Sensitivity of Normal, Paramalignant, and Malignant Human Urothelial Cells to Inhibitors of the Epidermal Growth Factor Receptor Signaling Pathway

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Abstract
Bladder cancer evolves via the accumulation of numerous genetic alterations, with loss of p53 and p16 function representing key events in the development of malignant disease. In addition, components of the epidermal growth factor receptor (EGFR) signaling pathway are frequently overexpressed, providing potential chemotherapeutic targets. We have previously described the generation of “paramalignant” human urothelial cells with disabled p53 or p16 functions. In this study, we investigated the relative responses of normal, paramalignant, and malignant human urothelial cells to EGFR tyrosine kinase inhibitors (PD153035 and GW572016), a mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) kinase (MEK) inhibitor (U0126), and a phosphatidylinositol 3-kinase inhibitor (LY294002). The proliferation of normal human urothelial cells was dependent on signaling via the EGFR and MEK pathways and was abolished reversibly by inhibitors of EGFR or downstream MEK signaling pathways. Inhibitors of phosphatidylinositol 3-kinase resulted in only transient cytostasis, which was most likely mediated via cross-talk with the MEK pathway. These responses were maintained in cells with disabled p16 function, whereas cells with loss of p53 function displayed reduced sensitivity to PD153035 and malignant cell lines were the most refractory to PD153035 and U0126. These results indicate that urothelial cells acquire insensitivity to inhibitors of EGFR signaling pathways as a result of malignant transformation. This has important implications for the use of EGFR inhibitors for bladder cancer therapy, as combination treatments with conventional chemotherapy or radiotherapy may protect normal cells and enable better selective targeting of malignant cells. (Mol Cancer Res 2008;6(1):53–63)

Introduction
The urinary bladder and associated tract is lined by a specialized transitional epithelium, the urothelium, which functions as a urinary barrier. Urothelium is mitotically quiescent, reflected in a slow rate of turnover and a low proportion of cells in the cell cycle (1, 2), but has a high proliferative capacity in response to damage (3). In normal urothelium, the regenerative response is considered a crucial feature that contributes to the maintenance and repair of urinary barrier integrity and is driven, at least in part, through the autocrine production of epidermal growth factor (EGF) family ligands acting on the EGF receptor (EGFR; ErbB1 or Her1; refs. 4–8).

Unsurprisingly, a signaling pathway so intimately involved in driving normal urothelial cell proliferation is subverted in urothelial cell carcinoma (UCC). The overexpression of EGFR is associated with high tumor grade and stage (9, 10) and is an independent predictor of recurrence and poor prognosis (11, 12). ErbB2 (or Her2/neu) overexpression has also been associated with bladder tumorigenesis (13). The importance of the EGF signaling cascade in many human cancers has provided an attractive therapeutic target, and several antibody and small-molecule inhibitors have been developed against the receptors, receptor-associated tyrosine kinases, and other downstream signaling components, including mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) kinase (MEK) and phosphatidylinositol 3-kinase (PI3K). Despite encouraging preclinical studies, typically, these agents have not been as effective as anticipated in clinical trials (reviewed in refs. 14–17). In part, this may reflect the presence of mutations in one or more components of the EGFR signaling pathway and use of alternative growth factor signaling pathways (reviewed in refs. 14–17), and it is now acknowledged that receptor expression levels alone cannot predict the response to inhibitors (18). In bladder cancer, recent work has suggested that the prognostic significance of EGFR or ErbB2 overexpression is modulated by expression of ErbB3 (Her3) and ErbB4 (Her4; ref. 19), indicating the complexity of interactions between the different EGFRs and their ligands.
To address how EGF signaling is altered as a consequence of malignant transformation in human urothelial cells, we have investigated the response of normal, genetically modified (“paramalignant”), and UCC-derived human urothelial cells to EGF pathway inhibitors. Normal human urothelial (NHU) cells were grown as proliferating finite cell lines in a well-characterized cell culture system (20, 21) and used to derive engineered paramalignant human urothelial cells with disabled p53 or p16 tumor suppressor function (22) to replicate molecular events in the development of UCC (23). Functional loss of p53 is considered an initiating event in the development of carcinoma in situ (24), and loss of both p53 and p16 is involved in the development of invasive disease (24-26). In addition, loss of part of chromosome 9 is an initiating event in the development of both carcinoma in situ and superficial papillary UCC (reviewed in ref. 27), and p16 is a candidate gene targeted by such chromosomal alterations (28-30). Using this approach, we have shown previously that loss of p53 and p16 functions is associated, respectively, with a small increase in proliferation rate and an increased apoptotic susceptibility to membrane-presented CD40 ligand (22). For comparison, the established UCC-derived RT4, RT112, and EJ cell lines were included in our study as representative of different grades and stages of UCC (31). The different cell lines were assessed for response to inhibitors designed to different components of the EGF signaling pathway: PD153035 to the EGFR-specific tyrosine kinase, GW572016 (Tykerb or lapatinib) with dual activity against the EGFR and ErbB2 tyrosine kinases, U0126 the MEK1/MEK2 inhibitor, and LY294002 as a PI3K inhibitor.

**Results**

**Titration of Inhibitors in NHU Cells**

Effective concentrations of inhibitors were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in NHU cells as the highest concentration of inhibitor at which cytostasis, but no loss of cell biomass due to cytotoxicity, was observed over a 10-day time course compared with DMSO vehicle controls (Fig. 1). The concentrations selected for further study were 1 μmol/L PD153035, 5 μmol/L U0126, 0.75 μmol/L GW572016, and 5 μmol/L LY294002.

**Morphology of Normal and Paramalignant Human Urothelial Cells**

Under normal growth conditions, or with 0.1% DMSO as a vehicle control, NHU cells were highly migratory and grew as a dispersed monolayer rather than colonies. At confluence, the cultures formed a typical epithelioid pavement, defined by phase-bright intercellular borders (Fig. 2A). NHU cells treated with 1 μmol/L PD153035 for 3 days were sparser than control...
cultures and preferentially formed colonies containing enlarged, flattened, and sometimes vacuolated cells (Fig. 2B). Treatment with 5 μmol/L U0126, 0.75 μmol/L GW572016, or 5 μmol/L LY294002 also induced the formation of colonies that contained enlarged, flattened, and often vacuolated cells (Fig. 2C-E, black arrowheads). In addition, signs of stratification were visible in the form of elongated, phase-bright cells after treatment with U0126 (Fig. 2C, white arrowhead), and some smaller cells were present in cultures treated with GW572016 or LY294002 (Fig. 2D and E). Mock-transduced NHU cells and the paramalignant human urothelial cell lines HU-p53DD and HU-CDK4mut showed a similar morphology to NHU cells when grown in the presence of inhibitors (data not shown).

Growth, Proliferation, and Survival of Normal and Paramalignant Human Urothelial Cells

The growth of mock-transduced NHU, HU-p53DD, and HU-CDK4mut cell cultures was studied over a 10-day period, and as described previously (22), cultures of HU-p53DD cells showed the highest rate of growth and acquired the greatest biomass (Fig. 3), whereas cultures of HU-CDK4mut cells displayed the lowest rate of growth and a reduced biomass compared with mock-transduced controls (P < 0.05; Fig. 3E). Cultures of mock-transduced NHU cells treated with 1 μmol/L PD153035, 5 μmol/L U0126, or 0.75 μmol/L GW572016 showed no increase in growth after 3 days (Fig. 3A-C), resulting in a significant difference in biomass at day 5 relative to the vehicle control (P < 0.01); the growth inhibition was sustained over the full 10-day period. By contrast, cultures treated with LY294002 showed only a temporary inhibition of growth despite frequent replenishment of the inhibitor (Fig. 3D).

Similar findings were found when HU-p53DD and HU-CDK4mut sublines were treated with inhibitors, with the exception that HU-p53DD cells showed continued, albeit reduced, growth in the presence of PD153035 for up to 7 days after application of the inhibitor (Fig. 3A). This was reflected in the significant difference in biomass displayed by HU-p53DD cells relative to mock-transduced controls at the 5-day time point after treatment with 1 μmol/L PD153035 (P < 0.01; Fig. 3E).

The proliferation of mock-transduced NHU, HU-p53DD, and HU-CDK4mut cells was assessed by 5-bromo-2’-deoxyuridine (BrdUrd) assay (Fig. 4) and showed a significant reduction at the 3-day time point after treatment with 1 μmol/L PD153035, 5 μmol/L U0126, 0.75 μmol/L GW572016, or 5 μmol/L LY294002 compared with the DMSO vehicle controls (P < 0.05 at least). In agreement with the trend in cell biomass observed by MTT assay, the proliferation of all three sublines was nearly abolished by PD153035, U0126, or GW572016 (Fig. 4), although PD153035 and GW572016 to a lesser extent were significantly less effective in HU-p53DD cells compared with mock-transduced controls (P < 0.1; Fig. 4). In addition, some proliferation was observed after treatment of all three sublines with LY294002 (Fig. 4), although still significantly less than in the solvent controls (P < 0.05 at least).

An apoptosis assay was used to determine if cell loss was a contributory factor to the reduction in biomass induced by the inhibitors. Less than 1% of mock-transduced NHU, HU-p53DD, and HU-CDK4mut cells were positive for the cleaved forms of either caspase-3 or cytokeratin 18 (CK18) produced by caspase-mediated cleavage during apoptosis when cultured in the presence of DMSO (vehicle control), 1 μmol/L PD153035, 5 μmol/L U0126, or 5 μmol/L LY294002 for 3 days (Table 1). By contrast, 24-h treatment with 20 μmol/L 15-deoxy-A<sub>12,14</sub>-prostaglandin J<sub>2</sub> (15dPGJ<sub>2</sub>) acted as a positive control for apoptosis, with 48% to 59% of cells showing expression of the cleaved forms of either caspase-3 or CK18 (Table 1). In agreement with the results obtained by BrdUrd incorporation, the percentage of cells expressing the proliferation marker Ki67 was greatly reduced in all three sublines by treatment with PD153035, U0126, or, to a lesser extent, LY294002 compared with the relevant vehicle-only control (Table 1).

Activity of Signaling Pathways in Normal and Paramalignant Human Urothelial Cells

The expression of phosphorylated ERK1/2 (pERK1/2), indicative of active signaling via the MEK pathway, was virtually undetectable after treatment of mock-transduced NHU, HU-p53DD, or HU-CDK4mut cells with 1 μmol/L PD153035, 5 μmol/L U0126, or 0.75 μmol/L GW572016 for 30 min (Fig. 5A, top, lanes 2-4), whereas total ERK1/2 levels remained constant (Fig. 5A, bottom). pERK1/2 levels were very slightly reduced after treatment with 5 μmol/L LY294002 compared with the vehicle-only control (Fig. 5A, top, lane 5). The expression of phosphorylated Akt (pAkt), on the other hand, was unaffected by 5 μmol/L U0126 and only slightly reduced...
by treatment with 1 μmol/L PD153035 or 0.75 μmol/L GW572016, indicating active signaling via the PI3K pathway (Fig. 5B, top, lanes 2-4). pAkt levels were most reduced after treatment with 5 μmol/L LY294002 compared with the solvent control (Fig. 5B, top, lane 5).

Reversible Nature of EGFR Signaling Pathway Inhibitors in Normal and Paramalignant Human Urothelial Cells

To determine whether the inhibitor-induced cytostasis was reversible, the effect of removing inhibitors after 3 days of treatment was examined. NHU cells started to recover by 24 h after removal of 1 μmol/L PD153035 (day 4), with the appearance of small, proliferative cells and reversion from a colony-forming phenotype to dispersed monolayers, as cells once again became migratory (data not shown). After 3 days (day 6), cultures were repopulated by small cells, which proliferated rapidly to form the confluent monolayers of typical pavement morphology described above. Such cultures were interspersed with occasional enlarged, flattened cells that eventually detached (data not shown).

Within 2 days of removal of PD153035 (day 5), cultures of NHU cells began to show an increase in biomass compared with those cultures maintained on PD153035 for the whole time course (Fig. 6A), which was significant from the 7-day time point onwards ($P < 0.001$; Fig. 6B). The biomass data were supported by BrdUrd uptake measurements, which showed an increase in proliferation within 48 h of PD153035 removal ($P < 0.001$; day 5; Fig. 6C). Although there was some
human urothelial cells. BrdUrd incorporation assays were done on mock-transduced NHU, HU-p53DD, and HU-CDK4mut cells after 3-d treatments with 0.1% (v/v) DMSO, 1 μmol/L PD153035, 5 μmol/L U0126, 0.75 μmol/L GW572016, or 5 μmol/L LY294002. Columns, average of six replicates normalized to the cell biomass of the DMSO controls; bars, SD. Representative results from duplicate experiments using two independently transduced NHU cell lines are shown. **, P < 0.01, one-way ANOVA with Dunnett’s post test. Note the reduced sensitivity of HU-p53DD cells to PD153035 and, to a lesser extent, GW572016 compared with mock-transduced NHU cells.

heterogeneity between individual cultures in terms of how quickly they reacquired a proliferative phenotype (reflected in the large error bars in Fig. 6C), all cultures released from PD153035-mediated cytostasis recovered and grew to confluence.

ERK1/2 was reprophorylated within 30 min of PD153035 being removed from the growth medium, whereas cells treated continuously with PD153035 displayed virtually no pERK1/2 expression (Fig. 6D).

NHU cells were also able to recover fully from cytostasis induced by 3-day treatments with 5 μmol/L U0126 or 0.75 μmol/L GW572016 (data not shown). The recovery of NHU cells from 3-day treatments with 5 μmol/L LY294002 was not tested, as cells were able to recover from LY294002 during treatment (see above). Mock-transduced NHU, HU-p53DD, and HU-CDK4mut cells showed a similar ability to NHU cells to recover from cytostasis induced by 3-day treatments with 1 μmol/L PD153035 (data not shown).

Growth, Proliferation, and Survival of Malignant Human Urothelial Cells

The biomass of RT4, RT112, and EJ cultures was slightly reduced after treatment with 1 μmol/L PD153035, 5 μmol/L U0126, or 5 μmol/L LY294002 compared with the vehicle-only controls (Fig. 7A-C). The effects of PD153035 and U0126 were significant from the 5-day time point onwards in all three cell lines (P < 0.05 at least; Fig. 7D), except for RT4 cells, which were not significantly affected by PD153035. Although the biomass of all three cell lines was significantly reduced at the 5-day time point when treated with LY294002 (P < 0.05 at least; Fig. 7D), the biomass then increased to a level that was comparable with that of cells cultured in a DMSO vehicle control by the 10-day time point (P > 0.05).

The proliferation of RT4, RT112, and EJ cells was not substantially affected by a 3-day treatment with 1 μmol/L PD153035 compared with a DMSO control (P > 0.05) but was reduced after treatment with 5 μmol/L U0126 in RT112 and EJ cells or 5 μmol/L LY294002 in RT4 and RT112 cells (Fig. 8). However, these reductions in proliferation were only significant for LY294002 in RT112 cells (P < 0.01), and U0126 actually caused a significant increase in proliferation in RT4 cells (P < 0.01; Fig. 8).

The treatment of RT4, RT112, or EJ cells with 1 μmol/L PD153035, 5 μmol/L U0126, or 5 μmol/L LY294002 for 3 days did not induce the expression of the cleaved forms of either caspase-3 or CK18, with <1% of cells shown to be positive for these apoptosis markers (Table 2). A 24-h treatment with 20 μmol/L 15dPGJ2 acted as a positive control for apoptosis, with up to 54% of cells showing expression of the cleaved forms of either caspase-3 or CK18 (Table 2). In agreement with the majority of results obtained by BrdUrd incorporation, the percentage of expression of the cell proliferation marker Ki67 was slightly reduced in all three cell lines by treatment with U0126, LY294002, or, to a lesser extent, PD153035 compared with a DMSO control (Table 2).

Discussion

We have shown that the proliferation of NHU cells is sensitive to inhibition of the EGFR signaling cascade,
specifically through the MEK pathway. This sensitivity is maintained in cells with p16 loss, and although we found some evidence for reduced sensitivity in cells with disabled p53 function, the UCC-derived cell lines were the most refractory, implying that other desensitizing mechanisms are acquired during malignant progression. The three UCC cell lines, RT4, RT112, and EJ, have previously been shown to express EGFR and are therefore suitable candidates for treatment with EGFR inhibitors (32-35). Although numerous studies have reported an inhibition of proliferation induced by EGFR tyrosine kinase inhibitors in bladder tumor cell lines (34-36), few studies have investigated the relative effects of inhibitors on normal and malignant-derived cells.

Urothelial cell proliferation is promoted through stimulation of EGFR and other growth factor receptors (e.g., fibroblast growth factor receptors; reviewed in ref. 37); however, only EGFR is implicated in autocrine regulation, which is mediated, at least in part, through juxtacrine or direct cell:cell interactions (4-8). Loss of p53 function is associated with carcinoma in situ, and our results suggest that cells with impaired p53 expression could acquire an early growth advantage over normal urothelial cells, both through increased proliferation (22) and potentially through a reduced responsiveness to negative regulatory feedback operating between the MEK and PI3K pathways (47). This sensitivity is maintained in cells with disabled p53 function, which is associated with carcinoma in situ, and our results suggest that cells with impaired p53 expression could acquire an early growth advantage over normal urothelial cells, both through increased proliferation (22) and potentially through a reduced responsiveness to negative regulatory feedback operating between the MEK and PI3K pathways (47). This sensitivity is maintained in cells with disabled p53 function, which is associated with carcinoma in situ, and our results suggest that cells with impaired p53 expression could acquire an early growth advantage over normal urothelial cells, both through increased proliferation (22) and potentially through a reduced responsiveness to negative regulatory feedback operating between the MEK and PI3K pathways (47).

Inhibition of EGFR signaling in cultures of NHU cells revealed pleiotropic effects that were consistent irrespective of whether signaling was inhibited at the level of the receptor-associated tyrosine kinase (PD153035 or GW572016) or in the downstream MEK cascade (U0126). With all three inhibitors, the major effect was reversible cytostasis, which was consistent with our previously published findings that PD153035-treated NHU cells accumulated in the G1 phase of the cell cycle (8). In addition to cytostasis, which was accompanied by acquisition of a large quiescent cell morphology, the same effective but noncytotoxic concentrations produced other profound changes to cell phenotype, including inhibition of cell migration (see ref. 8 for a more detailed discussion on cell motility), colony formation, a tendency for stratification (particularly with U0126 and PD153035), and a greatly increased adhesion to the substrate, which made the harvest of cells difficult using routine trypsin harvesting protocols. As EGFR activation induces the disassembly of focal adhesions and adherens junctions (45, 46), it is likely that reassembly of these features accounted for the observed changes in adherence and migration.

The PI3K inhibitor LY294002 induced an inhibitory effect on proliferation and growth of urothelial cell cultures that was only transient and was accompanied by an incomplete inhibition of ERK1/2 phosphorylation. This implies that although there was some feedback from the PI3K pathway onto the MEK pathway, cells were not dependent on PI3K signaling for proliferation or survival and that continued stimulation of the MEK pathway by the EGFR autocrine loop was able to overcome the inhibitory feedback. In support of this, PI3K inhibitors have previously been shown to inhibit insulin-like growth factor–induced, but not EGF-induced, phosphorylation of ERK1/2 by a cross-talk mechanism (47). Other studies have also found evidence of both positive and negative feedback operating between the MEK and PI3K pathways, but a consensus has yet to be reached on the mechanism of action (48-52). We also have previous evidence of such a cross-talk mechanism, as blockade of MEK signaling,
which is permissive for NHU cell differentiation induced by activation of peroxisome proliferator-activated receptor γ, can be substituted for by PI3K inhibition (53). The three UCC cell lines were less susceptible to growth inhibition imparted by PD153035 or U0126 compared with normal or paramalignant human urothelial cells and were especially refractory to the inhibitory effects of PD153035. This suggests that UCC cell lines may have acquired constitutively activating mutations in signaling components downstream of EGFR but upstream of MEK. In EJ cells, the H-Ras oncogene may be partially responsible for the resistance to EGF pathway inhibitors, as K-Ras has been associated with resistance to Iressa or Tarceva in lung adenocarcinoma (54). Another possibility is that mutations in EGFR itself may confer resistance to EGFR tyrosine kinase inhibitors, as has been previously shown in glioblastoma multiforme and non–small cell lung cancer (55, 56). Alternatively, UCC cell lines may signal via many other growth factor receptors that converge on the same downstream signaling cascades (e.g., MEK and PI3K) as EGFR, thereby rendering UCC cell lines less susceptible to inhibitors that target a receptor (such as PD153035) rather than a downstream signaling cascade (such as U0126). Interestingly, because HU-p53DD cells also showed some loss of sensitivity to PD153035, part of the mechanism may be p53 dependent.

Although malignant cells were not as dependent as normal cells on signaling via EGFR for their proliferation, they were still at least partially dependent on the MEK signaling pathway, as U0126 treatment reduced cell proliferation (especially in RT112 and EJ cells). Nevertheless, inhibition of the MEK pathway was not sufficient to cause sustained growth arrest, suggesting that UCC cells use other downstream signaling pathways to mediate growth, with PI3K, stress-activated protein kinase/c-Jun NH2-terminal kinase MAPK, p38 MAPK, or signal transducers and activators of transcription representing examples.

Our results agree with another study, in which the proliferation of UCC cell lines was not as dependent on MEK activation as NHU cells (57), thereby calling for caution in the use of inhibitors designed to EGFR signaling pathways for the treatment of bladder cancer, as they are most likely to inhibit

**FIGURE 6.** Recovery of NHU cells from PD153035-induced cytostasis. A and B, MTT assays were done on NHU cells at the 0-, 3-, 5-, 7-, and 10-d time points. A, Cells were treated with 0.1% (v/v) DMSO or 1 μmol/L PD153035 for 3 d followed by 7 d of culture in KSFMc, 0.1% (v/v) DMSO, or 1 μmol/L PD153035. Points, average of six replicates; bars, SD. Representative results from triplicate experiments using independent cell lines are shown. B, The 7-day time point after the start of treatment, or the 4th day of recovery, is shown in more detail. **,** P < 0.001, one-way ANOVA with Bonferroni multiple comparisons post test. C, BrdUrd incorporation assays were done on NHU cells after 3-d treatments with 0.1% (v/v) DMSO or 1 μmol/L PD153035 followed by 2 d of culture in KSFMc, 0.1% (v/v) DMSO, or 1 μmol/L PD153035. Columns, average of six replicates normalized to the cell biomass of the DMSO control; bars, SD. Representative results from triplicate experiments using independent cell lines are shown. **,** P < 0.001, one-way ANOVA with Bonferroni multiple comparisons post test. D, NHU cells were treated with 1 μmol/L PD153035 for 3 d, and either fresh 1 μmol/L PD153035 or KSFMc was replenished 30 min before lysis. Protein lysates were subjected to 8% to 16% (w/v) SDS-PAGE and immunoblotting with anti-pERK1/2 (top) or anti-ERK1/2 (bottom), and antibody binding was visualized by epifluorescence illumination at 800 or 700 nm, respectively. Lane 1, 1 μmol/L PD153035; lane 2, KSFMc. Representative results from triplicate experiments are shown. Note the increased biomass (A and B), proliferation (C), and pERK1/2 expression (D) of cells cultured in KSFMc after a 3-d treatment with PD153035 compared with cells grown in the presence of PD153035 for the whole time course. The large error bars (C) are indicative of heterogeneity between cultures in the time taken to reenter the cell cycle.
normal regenerating urothelial cells rather than malignant cells that have lost sensitivity to normal regulatory controls or have acquired constitutively activating mutations in downstream signaling pathways. An alternative use for EGFR tyrosine kinase inhibitors could be to suppress the regenerative response of normal urothelial cells before exposing the bladder to chemotherapeutic compounds designed to target proliferating cells. Combination treatments of EGFR tyrosine kinase inhibitors and conventional chemotherapeutic agents or radiotherapy may therefore enable selective targeting of proliferative, malignant cells over cell cycle-withdrawn, normal cells.

In conclusion, signaling via EGFR and the MEK pathway, but not the PI3K pathway, was essential for the proliferation of normal and paramalignant cells. Although HU-p53DD cells exhibited a delay in the induction of growth arrest mediated by PD153035, indicating that certain growth factor pathway-targeted therapies may be less effective against cells with disabled p53 tumor suppressor function, the sensitivity of paramalignant human urothelial cells to inhibitors of the EGFR signaling pathway was otherwise maintained as for NHU cells. The partial independence from EGFR and MEK signaling displayed by UCC cell lines, reliant mostly on alterations in addition to inactivation of p53 or p16, highlights the importance and necessity of carefully assessing inhibitors designed to EGFR signaling pathways for the treatment of bladder cancer and suggests that combination treatments of EGFR tyrosine kinase inhibitors and conventional chemotherapy or radiotherapy may be a more realistic treatment option for bladder cancer patients.

FIGURE 7. Effect of inhibitors on the biomass of malignant human urothelial cells. MTT assays were done on RT4, RT112, and EJ cells after 0, 3, 5, 7, and 10 d of culture in 0.1% (v/v) DMSO in the presence (dashed lines) or absence (solid lines) of 1 μmol/L PD153035 (A), 5 μmol/L U0126 (B), and 5 μmol/L LY294002 (C). Treatments were replenished every 2 to 3 d. Points, average of six replicates; bars, SD. Representative results from duplicate experiments are shown. D. The 5-day time point after treatment is shown in more detail. *, P < 0.05; **, P < 0.01, one-way ANOVA with Dunnett’s post test. Note the slightly reduced biomass of cells treated with any of the inhibitors compared with the vehicle-only control.

FIGURE 8. Effect of inhibitors on the proliferation of malignant human urothelial cells. BrdUrd incorporation assays were done on RT4, RT112, and EJ cells after 3-day treatments with 0.1% (v/v) DMSO, 1 μmol/L PD153035, 5 μmol/L U0126, or 5 μmol/L LY294002. Columns, average of six replicates normalized to the cell biomass of the DMSO controls; bars, SD. Representative results from duplicate experiments are shown. ***, P < 0.01, one-way ANOVA with Dunnett’s post test. Note the decreased proliferation of two of the three cell lines when treated with U0126 or LY294002 compared with the vehicle-only controls.
experiments were done on cell lines between passages 2 and 7. A total of six independent NHU cell lines were generated in parallel and acted as a parental control. ‘Mock-transduced’ p16-insensitive cyclin-dependent kinase 4 mutant (CDK4R24C) urothelial cell lines with disabled p53 or p16 tumor suppressor genes were obtained from CalBiochem (supplied by VWR International). GW572016, a dual EGFR and ErbB2 tyrosine kinase inhibitor, was supplied by GlaxoSmithKline. The apoptosis-inducing agent 15dPGJ2 was also obtained from Calbiochem. These reagents were all reconstituted in DMSO; the final concentration of DMSO was adjusted to 0.1% (v/v) in growth medium for all experiments, including the vehicle-only control.

Table 2. Effect of Inhibitors on the Expression of Apoptosis and Proliferation Markers in Malignant Human Urothelial Cells

<table>
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<th>Marker</th>
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<th>1 μmol/L PD153035 (%)</th>
<th>5 μmol/L U0126 (%)</th>
<th>5 μmol/L LY294002 (%)</th>
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NOTE: Immunofluorescence labeling was done on RT4, RT112, and EJ cells after 3-day treatments with 0.1% (v/v) DMSO. 1 μmol/L PD153035, 5 μmol/L U0126, and 5 μmol/L LY294002 or after a 24-h treatment with 20 μmol/L 15dPGJ2. The mean percentage of cells expressing cleaved caspase-3, cleaved CK18, or Ki67 is shown. Each data point is the mean from three replicate fields, and representative results from duplicate experiments are shown. Note the low levels of expression of the markers for apoptosis, except in cells treated with 15dPGJ2, and reduced expression of the proliferation marker after treatment with any of the inhibitors compared with the vehicle-only control.

Materials and Methods

All reagents were purchased from Sigma-Aldrich, unless otherwise stated.

Inhibitors

The tyrosine kinase EGFR inhibitor PD153035, the MEK1 and MEK2 inhibitor U0126, and the PI3K inhibitor LY294002 were obtained from Calbiochem (supplied by VWR International). GW572016, a dual EGFR and ErbB2 tyrosine kinase inhibitor, was supplied by GlaxoSmithKline. The apoptosis-inducing agent 15dPGJ2 was also obtained from Calbiochem. These reagents were all reconstituted in DMSO; the final concentration of DMSO was adjusted to 0.1% (v/v) in growth medium for all experiments, including the vehicle-only control.

Urothelial Cell Culture

Finite NHU cell lines were established from surgical specimens from patients with no history of urothelial malignancy and maintained as previously described (20, 21). The use of surgical specimens was carried out with the permission of the Local Research Ethics Committee and with the informed consent of patients. Paramalignant human urothelial cell lines with disabled p53 or p16 tumor suppressor protein function were generated by retroviral transduction to introduce a dominant-negative p53 miniprotein (p53DD) or a p16-insensitive cyclin-dependent kinase 4 mutant (CDK4R24C) and were functionally characterized (22). “Mock-transduced” NHU cells were generated in parallel and acted as a parental cell control (22). Normal and paramalignant human urothelial cells were maintained in complete keratinocyte serum-free medium (KSFMc), consisting of KSF containing recombinant human EGF and bovine pituitary extract at the manufacturer’s recommended concentrations (Invitrogen) and 30 ng/mL cholera toxin. The addition of 0.1 mg/mL genetin and 2 μg/mL puromycin to the culture medium of HU-p53DD and HU-CDK4mut cells, respectively, was used to maintain selection pressure on the cell lines but was omitted during experiments. A total of six independent NHU cell lines were used, including two retrovirally transduced NHU cell lines, and experiments were done on cell lines between passages 2 and 7.

Three established UCC-derived cell lines, RT4, RT112, and EJ, were obtained from the European Cell Culture Collection. RT4, RT112, and EJ cell lines were derived from UCC of grades 1, 2, and 3, respectively, and have been well characterized (31). UCC cell lines were cultured in a 1:1 mixture of DMEM and RPMI 1640 (Invitrogen) with 5% (v/v) fetal bovine serum (Harlan Sera-Lab).

Cell Proliferation Assay

Cell proliferation was determined by measuring the amount of BrdUrd incorporated into cellular DNA over a 4-h period. Cells were seeded in 96-well plates at a density of 2 × 10^3 per well for NHU and paramalignant human urothelial cells, 1.2 × 10^3 per well for RT4 cells, 1 × 10^3 per well for RT112 cells, and 4 × 10^2 per well for EJ cells and cultured for 18 h. Cells were then treated with inhibitors for up to 5 days, with all treatments replenished in fresh growth medium every 2 to 3 days. BrdUrd incorporation assays were carried out at the 3- or 5-day time points after treatment according to the manufacturer’s instructions (BrdUrd Labeling and Detection Kit III, Roche Diagnostics). BrdUrd uptake was estimated by measuring the absorbance of samples at 405 nm following background subtraction of blank values. All experiments were conducted in six replicate wells.

Cell Viability Assay

The relative biomass of a culture was determined by MTT assay, whereby a yellow formazan salt (MTT) is reduced to insoluble purple crystals by mitochondrial dehydrogenase. Cells were seeded as for BrdUrd incorporation and cultured for 18 h. Cells were then treated with inhibitors for up to 10 days, with treatments replenished in fresh growth medium every 2 to 3 days, and assayed at the 0-, 3-, 5-, 7-, and 10-day time points after treatment. MTT (0.5 mg/mL for NHU and paramalignant human urothelial cells or 0.25 mg/mL for UCC cell lines) was added and cells were incubated at 37°C for 4 h. Formazan crystals were dissolved in DMSO, and the absorbance of samples was measured at 570 nm against a DMSO control. All experiments were conducted in six replicate wells.
**Indirect Immunofluorescence Apoptosis Assay**

Cells were seeded on Teflon-coated 12-well slides at a density of $5 \times 10^5$ per well for NHU, paramalignant human urothelial, and RT4 cells, $4.5 \times 10^5$ per well for RT112 cells, and $4 \times 10^5$ per well for EJ cells and cultured for 18 h. Cells were then treated with inhibitors for 3 days or with 15dpGJ2 for 24 h, which acted as a positive control for apoptosis, and fixed in a 1:1 mixture of methanol and acetone. Slides were probed with primary antibodies to cleaved caspase-3 (Promega), cleaved CK18 (Roche Diagnostics), or Ki67 (Novocastra Laboratories) followed by secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes, supplied by Cambridge Bioscience). Nuclei were stained with 0.1 μg/mL Hoechst 33258. Slides were examined under epifluorescence illumination, and cells stained with secondary antibody only were included as negative controls. All experiments were conducted in three replicate wells. The mean percentage of positive cells was calculated by expressing the number of positive cells relative to the total number of Hoechst 33258–stained nuclei in each of three random fields of view.

**Western Blotting**

Cells were seeded in 25 cm² flasks at a density of $1 \times 10^5$ per flask and cultured for 18 h. Cells were then treated with inhibitors for up to 3 days, and cell lysates were collected *in situ* in 125 mmol/L Tris-HCl (pH 6.8), 20% (v/v) glycerol, 2% (w/v) SDS, 200 mmol/L NaF, 2 mmol/L Na3VO4, 40 mmol/L Na2P2O7, and 13 mmol/L DTT, and 0.2% (v/v) Protease Inhibitor Cocktail Set III (Calbiochem). Lysates were sonicated, incubated on ice for 30 min, and collected after centrifugation at 25,000 × g for 30 min at 4°C. Protein lysates (12.5 μg) were resolved by electrophoresis through 8% to 16% (w/v) Novex Tris-glycine gels (Invitrogen) and electroblotted onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences). Phosphorylated at Ser473 (anti-pAkt; Cell Signaling Technology) and the total number of Hoechst 33258–stained nuclei in three replicate wells. The mean percentage of positive cells was calculated by expressing the number of positive cells relative to the total number of Hoechst 33258–stained nuclei in each of three random fields of view.

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**References**

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Molecular Cancer Research

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