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The combination of an mTORc1/TORc2 inhibitor with lapatinib is synergistic in bladder cancer in vitro¹

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Abstract

Objective—To examine the ability of dual mTORc1/c2 inhibitors in conjunction with lapatinib to function in a synergistic manner to inhibit cell proliferation and anchorage-independent growth in bladder cancer cell lines.

Materials and methods—We examined patient tumor samples for overexpression of pS6, p4EBP1, pAkt, and phosphorylated epidermal growth factor receptor (pEGFR) using a tissue microarray containing 84 cases. Three bladder cancer cell lines, T24, HT1376, and UM-UC-3, were analyzed for cell proliferation after treatment with mTORc1/c2 inhibitors OSI-027 or PP242. Western blots were used to verify that the drugs were inhibiting phosphorylation of target proteins within the mTOR pathway, and they were compared with rapamycin inhibition. We also analyzed cell proliferation and anchorage-independent growth after treatment with OSI-027 and lapatinib in combination. PARP cleavage and autophagic flux were measured by examining levels of LC3B and p62 by western blotting.

Results—Tumor samples show increased expression of pEGFR (38% vs. 8%) and HER2 (38% vs. 4%) and decreased expression of pAkt S473 (7.5% vs. 29%) and pAkt T308 (50% vs. 84%) relative to normal tissue. Significant differences between normal and tumor samples for staining with pEGFR ($P = 0.0188$), HER 2 ($P = 0.0017$), pATK S473 ($P = 0.0128$), and pAkt T308 ($P = 0.0015$) is observed. Expression of proteins within the EGFR/HER2 pathway or within the mTOR pathway is correlated. No correlation was found between staining and tumor stage. OSI-027 and PP242 diminish cell proliferation in all 3 cell lines with IC₅₀ values ranging from 0.63 to 17.95 μ M. Both drugs inhibit phosphorylation of both mTORc1 and mTORc2 pathway components. OSI-027 and lapatinib inhibit cell proliferation and anchorage-independent growth in a synergistic

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manner. One cell line exhibited apoptosis in response to combination drug treatment, whereas the other 2 cell lines have increased levels of autophagy indicative of resistance to apoptosis.

Conclusions—The combination of OSI-027 and lapatinib results in antitumor synergy and further exploration of this combination should be undertaken.

Keywords

Bladder cancer; mTOR; EGFR; Lapatinib; Antitumor synergy

1. Introduction

Bladder cancer (BC) is the sixth most common cancer, with an annual estimated incidence of 73,510 with 14,880 deaths in the United States [1]. Radical cystectomy with or without chemotherapy is the usual treatment for muscle-invasive BC (MIBC). Surgical management of MIBC results in approximately 50% rate of distant metastasis and BC-related death [2,3]. The most common chemotherapy regimens are platinum-based combinations and show a survival benefit in clinical trials [4]. Side effects of chemotherapy are many and may be severe, e.g., bone marrow suppression, neuropathy, and nephropathy. Therefore, the search for less toxic agents continues.

The mTOR protein is a serine-threonine kinase that forms 2 protein complexes, mTORc1 and mTORc2, to regulate cell growth, motility, and proliferation [5]. BC tumors exhibit increased p4EBP1 T37/46 expression, a downstream substrate of mTORc1 [6,7]. There is conflicting evidence for overexpression of pS6 and p-mTOR [6–8]. Inhibition of mTORc1 by rapamycin or RAD001 in vitro reduces cell proliferation in BC and decreases pS6 [9–11]. In PTEN/p53 knockdown mice, an invasive BC model, activation of the mTORc1 pathway includes up-regulation of mTOR, p-mTOR, pAkt, and pS6, and rapamycin treatment decreased tumor growth and lowered expression of pS6 [12,13].

Dual mTORc1/c2 inhibitors are more effective in preventing cell proliferation than the rapalog class of inhibitors [14]. PP242, the first in this class of inhibitors, reduces the onset of disease and is synergistic with imatinib in a murine model of leukemia [15]. OSI-027 inhibits phosphorylation of the mTORc2 target Akt and mTORc1 targets S6K, S6 riboprotein, 4EBP1, and mTOR itself [16]. OSI-027 is effective in in vitro models of lymphoma, colon cancer, and breast cancer [16,17]. To date, no one has examined the efficacy of dual mTORc1/c2 inhibitors in BC despite evidence for misregulation of the mTOR pathway in BC.

The HER (ErbB) family consists of 4 receptor tyrosine kinases and this pathway is altered in BC [18]. Epidermal growth factor receptor (EGFR) is overexpressed in BC and overexpression is correlated with tumor stage, grade, and disease outcome [19]. Reports of HER2 overexpression are variable, but a recent large study found only 9.2% of muscle-invasive tumors overexpressed HER2 protein [20]. Lapatinib inhibits both EGFR and HER2 and has been tested in a clinical trial as a second-line therapy for advanced BC [21].

Given that both the mTOR and EGFR/HER2 pathways are misregulated in many BC tumors, we chose to examine the antitumor activity of mTORc1/c2 inhibitors combined with

lapatinib in BC in vitro models. Owing to feedback reactivation of Akt in the presence of some dual mTORc1/c2 inhibitors, combinations of mTORc1/c2 inhibitors with receptor tyrosine kinase (RTK) inhibitors increase efficacy [22–24]. We explored the effects of PP242 and OSI-027 on proliferation of BC cell lines and subsequently examined the combination effect of OSI-027 and lapatinib on cell proliferation, anchorage-independent growth, apoptosis, and autophagy.

2. Patients and methods

2.1. Tissue microarray (TMA) and immunohistochemistry

A TMA was made from archival formalin-fixed paraffin samples obtained at Mayo Clinic under Institutional Review Board approval and histologically verified by a pathologist (K.W.). A total of 84 cases of high-grade (World Health Organization 1973, grade 3) urothelial carcinoma were identified and 2 punches from each case were arrayed in paraffin. A paired normal single punch from 71 of the 84 samples was also arrayed. Population demographics are given in Table 1. TMA sections (5 µm) were deparaffinized, hydrated, subjected to antigen-retrieval solution (DakoCytomation, Denmark) with steam heat for 20 minutes, and then blocked with diluent containing background-reducing components (DakoCytomation). Immunostaining was performed with HER2 (DakoCytomation, prediluted); phosphorylated EGFR (pEGFR) Y1068 at 1:1,200; EGFR at 1:75 (Epitomics, Burlingame, CA); pAkt T308 at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA); pRPS6 S235/236 at 1:100, p4EBP1 T37/46 at 1:1,000, and pAkt S473 at 1:20 in high pH buffer (Cell Signaling, Danvers, MA). The EnVision Dual Labeled Polymer kit (DakoCytomation) was used according to the manufacturer's instructions for 3,3'-diaminobenzidine staining. Slides were lightly counterstained with Gill I hematoxylin, dehydrated, and mounted. Positive control sections were as follows: breast cancer tissue for pRPS6 S235/236 (cytoplasmic), p4EBP1 T37/46 (cytoplasmic), and HER2 (membrane); pancreatic cancer tissue for pEGFR Y1068 (membrane) and EGFR (membrane); and lung cancer tissue for pAkt S473 (nuclear and cytoplasmic) and pAkt T308 (nuclear). Negative sections were incubated with mouse or rabbit IgG replacing the primary antibody. Images were obtained at 20× using ScanScope XT (Aperio Technologies, Vista, CA), and staining was scored with an algorithm in the ImageScope software (Aperio Technologies) created by a histologist based on signal intensity (0, 1+, 2+, and 3+) and percentage of positive cells (0 < 5%, 1+ = 5%–20%, 2+ = 20%–50%, and 3+ > 50%). A score of 2+ or 3+ was used to indicate protein overexpression. Punches were excluded if insufficient tissue was present for scoring. If 2 tumor punches per patient were scorable, the scores of both tumor punches were averaged for statistical analysis.

2.2. Cell lines and cell culture

High-grade BC cell lines T24, HT1376, and UM-UC-3 were purchased from ATCC and STR verified to match the STR profiles provided (Genotyping Core Facility, Mayo Clinic, Rochester, MN). T24 cells were maintained in Roswell Park Memorial Institute medium containing 10% fetal bovine serum and 10 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 µg/ml amphotericin. HT1376 and UM-UC-3 were maintained in Minimum Essential Medium, supplemented with nonessential amino acids, 1 mM sodium pyruvate, 10% fetal

bovine serum, penicillin, streptomycin, and amphotericin. Cell lines were grown in the presence of 5% CO₂.

2.3. Reagents

PP242 was from Sigma (St. Louis, MO), OSI-027 from Selleck (Houston, TX), and lapatinib from LC Laboratories (Woburn, MA). Inhibitor stocks were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. Hydroxychloroquine (HCQ) (Fisher Scientific) was dissolved in water, filter sterilized, and stored at 4°C.

2.4. Cell proliferation assays

Cells were seeded at 20,000 per well in 12-well dishes and treated the next day with inhibitors. The final DMSO concentration was 0.1%. At 72 hours after treatment, cells were counted by Coulter counter. Results are presented as a percentage of DMSO-treated cells. Assays were performed in triplicate, with 3 replicates. For synergy studies, the dose of lapatinib was held constant at either 2.5 or 5 µM, while varying the amount of OSI-027. The assay was performed twice in triplicate. Single-drug IC₅₀ values, combinatorial index (CI) values for synergy, and the corresponding graphs were calculated with Calcsyn (BioSoft, Cambridge, UK).

2.5. Immunoblotting

Antibodies to p4EBP1 T37/46, S65, T70, 4EBP1, pRPS6 S235/236, S240/244, RPS6, pAkt S473, Akt, PARP, caspase 3, cleaved caspase 3, and LC3B were obtained from Cell Signaling. Antibody to p62 was obtained from Santa Cruz Biotechnology. GAPDH antibody was obtained from Millipore (Billerica, MA). Proteins were isolated in radio immunoprecipitation assay buffer containing Halt protease and phosphatase inhibitors (Pierce, Rockford, IL) and quantitated by bicinchoninic acid assay. Equal amounts of protein were separated on 4% to 12% Bis-Tris gels (Invitrogen, Grand Island, NY), transferred to polyvinylidene fluoride, blocked in 5% nonfat milk in TBST (50 mM Tris, 150 mM NaCl, and 0.1% Tween-20), and incubated overnight in primary antibodies as per manufacturer's instructions at 4°C. Blots were washed in TBST, incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized with SuperSignal WestPico chemiluminescent substrate (ThermoScientific) followed by exposure to x-ray film. Blots were quantitated with ImageJ (National Institute of Health).

2.6. Clonogenic assays

Cells were suspended in 0.75% low-melting-temperature agarose in supplemented Minimum Essential Medium and overlaid on a bottom agar layer of the same composition. HT1376 cells were plated at 1,500 per 60-mm dish. UM-UC-3 cells were plated at 800 per well in 6-well dishes. OSI-027 or lapatinib or both were added to both the bottom and top agarose layers. Drugs were added weekly by dilution in medium and overlaying each dish. Colonies were stained at 3 to 4 weeks after plating with 0.005% crystal violet and were counted after photographing. Data are reported as a percentage of DMSO controls. CI values were calculated with Calcsyn.

2.7. Statistics

Spearman Rank Correlation was used to determine correlation between immunohistochemical stains. Correlation between stage and immunohistochemical stain was determined with Fisher exact test. The difference between staining of normal and tumor tissue was analyzed with the Mann-Whitney *U* test. In all cases, $P < 0.05$ was considered significant.

3. Results

3.1. Expression of mTOR and EGFR pathway components in patient samples

Representative staining of the TMA is shown in Fig. 1. Significant differences between normal and tumor samples for staining with pEGFR ($P = 0.0188$), HER 2 ($P = 0.0017$), pAkt S473 ($P = 0.0128$), and pAkt T308 ($P = 0.0015$) were found. High levels of pEGFR, as defined by scores 2+, were seen in 38% (30/79) of tumors vs. 8% (2/25) of normal tissues. HER2 was highly expressed in 38% (30/79) of tumors vs. 4% (1/24) of normal tissue samples. Conversely, the number of tumors overexpressing either pAkt S473 or pAkt T308 was decreased compared with normal, 7.5% (6/80) vs. 29% (7/24) for S473 and 50% (39/78) vs. 84% (16/19) for T308.

Correlations between staining patterns were examined and data are shown in Table 2. Correlations between the following proteins were seen: EGFR and pEGFR; HER2 and EGFR; HER2 and pAkt T308; HER2 and pRPS6; pAkt S473 and p4EBP1; pAkt S473 and pAkt T308; pAkt T308 and p4EBP1; pRPS6 and p4EBP1; and pRPS6 and pAkt S473. No correlations between tumor stage T2 ($n = 15$), T3 ($n = 44$), and T4 ($n = 18$), and staining were found.

3.2. Dual mTOR inhibitors inhibit cell proliferation

We examined whether OSI-027 and PP242 would inhibit BC cell growth. Both inhibitors reduce the proliferation of BC cell lines in a dose-dependent fashion (Fig. 2) with IC_{50} values in the low micromolar range (Table 3), suggesting that dual mTOR inhibitors might be effective treatments for BC.

3.3. OSI-027 and PP242 target mTORc1 and mTORc2

We compared the specificity of OSI-027, PP242, and rapamycin to target components of the mTORc1/c2 pathways by analyzing the phosphorylation status of those proteins. Phosphorylation of 4EBP1 is decreased at an early time point and continues to be suppressed at 24 hours by OSI-027 or PP242. Phosphorylation inhibition by rapamycin is not as robust at either time point (Fig. 3A). Phosphorylation of RPS6, another direct downstream target of mTORc1, is decreased by all 3 inhibitors at the later time point but only in UM-UC-3 cells at the early time point (Fig. 3B).

Both OSI-027 and PP242 completely abolish phosphorylation of Akt at S473 at 1 hour after treatment, demonstrating mTORc2 inhibition, and this effect is sustained for 24 hours. After prolonged incubation with rapamycin, Akt S473 phosphorylation levels decline (Fig. 3C).

Thus, in comparison with rapamycin, OSI-027 and PP242 target both mTORc1 and mTORc2 in BC cell lines.

3.4. OSI-027 and lapatinib act synergistically

HER2 and pEGFR expressions are up-regulated in BC, making them potential therapeutic targets. Lapatinib targets both EGFR and HER2 and synergizes with OSI-027 to reduce cell proliferation. Different drug ratios were tested for synergy by cell proliferation (Fig. 4A, C, and E). CI values for each ratio were calculated (Fig. 4B, D, and F). $CI < 1$ indicates synergy and $CI = 1$ suggests an additive effect [25]. CI values in the cell proliferation assay were less than 0.2 for HT1376, less than 0.55 for T24, and between 0.65 and 1.1 for UM-UC-3.

The ability of many cancer cell lines to grow in an anchorage-independent fashion is used as a hallmark of cancer invasiveness. The combination of OSI-027 plus lapatinib is highly effective at reducing colony formation in HT1376 and UM-UC-3 cells. CI values for different drug doses in both cell lines (Supplemental Table 1) indicate strong synergy. CI values for HT1376 range from 0.128 to 1.075 with an average of 0.374. For UM-UC-3 cells, the range was 0.177 to 1.383 with an average of 0.54, a value lower than the lowest CI value derived from the cell proliferation assay. CI values varied with both dose and drug ratio.

3.5. Evidence of apoptosis and autophagy as a result of combination therapy

We explored whether the OSI-027 and lapatinib combination was inducing apoptosis as a mechanism of cell death. HT1376 cells showed evidence of increased apoptosis as evaluated by PARP and caspase 3 cleavage in response to either lapatinib or combination treatment (Fig. 5A). Neither T24 nor UM-UC-3 cells exhibited increased levels of apoptosis. T24 cells showed a background level of apoptosis with all treatment conditions, whereas UM-UC-3 cells did not exhibit any signs of apoptosis. We evaluated whether autophagy was induced by the drug combination and found that all of the cell lines demonstrated increased levels of LC3B-II in response to combination treatment, indicative of increased autophagy. As a measure of autophagic flux, we blocked autophagy with HCQ and found that there is a basal increase in the amount of LC3B-II present in DMSO-treated cells. Additionally, there is increased accumulation of LC3B-II in all cell lines treated with HCQ regardless of additional drug treatment, indicating autophagic flux and a properly functioning autophagy pathway (Fig. 5B–D). The combination drug treatment plus HCQ had no effect on the levels of p62 in HT1376 and UM-UC-3 cells but increased the level of p62 in T24 cells. Blocking autophagy with HCQ did not induce apoptosis in T24 or UM-UC-3 cells as measured by PARP cleavage, whereas levels of PARP cleavage in HT1376 cells were slightly increased by HCQ.

4. Discussion

Treatment options for BC have not changed significantly in more than a decade and more effective therapies with better side effect profiles are needed for MIBC. A number of BC tumors overexpressed the mTOR pathway components pRPS6 (22%) and p4EPB1 (42%); however, expression was not significantly different than normal tissue. These levels are similar to what has been previously reported [7]. Despite having 71 normal samples, only a

small number of those were scorable owing to a lack of sufficient epithelial tissue in many samples, which may account for the lack of significant difference between tumor and normal staining in some cases.

Positive correlations for staining were found between members of the mTOR pathway, p4EBP1, and pRPS6. Activation of Akt (T308 and S473) was correlated with 4EBP1 staining, whereas only Akt S473 staining was correlated with pRPS6. Akt activation would be predicted to increase the levels of both pRPS6 and p4EBP1, and these correlations indicate that the Akt/mTOR pathway is a viable therapeutic target in BC.

EGFR was overexpressed in a large number of tumor samples (76.5%), although we found no correlation with tumor grade in contrast to other reports [19]. The lack of correlation of any stain with tumor grade is likely the result of the small sample size for T2 and T4 tumors. EGFR expression was strongly correlated with expression of pEGFR and negatively correlated with HER2 expression. Although pEGFR and HER2 were overexpressed compared with normal tissue, there was no correlation between these 2 stains, perhaps indicating a difference in tumor types.

Our results indicate that targeting both the mTOR pathway and EGFR/HER2 produces a synergistic combination with potential for BC treatment both chemotherapeutically and intravesically. Currently, a phase I trial of OSI-027 is underway (NCT 00698243) for solid tumors or lymphoma. We demonstrate that dual mTORc1/c2 inhibitors reduce BC cell proliferation. Combining OSI-027 with lapatinib produces a highly synergistic combination, reducing both cell proliferation and anchorage-independent growth. Our rationale for adding an EGFR/HER2 inhibitor to the mTOR inhibitor was based on reports that inhibition of mTOR can cause feedback activation of Akt via receptor tyrosine kinases [22].

We are the first to report the combination of a dual mTORc1/c2 inhibitor and an EGFR/HER2 inhibitor in BC, although other drugs from these classes have been combined to treat animal models of breast cancer and leukemia [15,22,23]. OSI-027 combined with erlotinib reduced the proliferation of head and neck cancers in vitro, whereas OSI-027 and cetuximab reduced tumor growth in mouse models [24]. Clearly, a number of drug combinations from within the mTORc1/c2 and RTK categories can be effective, and the optimal choice of drug combination and drug ratio would need to be determined experimentally based on the genetic makeup of each type of cancer.

The molecular mechanism that produces synergy between lapatinib and OSI-027 in BC is not clear. OSI-027 induced apoptosis in 3 of 22 cell lines in one study and caused apoptosis in lymphoma cells in a separate study [16,17]. Apoptosis does not appear to be a ubiquitous response to this drug. Our BC cell line with the highest IC₅₀ value for OSI-027, HT1376, also exhibited the highest degree of synergy, and demonstrated apoptosis in response to the drug combination.

The use of mTOR inhibitors alone is expected to increase the levels of autophagy owing to the negative regulation of mTOR on the autophagy pathway. The mTOR inhibitor AZD8055 induces protective autophagy in colon cancer cells, decreasing the effectiveness of chemotherapy [26]. Tumors containing Ras mutations require autophagy for survival and

have an elevated level of basal autophagy [27]. Both T24 and UM-UC-3 cells have Ras mutations but HT1376 does not [28]. In T24 and UM-UC-3 cells, we observed a background level of autophagy in the absence of drug treatment. Ras mutations are found in only 11% of BC tumor samples suggesting that combination drug treatment would potentially be effective in many more cases than not [29]. Because the levels of synergy observed for T24 and UM-UC-3 cells were lower than those seen for HT1376, we propose that these cells are using autophagy as a protective mechanism, and that if apoptosis levels can be increased, then levels of synergy would also increase. The role autophagy plays in cancer is a confusing one and an area that has not been adequately explored in BC. In some cases, autophagy protects tumor cells from cell death [26,30,31]. Drugs that can attenuate autophagy might sensitize cells toward cellular death, and a number of clinical trials are underway to address this question. A better understanding of the genetic basis underlying the apoptotic or autophagy response in different tumor types is needed.

5. Conclusions

The combination of OSI-027 and lapatinib is a highly synergistic drug combination in BC. Both OSI-027 and lapatinib are orally available, making them an easily administered and logical chemotherapeutic approach to MIBC. This combinatorial therapy bears further study in both xenograft and orthotopic models of BC as a potential novel therapeutic strategy in the treatment of BC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.urolonc.2013.06.004>

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Fig. 1. IHC staining of patient tumor and normal samples. Representative patient tumor (T) with paired normal (N) tissue stained as indicated. All tumor samples shown are T3. Scale bar = 100 μ m. IHC = immunohistochemical.

Fig. 2. Dual mTOR inhibitors inhibit bladder cancer cell growth in a dose-dependent manner. HT1376, T24, and UM-UC-3 cells were treated for 72 hours with either OSI-027 or PP242 and then counted via Coulter counter. Results are expressed as a percentage of DMSO control. Three replicate experiments were performed in triplicate. (A) Dose-response curves for OSI-027 for treatments from 25 to 0.1 μM . (B) Dose-response curves for PP242 treatments from 2 to 0.1 μM .

Fig. 3.

Phosphorylation of mTOR downstream targets 4EBP1, RPS6, and Akt is inhibited by mTORc1/c2 inhibitors. Cells were treated for either 1 or 24 hours with OSI-027, PP242, or rapamycin. Both 4EBP1 and RPS6 are mTORc1 targets, Akt is an mTORc2 target. (A) 4EBP1: phosphorylation at T37/46, S65, and T70 is greatly reduced by OSI-027 and PP242. The effect with rapamycin is reduced and variable. Total 4EBP1 levels are unchanged. (B) RPS6: pRPS6 S235/236 and S240/244 are both reduced by all 3 inhibitors at 24 hours. (C) Akt: pAkt S273 levels are reduced by OSI-027 and PP242 but not affected by rapamycin.

Fig. 4. Inhibition of mTORc1/c2 and EGFR produces a synergistic combination. Cell proliferation assays were performed after 72-hours treatment with OSI-027, lapatinib, or both drugs. For combination studies, the dose of lapatinib was held constant at either 5 or 2.5 μ M as indicated in the legend, whereas the dose of OSI-027 was varied and is plotted on the x-axis. (A) HT1376, (C) T24, (E) UM-UC-3. Corresponding CI values were calculated and are graphed in (B) HT1376, (D) T24, (F) UM-UC-3. Values less than 1 represent synergy whereas a value of 1 is additive.

Fig. 5.

Evidence for apoptosis and autophagy. Cells were treated for 24 hours with OSI-027 or lapatinib or both and proteins were isolated. PARP and caspase 3 cleavage were indicators of apoptosis and LC3B as an indicator of autophagy. (A) Only HT1376 cells showed increased evidence of apoptosis after treatment. (B–D) Cells were treated with HCQ in addition to OSI-027 or lapatinib and the resultant protein was analyzed via immunoblotting. Blots were quantitated with ImageJ, and the results shown in the graph. The signal for each probe was normalized to GAPDH. Treatment of cells with HCQ increased the levels of

LC3B-II; however, it did not significantly alter the levels of apoptosis seen. Increased levels of both LC3B-II and p62 indicate autophagic flux.

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Table 1

Demographics of patient samples

	Total (%)
No. of patients	84 (100)
Age (mean $\pm$ SD)	73.9 $\pm$ 9
Male/female	64/20
T-stage	
pTis	2 (2.3)
pT2	17 (20.2)
pT3	47 (55.9)
pT4	18 (21.4)
Grade	
High grade	84

SD = standard deviation.

Table 2

Spearman rank correlation coefficients between IHC stains

	pEGFR	EGFR	p4EBP1	pAKT S473	pAKT T308	pRPS6	HER2
pEGFR	r	0.7630	0.0866	-0.0326	-0.0426	0.1164	-0.1930
	p	1.92E-16	0.4450	0.7753	0.7094	0.3069	0.0884
EGFR	r		0.0969	-0.0579	-0.0823	0.1141	-0.3197
	p		0.3956	0.6078	0.4652	0.3103	0.0036
p4EBP1	r			0.4606	0.5830	0.3950	0.0861
	p			1.95E-05	1.72E-08	0.0003	0.4508
pAKT S473	r				0.4273	0.3609	0.1272
	p				8.60E-05	0.0011	0.2607
pAKT T308	r					0.1753	0.2947
	p					0.1198	0.0080
pRPS6	r						0.7630
	p						1.92E-16
HER2	r						
	p						

IHC = immunohistochemical; r = Spearman Rank Correlation Coefficient; p = P value. Bolded P values are significant at $P < 0.05$.

Table 3IC₅₀ values for PP242 and OSI-027 in Bladder Cancer Cell Lines

	PP242 (μM)	OSI-027 (μM)
HT1376	1.88 ± 1.1	17.95 ± 1.7
T24	1.37 ± 0.4	3.31 ± 1.3
UM-UC-3	0.63 ± 0.1	4.14 ± 0.8