Critical Role of Bad Phosphorylation by Akt in Cytostatic Resistance of Human Bladder Cancer Cells

ARPAD SZANTO[1], ZITA BOGNAR[2], ANDRAS SZIGETI[3], ALIZ SZABO[2], LASZLO FARKAS[1] and FERENC GALLYAS Jr.[2]

Departments of [1]Urology, [2]Biochemistry and Medical Chemistry, and [3]Oncotherapy, Faculty of Medicine, University of Pecs, Hungary

Abstract. *Background: Taxol is the most commonly used agent for salvage chemotherapy in transitional cell carcinoma of the urothelium. We examined mechanisms responsible for taxol resistance by using T24 human bladder carcinoma cells. Materials and Methods: We used an inhibitor and an activator of the phosphatidylinositol-3 kinase-Akt pathway in cell survival and caspase-3 assays, an HPLC method for determining released cytochrome c and immunoblotting for detecting protein phosphorylation. Results: Activation of Akt increased paclitaxel resistance by increasing Bad phosphorylation, leading to decreased release of mitochondrial cytochrome c and caspase-3-mediated apoptosis. On the other hand, inhibition of Akt prevented paclitaxel resistance by enhancing the effects of paclitaxel on Bad phosphorylation, mitochondrial cytochrome c release and caspase-3-mediated apoptosis, besides diminishing or abolishing the opposing effects of Akt activation. Conclusion: Akt-mediated Bad phosphorylation plays an important role in preservation of mitochondrial membrane systems leading to paclitaxel resistance in T24 cells.*

Taxane-based chemotherapy is currently the most commonly used remedy for salvage chemotherapy in transitional cell carcinoma of the urothelium (1, 2). Paclitaxel (taxol) interferes with the mitotic spindle during mitosis of cells, stabilizing the microtubule by inhibiting tubulin dimerisation, and so inhibiting the separation of the sister chromatids (3-5). Paclitaxel can affect kinases (6) that play important roles in cell death processes, and regulate the expression of tumour suppressor genes and cytokines (7). In the paclitaxel-induced cell death process, activation of c-Jun *N*-terminal kinase (JNK) plays a critical role by suppressing Akt activation and promoting the nuclear accumulation of forkhead-related transcription factor-3a (FoxO3a) (8). Nuclear translocation of FoxO3a can facilitate apoptosis by inducing the expression of Bim, a BH3-only proapoptotic Bcl-2 homolog protein (9). It has also been demonstrated that Akt overexpression prevented paclitaxel-induced cell death (10-14), probably by processes such as inactivation of caspase-3, caspase-9, Bad or forkhead homologue rhabdomyosarcoma (FKHR) transcription factors (15) leading to cytostatic resistance. Bad belongs to the pro-apoptotic members of the Bcl-2 family, which forms a complex with the antiapoptotic member of the family, Bcl-xL. The bound Bcl-xL is not able to exert its antiapoptotic effects. When Bad is phosphorylated at Ser^{112}, Ser^{136} or Ser^{155}, it forms a complex with the 14-3-3 protein in the cytosol thereby preventing binding to Bcl-2/Bcl-xL and so promoting cell survival (16). Phosphorylation of Bad can be triggered by survival factors, such as protein kinase A (PKA) and Akt (17). In the present paper, we studied involvement of Akt-dependent Bad phosphorylation and preservation of the integrity of mitochondrial membrane systems in paclitaxel resistance of T24 human urine bladder transitional cancer cell line.

Materials and Methods

Materials. Phosphatidylinositol-3 kinase (PI-3K) inhibitor LY-294002, poly(ADP-ribose) polymerase (PARP-1) inhibitor PJ34, protease inhibitor cocktail, and all chemicals for cell culture were purchased from Sigma-Aldrich Kft (Budapest, Hungary). The following antibodies were used: anti-Akt, anti-phospho-Akt, anti-phospho-glycogen synthase kinase-3β (GSK), anti-phospho Bad, anti-Bad (Cell Signalling Technology, Beverly, MA, USA); anti-mouse IgG and anti-rabbit IgG (Sigma-Aldrich Kft).

Cell culture. T24 human bladder carcinoma cells were from the American Type Culture Collection (Wesel, Germany). The cells were maintained as monolayer adherent culture in minimum essential Eagle's medium containing 1% antibiotic-antimycotic solution and 10% fetal calf serum (MEM/FCS) in humid 5% CO_2 atmosphere at 37°C.

Correspondence to: Ferenc Gallyas Jr., Department of Biochemistry and Medical Chemistry, University of Pecs, Szigeti st. 12., 7624-Pécs-Hungary. E-mail: ferenc.gallyas@aok.pte.hu

Cell viability assay. The cells were seeded into 96-well plates at a starting density of 10^4 cells per well and cultured overnight before paclitaxel (50 or 100 nM) and PJ34 (10 μM) or LY-294002 (5 μM) were added to the medium for 24 h. The medium was changed to a fresh one containing 0.5% of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT$^+$) for an additional 3 hours, then the MTT$^+$ reaction was terminated by adding HCl to 10 mM final concentration. The amount of blue formazan dye formed from MTT$^+$ was proportional to the number of live cells, and was determined with an Anthos Labtech 2010 ELISA reader at 550 nm. All experiments were run in at least 4 replicates and repeated 3 times.

Western blot analysis. Cells were seeded and treated as for the cell viability assay. Cells were harvested at intervals in a chilled lysis buffer containing 0.5 mM sodium-metavanadate, 1 mM ethylenediaminetetraacetic acid (EDTA) and protease inhibitor cocktail in phosphate-buffered saline (PBS). Immunoblotting was performed as described elsewhere (18). All experiments were repeated 3 times.

Caspase-3 activity assay. The cells were treated with paclitaxel in the presence or absence of PJ34 or the PI3 kinase inhibitor LY294002 for 16 h. The cells were harvested and determination of caspase-3 activity was carried out as described elsewhere (18). All experiments were repeated 3 times.

Determination of cytochrome c level by a high pressure liquid chromatography (HPLC) method. The analysis of cytochrome *c* from the cytosol fraction of T24 cells treated with paclitaxel in the presence or absence of PJ34 or the PI3 kinase inhibitor LY294002 for 16 h was perfomed as described elsewhere (18). Data acquisition was performed from at least three independent experiments.

Statistical analysis. Data are presented as means±S.E.M. For multiple comparisons of groups, ANOVA was used. Statistical difference between groups was established by paired Student's *t*-test with Bonferroni's correction.

Results

To examine the possible role of Akt in paclitaxel resistance in bladder cancer cells, we used an activator and an inhibitor of the enzyme. For inhibiting Akt, we used LY-294002, a commercially available inhibitor of the upstream activating enzyme, PI-3K. For activating Akt, we used the PARP-1 inhibitor PJ34 that was previously shown to induce Akt phosphorylation (19-21).

First, we wanted to establish whether PJ34 had any direct effect on mitochondrial permeability transition (mPT). To this end, we monitored high amplitude swelling of isolated, Percoll gradient-purified rat liver mitochondria in the presence of either 2.5 μM Ca^{2+} and PJ34 or 2.5 μM Ca^{2+}, 20 μM paclitaxel and PJ34. As we found previously (22), this amount of Ca^{2+} did not induce mPT, however 20 μM paclitaxel caused maximal swelling of mitochondria that was prevented completely by 2.5 μM cyclosporine A. PJ34 did
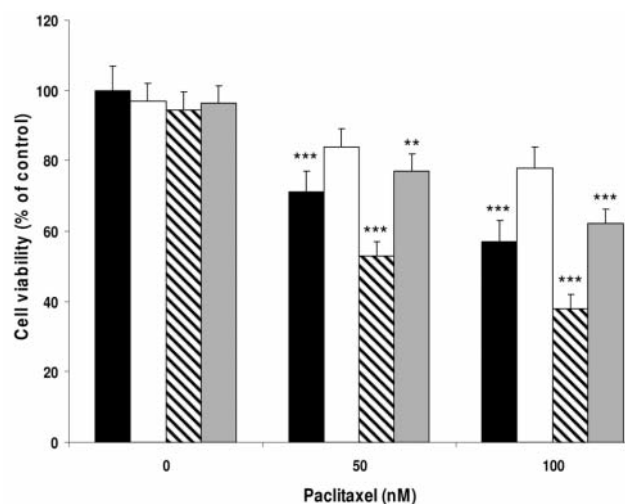

Figure 1. *Effect of PI-3K modulators on paclitaxel-induced cell death in T24 cells. T24 cells were exposed for 16 h to 50 or 100 nM of paclitaxel alone (filled bars) or together with 10 μM of PJ34 (empty bars), 5 μM of LY-294002 (striped bars) or a combination of PJ34 and LY-294002 (gray bars). Viability of the cells was detected by the MTT$^+$ method. Data represent means±S.E.M. of three independent experiments, each running in four replicates. Significantly different from paclitaxel + PJ34 treated group at **p<0.01, ***p<0.001.*

not induce mPT on its own and did not affect paclitaxel-induced mPT (data not shown). These results proved that PJ34 was indifferent to mPT, and allowed us to use this drug as an Akt-activating agent further on.

Role of PI-3K-Akt pathway in paclitaxel-iduced cell death in T24 cells. Activation of Akt by 10 μM PJ34 indeed induced paclitaxel resistance as was revealed by increased cell viabilities. On the other hand, 5 μM of the PI-3K inhibitor LY-294002 significantly enhanced cytotoxicity of paclitaxel, and reduced PJ34-induced paclitaxel resistance (Figure 1). These results suggest that Akt activation was strongly involved in paclitaxel resistance in T24 cells.

Role of PI-3K-Akt pathway in paclitaxel-induced caspase-3 activation in T24 cells. Since according to previous studies (4, 5), paclitaxel administration induces mainly apoptotic cell death, we tested the effect of Akt activation on paclitaxel-induced caspase-3 activation. In T24 bladder carcinoma cells, sixteen hours of paclitaxel treatment at a concentration of 50 or 100 nM resulted in marked activation of caspase-3. This effect was significantly reduced (*p*<0.001) when the cells were pretreated with 10 μM PJ34. On the other hand, 5 μM of LY294002 significantly increased (*p*<0.001) paclitaxel-induced caspase-3 activation, and reduced the cytoprotective effect of PJ34 (Figure 2).
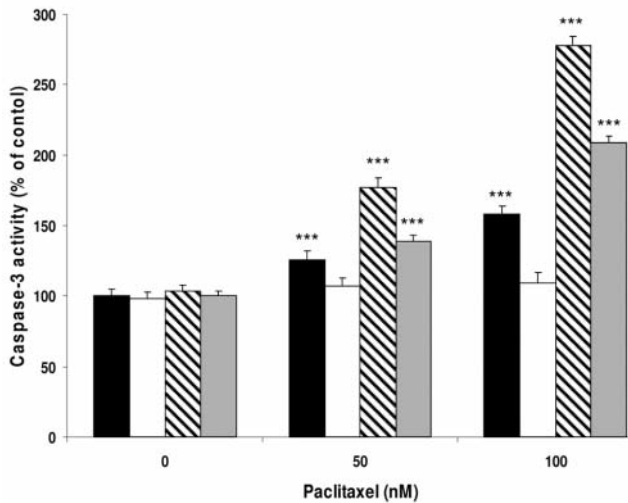
Figure 2. *Effect of PI-3K modulators on paclitaxel-induced caspase-3 activation in T24 cells. T24 cells were exposed for 16 h to 50 or 100 nM of paclitaxel alone (filled bars), or together with 10 μM of PJ34 (empty bars), 5 μM of LY-294002 (striped bars), or a combination of PJ34 and LY-294002 (gray bars). After subcellular fractionation, the cytosolic fractions of the cells were used for determination of caspase-3 activity by using a fluorescence plate reader and a fluorescent caspase-3 substrate, Ac-DEVD-AMC. Data represent means±S.E.M. of three independent experiments, each running in four replicates. ***p<0.001 significantly different from paclitaxel + PJ34 treated group.*
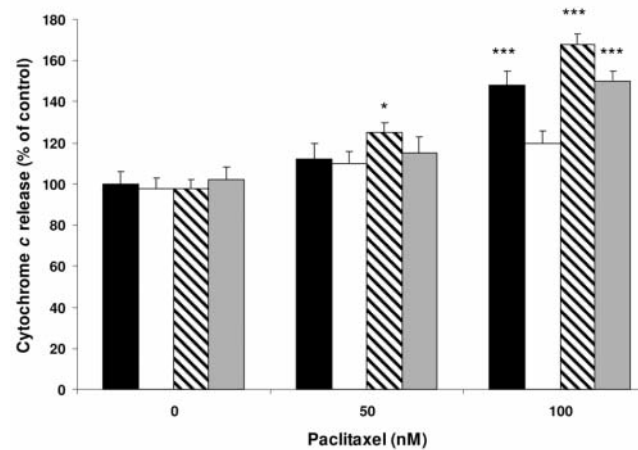

Figure 3. *Effect of PI-3K modulators on paclitaxel-iduced cytochrome c release in T24 cells. T24 cells were exposed for 16 h to 50 or 100 nM of paclitaxel alone (filled bars), or together with 10 μM of PJ34 (empty bars), 5 μM of LY-294002 (striped bars), or a combination of PJ34 and LY-294002 (gray bars). After subcellular fractionation, the cytosolic fraction of the cells was used for determination of cytochrome c release by using an HPLC method. Data represent means±S.E.M. of three independent experiments, each running in four replicates. *p<0.05, ***p<0.001 significantly different from paclitaxel + PJ34 treated group.*

Role of PI-3K-Akt pathway in paclitaxel induced cytochrome c release in T24 cells. As we found previously, paclitaxel induces mPT that could contribute to its apoptosis-inducing effect by releasing mitochondrial intermembrane pro-apoptotic proteins, such as cytochrome *c* (22). Accordingly, we determined cytochrome *c* release in the cytosolic fraction of T24 cells by using an HPLC method. In T24 bladder carcinoma cells, 16 h of 100 nM paclitaxel treatment resulted in a marked increase of released cytochrome c. This effect was significantly reduced ($p<0.001$) when the cells were pretreated with 10 μM PJ34. On the other hand, 5 μM of LY294002 significantly increased ($p<0.001$) paclitaxel-induced cytochrome *c* release and counteracted the effect of PJ34 (Figure 3). A similar pattern was observed when the cells were treated with 50 nM paclitaxel, however, the differences between the treated groups reached statistical significance only in the case of PI-3K inhibition ($p<0.05$).

Effect of Akt activation on Bad phosphorylation in paclitaxel-treated T24 cells. Glycogen synthase kinase 3β (GSK) and Bad are downstream targets for Akt, and Bad phosphorylation is an important mechanism in preservation of the integrity of mitochondrial membrane systems (23, 24). For these reasons, we studied the effect of paclitaxel, PJ34 and LY-294002 on expression level and phosphorylation of Akt, GSK and Bad.

Non of the treatments affected either Akt or Bad expression up to 6 h, hence total Akt and Bad immunoblot patterns were identical to that of the loading control actin (Figure 4). In paclitaxel-treated T24 cells, there was an early Akt(Ser[473]) phosphorylation that peaked at 3 h treatment, hence we studied the modulating effect of the other two drugs for this period of time. In complete agreement with their respective modulating effect on PI-3K, 5 μM LY-294002 markedly reduced while 10 μM PJ34 significantly increased Akt phosphorylation. The activation pattern of Akt was faithfully reflected in the phosphorylation of its downstream targets GSK(Ser[9]) and Bad(Ser[136]) as demonstrated by representative immunoblots shown in Figure 4. Since phosphorylation of Bad(Ser[136]) prevents its interaction with the mitochondrial membrane stabilizers Bcl-2 and Bcl-xL (23), these results suggest that Akt-mediated Bad phosphorylation represents an important mechanism in paclitaxel resistance in T24 bladder carcinoma cells.

Discussion

In the present report, we investigated the mechanisms involved in paclitaxel resistance in the T24 human bladder carcinoma cell line in order to improve therapeutic management of transitional cell carcinomas of the
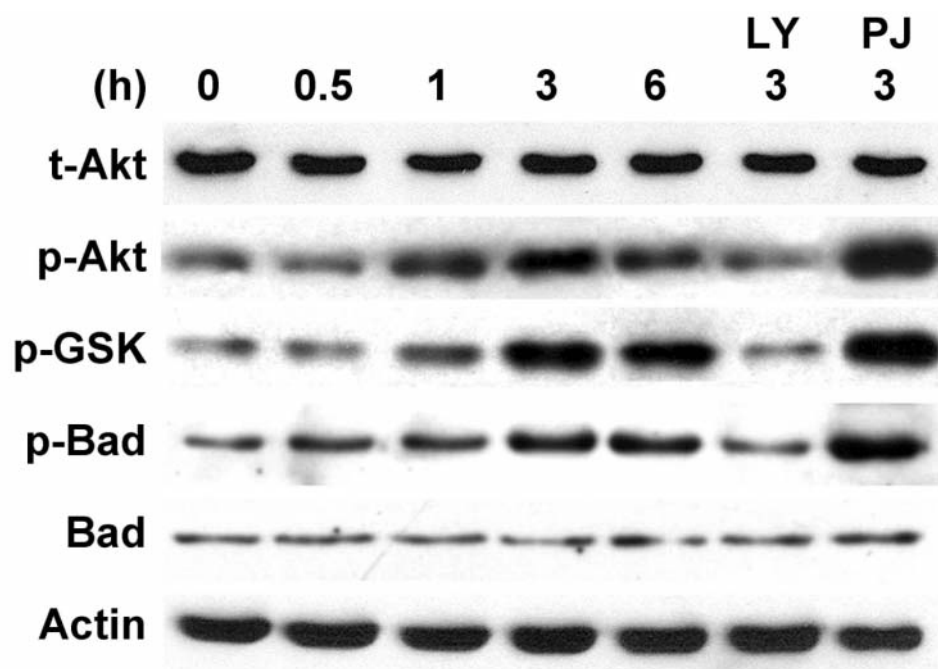
Figure 4. *Effect of PI-3K modulators on Akt and Bad phosphorylation in paclitaxel-treated T24 cells. T24 cells were treated with 100 nM of paclitaxel alone, or together with 5 μM LY294002 (LY), or 10 μM of PJ34 (PJ) for the indicated times (hours, h). Total cell extracts were analyzed by immunoblotting utilizing anti-Akt (t-AKT), anti-phospho-Akt (p-AKT), anti-phospho-GSK (p-GSK), anti-Bad (BAD) and anti-phospho-Bad (p-BAD) primary antibodies. Equal protein loading was confirmed by using anti-actin primary antibody and immunoblotting. Representative blots of three independent experiments are presented.*

urothelium. Several previous studies demonstrated that activation of the PI-3K-Akt system was strongly involved in mediating drug resistance under various conditions (10-14), thus we studied how activation and inhibition of this pathway modulates apoptotic effects of paclitaxel on this cell line. In order to inhibit the pathway, we used LY-294002, an inhibitor of PI-3K. Since PI-3K is an upstream activator of Akt, PI-3K-induced phosphorylation, that is activation of Akt, is fully inhibited in the presence of 5 μM of the drug. Although specificity and possible side-effects of a pharmacological agent is always an issue, LY 294002 has been reported to inhibit all isoforms of PI-3K while not affecting other kinases such as protein kinase C, protein kinase A, mitogen-activated protein kinases, S6 kinase, epidermal growth factor tyrosine kinase, c-src kinase, phosphatidylinositol-4 kinase and diacylglycerol kinase (25). In the absence of a commercially available activator, we used PJ34, an effective PARP-1 inhibitor for activating the PI-3K-Akt pathway. By inhibiting PARP-1, PJ34 indirectly activates Akt in various systems (19-21). Despite its accepted selectivity for PARP-1, it was reasonable to suppose that PJ34 might affect mPT since it interferes with the adenosine diphosphate (ADP) pocket of the enzyme (26) so it might have interacted with *e.g.* the adenine nucleotide translocase component of the mPT pore. We excluded this possibility by

mPT experiments utilizing isolated mitochondria. Although by its PARP-1 inhibiting and PI-3K-Akt pathway activating effects PJ34 attenuates oxidative damages in various systems, the molecule does not have considerable direct free-radical scavenging activity (26). Previously, we found that paclitaxel induced mPT and free radical formation (22), processes which could participate in its pro-apoptotic effect. PJ34 induced paclitaxel resistance by attenuating paclitaxel-induced apoptosis as was revealed by measuring cell viability and caspase-3 activation. On the other hand, LY-294002 enhanced the effect of paclitaxel on cell death and caspase-3 activation, and counteracted the paclitaxel resistance-inducing effect of PJ34, indicating that activation of the PI-3K-Akt pathway played a significant role.

During mPT, pro-apoptotic mitochondrial intermembrane proteins such as apoptosis-inducing factor, endonuclease G and cytochrome *c* are released that trigger caspase-independent and -dependent apoptosis, respectively (27). Furthermore, outer mitochondrial membrane permeabilization and cyclosporine A-independent cytochrome *c* release could be induced by pro-apoptotic Bcl-2 proteins (23, 28). We observed caspase-3-mediated apoptosis in paclitaxel-treated T24 cells that was attenuated by PJ34. Since PJ34 did not affect mPT nor scavenge free radicals, this antiapoptotic effect had to be the result of its direct inhibitory effect on

PARP-1 and of its indirect activatory effect on the PI-3K-Akt pathway. By inhibiting cytostatic-induced DNA damage, PARP-1 inhibition was reported to facilitate the efficacy of various cytostatic agents, even PARP-1 inhibition alone was found to be effective against breast cancer (BRCA) 1- and 2-deficient tumors (29). On the other hand, PARP-1 inhibition prevented depletion of its substrate, NAD^+, resulting in preservation of ATP energy and activation of the cytoprotective PI-3K-Akt pathway, both of which result in cytostatic resistance. NAD^+ depletion is usually associated with necrotic cell death (30), and inhibition of the PI-3K-Akt pathway by LY-294002 enhanced paclitaxel-induced caspase-3-mediated apoptosis, and antagonized the opposing effect of PJ34, indicating the significant involvement of the PI-3K-Akt pathway in the paclitaxel resistance-inducing effect of PJ34. Akt is a central regulator of cell survival. It induces phosphorylation of Bad at Ser^{136} that forms a complex with 14-3-3 protein in the cytosol, thereby preventing Bad binding to Bcl-2/Bcl-xL so promoting stabilization of the mitochondrial membrane system and cell survival (17, 31). We found that paclitaxel induced an early activation of Akt demonstrated by its phosphorylation, and by phosphorylation of its downstream targets GSK-3β and Bad. According to their respective theoretical roles, PJ34 activated and LY-294002 inhibited all these phosphorylations, indicating that Akt-mediated Bad phosphorylation and preservation of mitochondrial membrane integrity played significant roles in paclitaxel resistance of T24 cells.

Taken together, Bad phosphorylation resulting from activation of the PI-3K-Akt pathway plays a pivotal role in paclitaxel resistance of T24 cells, therefore, inhibition of this pathway could facilitate paclitaxel therapy in transitional cell carcinomas of the urothelium.

Acknowledgements

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