of Go6976 on desmosomes but resulted in an almost complete inhibition of adherens junction formation. Adherens junctions are attached to the actin cytoskeleton, making it likely that disruption of actin filaments has a more pronounced effect on adherens junctions than on desmosomes. Previous studies have also linked inhibition of PKC α to calcium independency of desmosomes (54). It is feasible to speculate that PKC balance has a central role in function of the adhesion zipper, which works toward stable cell-cell adhesion during cell differentiation. Based on the present study, one could

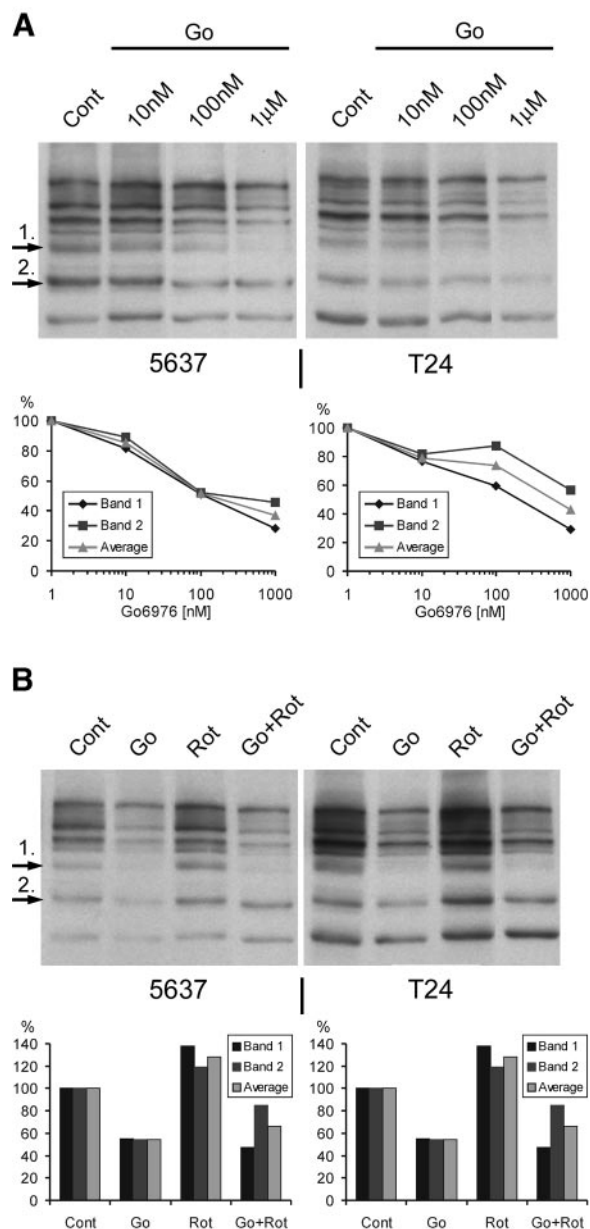


Fig. 9. Go6976, but not rottlerin, inhibits phorbol ester 12-myristate 13-acetate-stimulated classical PKC isoenzyme activation. Western blots were carried out using anti-phospho-PKC substrate antibody, which detects phosphorylated substrates specific for classical PKCs (α , β , and γ). *A*, 5637 and T24 cells treated with different concentrations of Go6976 (10 nM, 100 nM, and 1 μ M) or without Go6976 (*Cont*). *B*, 5637 and T24 cells treated with 1 μ M Go6976 (*Go*), 10 μ M rottlerin (*Rot*), or a combination of 1 μ M Go6976 and 10 μ M rottlerin (*Go+Rot*) or without inhibitors (*Cont*). A graph below each panel demonstrates results of densitometric quantification of two selected bands indicated with arrows. The vertical axis (%) demonstrates percentage of activation of classical PKCs when compared with control. The horizontal axis demonstrates concentration of Go6976 in logarithmic scale (*A*) or inhibitors that were used in the experiment (*B*).

speculate that high-grade carcinomas are able to construct the first phase of the adhesion zipper, in which the formation of filopodia and clustering of adherens junctional proteins in the tip of the filopodia occurs, but the cells are not able to proceed to the second phase of the zipper formation. In the second phase, desmosomes clamp the opposing cell surfaces together, which may be disrupted by changes in PKC activation balance. Previous studies have shown the importance of adherens junctions and desmosomes to stable cell-cell adhesion seen in normal epithelium (25–27).

The results of the current study showed that Go6976 efficiently inhibits the activity of classical PKCs. Rottlerin (PKC δ -inhibitor) did not inhibit classical PKC activity but, in contrast, caused a minor increase in its activity when used alone and in combination with Go6976. This finding may partially explain the disruption of PKC balance commonly seen in cancers. One can speculate that inhibition of certain isoenzyme could result in activation of other isoenzymes through several pathways. The mechanism of rottlerin-induced activation of classical PKCs remains to be elucidated.

The findings of the present study with aggressive TCC lines and Go6976 suggest that Go6976 can differentiate cells by inducing the formation of cell junctions and partially reversing the invasive phenotype. Go6976-induced differentiation in TCC cells occurs in highly similar manner as in cultured keratinocytes, a well-characterized model for epithelial cell differentiation. In keratinocytes, rise of the extracellular calcium levels causes rapid formation of adherens junctions and desmosomes (55, 56) and formation of β 4-integrin-containing hemidesmosomal structures and translocation of β 1-integrin from the cell-matrix adhesions sites (57). The results with normal urothelial cells showed that they behave similarly to cultured keratinocytes when induced to differentiate by increasing the extracellular calcium concentration. Specifically, a transient translocation of β 1 integrin to cell-cell contact zone and subsequent fading of immunosignal was observed. Furthermore, β 4-integrin-positive cell-matrix junctions were observed after elevation of extracellular calcium concentration.

Previous studies concerning Go6976 have demonstrated the chemotherapeutic potential of the substance using *in vivo* carcinoma models (18, 19). Our present study further enlightens the molecular mechanisms through which Go6976 acts as a chemotherapeutic agent. We have shown that Go6976 is a potent inducer of cell-cell and cell-matrix junctions, which play a pivotal role in the cancer progression, invasion, and metastasis but also cell signaling and tumor suppression. Restoration of prominent cell junctions by Go6976 can induce a less invasive phenotype of bladder cancer cells. Thus, our results encourage additional investigations on Go6976 as a chemotherapeutic agent in treatment of cancers.

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