Selective targeting of lectins and their macropinocytosis in urothelial tumours: translation from in vitro to ex vivo

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Abstract

Urinary bladder cancer can be treated by intravesical application of therapeutic agents, but the specific targeting of cancer urothelial cells and the endocytotic pathways of the agents are not known. During carcinogenesis, the superficial urothelial cells exhibit changes in sugar residues on the apical plasma membranes. This can be exploited for selective targeting from the luminal side of the bladder. Here we show that the plant lectins Jacalin (from *Artocarpus integrifolia*), ACA (from *Amaranthus caudatus*) and DSA (from *Datura stramonium*) selectively bind to the apical plasma membrane of low-grade (RT4) and high-grade (T24) cancer urothelial cells *in vitro* and urothelial tumours *ex vivo*. The amount of lectin binding was significantly different between RT4 and T24 cells. Endocytosis of lectins was observed only in cancer urothelial cells and not in normal urothelial cells. Transmission electron microscopy analysis showed macropinosomes, endosome-like vesicles and multivesicular bodies filled with lectins in RT4 and T24 cells and also in cells of urothelial tumours *ex vivo*. Endocytosis of Jacalin and ACA in cancer cells was decreased *in vitro* after addition of inhibitor of macropinocytosis 5-(N-ethyl-N-isopropyl) amiloride (EIPA) and increased after stimulation of macropinocytosis with epidermal growth factor (EGF). Clathrin, caveolin and flotillin did not colocalise with lectins. These results confirm that the predominant mechanism of lectin endocytosis in cancer urothelial cells is macropinocytosis. Therefore, we propose that lectins in combination with conjugated therapeutic agents are promising tools for improved intravesical therapy by targeting cancer cells.

Introduction

The urothelium is the stratified epithelium of the urinary bladder and is the main origin of cancer transformation leading to bladder cancer (Shin et al. 2014). In the healthy bladder, the highly differentiated superficial umbrella cells express large amounts of four uroplakins (UPIa, UPIb, UPII and UPIIIa) that form urothelial plaques in the apical plasma membrane (Yu et al. 1994; Wu et al. 1994a; Wu et al. 1990; Hudoklin et al. 2011; Kachar et al. 1999; Porter et al. 1967). UPII is not glycosylated, whereas UPIa, UPIb and UPIIIa are all glycosylated with N-linked glycans (Yu et al. 1994; Wu and Sun 1993). UPIa and UPIb are glycosylated by mannose-rich structures and complex glycans, respectively (Xie et al. 2006). UPIIIa contain complex glycans capped by sialic acid in α(2,3)- and α(2,6)-linkage with galactose (Gal) and by the Gal-α(1,3)-Gal (Malagolini et al. 2000). UP expression and glycosylation profiles are altered during urothelial preneoplastic changes and early urothelial carcinogenesis and are downregulated at later stages of urothelial carcinogenesis (Zupancic 2013; Zupancic et al. 2020; Zupančič et al. 2011a; Ogawa et al. 1999; Wu et al. 1998; Zupančič et al. 2011b; Kątnik-Prastowska et al. 2014). It is not known how these particular glycans are altered during bladder carcinogenesis, but it seems that the proteoglycans, glycoproteins and glycosphingolipids, together with their ligands, play a crucial role in the malignant transformation, invasion and metastasis of bladder cancer (Ohyama 2008) and could be exploited for targeted drug delivery by endocytosis into cancer urothelial cells.

Bladder cancer is divided into non-muscle invasive (NMIBC) and muscle invasive bladder cancer (MIBC), while benign lesions are usually papillomas. NMIBC tumours are confined to the urothelium (stage Ta) or

invade the lamina propria below the urothelium (stage T1). MIBC invade the muscle (stage T2) or perivesical adipose tissue and sometimes adjacent organs (stage T3 or T4, respectively). Useful *in vitro* models for NMIBC (stage Ta) are the low-grade human urothelial cell line RT4 and for MIBC (stage T2) the high-grade cell line T24. Despite successful removal of NMIBC tumours by transurethral resection of the bladder (TURB) and intravesical instillation of mitomycin C or Bacillus Calmette Guerin (BCG), the recurrence rate is high (50–70%) (Ritch et al. 2020). Approximately 20% of recurrences progress to MIBC, which has a high risk of metastasis with poor prognosis (Aragon-Ching et al. 2018). Despite numerous novel experimental approaches, there has been little progress in developing more selective therapies that could reduce local or systemic side effects. Therefore, more targeted delivery of cytotoxic drugs to cancer cells is beneficial to avoid drug toxicity and improve drug efficacy. In the case of bladder cancer, of which more than 75% are NMIBC at initial diagnosis (Kamat et al. 2016), it is possible to use the intravesical route for targeted delivery of site-specific drugs that could be administered through a catheter to avoid systemic use.

Intensive research is being conducted on various strategies for targeted drug delivery to urothelial tumours (Zacchè et al. 2015; Yoon et al. 2020; Heath and Rosenberg 2021). Among them, plant lectins such as WGA (wheat germ agglutinin from *Triticum vulgaris*), Jacalin (from *Artocarpus integrifolia*), ACA (from *Amaranthus caudatus*) and DSA (from *Datura stramonium*) are being investigated and proposed as a promising approach for selective drug delivery to bladder cancer cells due to changes in sugar residues on the cell surface (Neutsch et al. 2014, 2012; Neutsch et al. 2011; Neutsch et al. 2013; Plattner et al. 2008; Zupancic et al. 2014; Kreft et al. 2009b; Azevedo et al. 2017). Jacalin and ACA show high affinity for T-antigen (Core1) and Tn-antigen (alpha-N-acetylgalactosamine)-bound peptides and Jacalin also for Core3 and sialyl-T (ST)-bound peptides (Tachibana et al. 2006). DSA recognises galactosyl(β-1,4)N-acetylglucosamine oligomers and prefers chitobiose or chitotriose over a single *N*-acetylglucosamine residue. We have demonstrated in the rodent bladder cancer model that Jacalin, ACA and DSA show specific targeting of transformed cells. Jacalin and DSA discriminated between normal and cancer urothelial cells, while ACA showed differential binding during cancer progression (Zupancic et al. 2014).

It is known that cancer cells from different types of tumours engulf large amounts of extracellular fluid, particulate cargo and membranes with specific sugar residues (Xiao et al. 2021). We have shown that poorly differentiated urothelial cells have increased endocytotic activity compared to highly differentiated superficial urothelial cells (Kreft et al. 2009b; Tratnjek et al. 2017; Lojk et al. 2018). Since cancer urothelial cells and superficial cells of tumours do not reach terminal differentiation, it is important to understand the mechanism of lectin endocytosis in order to use them as targeted drug delivery system.

To investigate the selective targeting and endocytosis mechanism of lectins, the lectins Jacalin, ACA and DSA with different carbohydrate specificities were selected. To increase the significance of our results and bring them closer to a possible clinical application in urology, we combined experiments on urothelial cancer cells *in vitro* with urothelial tumours *ex vivo*. Our results show that lectins can be used for selective targeting of urothelial tumours and that the main mechanism of lectin endocytosis is macropinocytosis.

Material And Methods

Materials

FITC-conjugated lectins from *Artocarpus integrifolia* (Jacalin; FL-1151) and from *Datura stramonium* (DSA; FL-1181) were purchased from Vector Laboratories (Burlingame, USA) and FITC-conjugated lectins from *Amaranthus caudatus* (ACA; 21510290) were purchased from GlycoMatrix (Dublin Ohio, USA). Colloidal gold-conjugated lectins Jacalin-15 nm gold (GP-6301-15), ACA-10 nm gold (GP-8201-10), and DSA-5 nm gold (GP-5701-5) were purchased from EY Laboratories (San Mateo, USA). Rabbit polyclonal antibody anti-UP was a kind gift from Prof. Dr. Tung-Tien Sun, Department of Cell Biology, New York University Medical School (Wu et al. 1990). The following rabbit polyclonal antibodies were also used: anti-flotillin (ab41927, Abcam), anti-clathrin (610499, BD Transductions) and anti-caveolin (ab18199, Abcam). Goat anti-rabbit AlexaFluor 488 (A11008, Invitrogen) and goat anti-rabbit AlexaFluor 555 (A21428, Invitrogen) were used as secondary antibodies. Vectashield mounting medium with DAPI was obtained from Vector Laboratories (Burlingame, USA).

Cell cultures for *in vitro* experiments

Human bladder cancer cell lines RT4 and T24 (ATTC, Manassas, VA) were grown in Advanced Dulbecco's Modified Eagle's Medium (A-DMEM) / F12 medium (1:1) supplemented with 5% FBS, 10,000 U/mL penicillin and 10,000 µg/mL streptomycin (Gibco, Invitrogen, Vienna, Austria) at 37°C in a humidified atmosphere containing 5% CO_2. Experiments were performed after growing RT4 and T24 cells (5×10^4 cells/cm^2) for 1 week. Secondary cultures of normal porcine urothelial cells (NPU) were prepared from porcine urinary bladders (Višnjar et al. 2017; Visnjar et al. 2012). Briefly, NPU cells were cultured in UroM medium containing MCDB153 (Sigma, Taufkirchen, Germany)/A-DMEM (1:1), 2.5% FBS, 0.1 mM phosphoethanolamine (Sigma), 0.5 µg/mL hydrocortisone (Sigma), 5 µg/mL insulin (Sigma), 4 mM glutamax, 10,000 U/mL penicillin, and 10,000 µg/mL streptomycin solution. NPU cells (1×10^5 cells/cm^2) were grown to confluence in UroM medium and then for a further 3 weeks in UroM medium containing 2.7 mM calcium and no FBS to initiate urothelial differentiation. The cell cultures were used for immunofluorescence and *in vitro* lectin binding assays as well as for endocytosis analyses.

Patients and sampling for *ex vivo* experiments

The study was conducted in accordance with the 1964 Helsinki Declaration and its later amendments and approved by the National Medical Ethics Committee of Slovenia, No. 101/11/14. The study population consisted of 10 patients with bladder cancer who underwent TURB in the Department of Urology, University Medical Centre Ljubljana, Slovenia. Informed consent was obtained from all patients prior to tissue retrieval.

For immunofluorescence and lectin binding assays, two samples were obtained from each patient by cold cup biopsies: i) the urothelial tumour and ii) the cystoscopically normal urothelium 2 cm away from the tumour. For transmission electron microscopy (TEM), immunoelectron microscopy and *ex vivo* lectin analysis of endocytosis, a sample of the urothelial tumour was collected from each patient by cold cup biopsies. The biopsies captured the urothelium and lamina propria. For pathological staging and grading of all samples, the pathologist used the AJCC cancer staging manual (Amin et al. 2017). Urothelial cancers were diagnosed as papilloma (1 sample), non-invasive low-grade urothelial carcinoma - Ta, l. g. (3 samples), invasive low-grade urothelial carcinoma with invasion into the lamina propria - T1, l. g. (2 samples), invasive urothelial carcinoma high-grade with invasion into the lamina propria - T1, h. g. (2 samples), and invasive urothelial carcinoma high-grade with invasion into the muscularis propria – T2, h. g. (2 samples). Of ten cystoscopically normal urothelial specimens, six specimens were classified as normal because histopathological examination revealed that they showed no signs of hyperplasia or dysplasia.

Lectin binding assay

In vitro

NPU, RT4, and T24 cells were cooled to 4°C, washed with ice-cold PBS and incubated with FITC-conjugated lectins (20 µg/ml) for 30 min at 4°C in the dark. Then cells were washed three times with PBS. Cells were then either fixed with 4% formaldehyde for 15 min at 4°C to perform fluorescence microscopy and immunofluorescence of uroplakins, or fluorescence was measured in live cells to determine the amount of lectin binding.

Binding of lectins to the apical plasma membrane was imaged with a fluorescence microscope (AxioImager Z.1, Zeiss, Germany) using an oil immersion objective (63×oil/NA 1.40) and optical sectioning (ApoTome, Zeiss).

To determine the amount of lectin binding in live cells, the intensity of fluorescence was measured using a microplate reader (Saphire2, Tecan, Mannedorf, Switzerland). The amount of lectin binding to the cells was presented as the average of the absolute fluorescence intensity (a.u.) in each culture. The measurements were performed at least in three independent experiments, each experiment in triplicate.

Ex vivo

Biopsy samples were incubated with FITC-conjugated lectins (20 µg/ml) in UroM medium. After incubation in the dark for 30 min at 4°C, the samples were washed with PBS. Samples were then fixed in 4% formaldehyde for 30 min at 4°C, washed in PBS, placed in OCT embedding medium (Tissue Tek, The Netherlands) and frozen in liquid nitrogen. The frozen tissue blocks were cut perpendicular to the luminal surface of the urothelium, 7 µm serial sections were made and air-dried for 2 h at room temperature.

The first cryosection of each sample was mounted in Vectashield-DAPI and imaged using a fluorescence microscope (Eclipse TE300, Nikon, Japan). Consecutive cryosections from each biopsy sample were used

for immunofluorescence.

Immunofluorescence of uroplakins

In vitro

NPU, RT4 and T24 cells were fixed with 4% formaldehyde for 15 min at 4°C and then incubated with 0.5% BSA, 0.1% saponin, 0.1% gelatin, 50 mM NH_4Cl, 0.02% NaN for 30 min to block non-specific labelling. Then the cells were incubated with anti-UPs primary antibody (1:1,000). Cells were then washed with PBS and incubated with AlexaFluor 555-conjugated goat anti-rabbit secondary antibody (1:400) for 1 h at 37°C. Cells were mounted in Vectashield-DAPI and imaged with a fluorescence microscope (AxioImager Z.1).

Ex vivo

Cryosections obtained after the *ex vivo* lectin binding assay were washed in PBS and pre-incubated with 5% fetal calf serum (FCS) and 1% BSA in PBS for 1 h at 37°C. Then the sections were incubated with the anti-UP antibody (1:1,000) in 1% BSA in PBS at 4°C overnight. For the negative controls, incubation with the primary antibody was omitted or the specific antibody was replaced with a non-relevant antibody. After washing, sections were incubated with AlexaFluor 555-conjugated goat anti-rabbit secondary antibody (1:400) for 1 h at room temperature, washed in PBS and mounted in Vectashield-DAPI. Cryosections were examined with a fluorescence microscope (Eclipse TE300).

Immunoelectron microscopy of uroplakins

Biopsies were cut into 1 mm^3 blocks and fixed in 2% formaldehyde and 0.05% glutaraldehyde for 1h at RT. Samples were dehydrated in 30% ethanol at 0°C, 55% ethanol at -15°C, 70% ethanol at -30°C, 100% ethanol at -50°C, and gradually infiltrated in 100% Lowicryl HM20 (Polysciences). Samples were polymerized under UV light. Ultrathin sections were cut and collected on gold grids. Nonspecific labelling was blocked in PBS buffer containing 0.1% fish gelatine, 0.8% BSA and 5% fetal calf serum. Then UPs were immunolocalized by anti-UP antibody (1:5000). After washing with PBS, UPs were detected with anti-rabbit IgG secondary antibodies conjugated to 5-nm or 10-nm gold. Sections were washed in PBS followed by distilled water. Five nm gold was silver-enhanced for 4 min with IntenSE (Amersham, UK). Ultrathin sections were counterstained with uranyl acetate and lead citrate (Merck, Darmstadt, Germany). For negative controls the protocols were the same, except omitting the primary antibody or incubating the sections with rabbit serum. Sections were analysed with transmission electron microscope (Philips CM100, The Netherlands) at 80 kV and micrographs were taken with an AMT camera (Advanced Microscopy Techniques Corp., Woburn, MA, USA).

Endocytosis analysis

Endocytosis assays and immunofluorescence in vitro

To investigate flotillin-, caveolin- and clathrin-mediated endocytosis, NPU, RT4 and T24 cells were incubated with FITC-conjugated lectins for 1 h at 37°C. Cells were washed with PBS and fixed in 4% formaldehyde in PBS for 15 min at 4°C. After blocking with 0.5% BSA, 0.1% saponin, 0.1% gelatin, 50 mM NH4Cl, 0.02% NaN for 30 min, the cells were incubated with rabbit polyclonal anti-flotillin antibody (1:200), rabbit polyclonal anti-caveolin antibody (1:200) and mouse monoclonal anti-clathrin antibody (1:200) for 1h at 37°C. Cells were then washed with PBS and incubated with AlexaFluor555-conjugated anti-rabbit and anti-mouse secondary antibodies (1:400) for 30 min at 37°C. Cells were mounted in Vectashield-DAPI and imaged using a fluorescence microscope (AxioImager Z.1).

To analyse macropinocytosis of lectins, RT4 and T24 cells were incubated with each FITC-conjugated lectin and 0.5 mg/mL TRITC-dextran (3kDa, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at 37°C. Cells were fixed with 4% formaldehyde for 15 min at 4°C, washed and embedded in Vectashield-DAPI. Imaging was performed using a fluorescence microscope (AxioImager Z.1).

To inhibit macropinocytosis, 50 µM EIPA (5-(N-ethyl-N-isopropyl)amirolide, A3085, Sigma) or 100 µM Dyngo (ab120689, Abcam) was added to T24 and RT4 cells prior to incubation with the FITC-conjugated lectins for 1 h at 37°C and during incubation with the lectins for 1 h at 37°C. To stimulate macropinocytosis, 500 nM EGF was added before incubation with the FITC-conjugated lectins and during the 1-h incubation with the lectins at 37°C for 1 h. As a negative control, cells were incubated with FITC-conjugated lectins only.

The intensity of FITC fluorescence was measured using a microplate reader (Saphire2). The amount of lectins in the cells was expressed as the ratio between the average fluorescence intensity after treatment and an average fluorescence intensity of the control cells.

Endocytosis analysis by transmission electron microscopy in vitro and ex vivo

Biopsy samples and RT4 and T24 cells were incubated with gold-conjugated lectins (1 µg/ml) for 1 h at 37°C. They were washed in PBS and fixed with 4% formaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2.5 hours at 4°C. They were then washed in 0.33 M sucrose in cacodylate buffer and post-fixed with 1% OsO_4 for 1 h at room temperature. They were then dehydrated and embedded in Epon (Serva, Heidelberg, Germany). The ultrathin sections were stained with uranyl acetate and lead citrate. Samples were analysed with transmission electron microscope (Philips CM100 at 80 kV) and micrographs were taken with an AMT camera (Advanced Microscopy Techniques Corp., Woburn, MA, USA).

Statistics

Statistical analyses were performed using Microsoft Excel and Graph Pad Prism version 5 (Graph Pad Software Inc., La Jolla, CA). A two-way analysis of variance ANOVA (Bonferroni's post hoc test) was performed to test whether differences between groups were significant. Statistical significance was

defined as *$p < 0.05$ or **$p < 0.001$. All data were expressed as mean ± standard error, and experiments were performed at least in three independent experiments, each experiment in triplicate.

Results

Decreased expression of UPs is similar in in vitro and ex vivo models of bladder cancer

UPs, are differentiation-dependent and urothelial-specific transmembrane proteins that form urothelial plaques (Hu et al. 2005; Sun et al. 1999). It was shown, that their expression decreases during urothelial carcinogenesis (Huang et al. 2007; Ogawa et al. 1999). Here we investigated in *in vitro* and *ex vivo* models using UPs immunolabelling (Fig. 1). The strongest reaction to UPs was observed in NPU cells (Fig. 1a), while only individual RT4 cells were positive for UPs (Fig. 1b) and T24 cells were negative (Fig. 1c).

Ex vivo, the morphology of the urothelium in Ta and T1 tumours was altered in comparison to normal urothelium (Fig. 1g-i). Normal urothelial cells showed UP expression in the apical plasma membrane (Fig. 1d, j). The urothelium of bladder cancers showed reduced expression of UPs regardless of stage Ta or T1 (Fig. 1e, f, k-p). In addition, immunoelectron microscopy showed that UPs-positive superficial urothelial cells of the normal urothelium contained UPs in the apical plasma membrane and in discoidal vesicles (Fig. 1j), whereas in stages Ta and T1 fewer UPs than in normal tissue were found in the apical plasma membrane and only a few UPs were observed in round vesicles (Fig. 1k-p).

The results of immunolabelling of UPs confirmed that NPU cells reached a high differentiated state that is comparable to normal human urothelium. However, the expression of UPs was decreased in urothelial cancer cells *in vitro* and *ex vivo*. Therefore, we conclude that NPU cells correspond to normal urothelium, while RT4 and T24 cells correspond to Ta and T1 tumours, respectively and do not reach high differentiation state. These results are in accordance with our previously published data (Zupancic et al. 2014; Tratnjek et al. 2017; Kreft et al. 2009a; Visnjar et al. 2017b; Jerman et al. 2021; Visnjar et al. 2017a)

Lectins bind selectively to cancer urothelial cells in in vitro and ex vivo models

We have previously shown that Jacalin and ACA exhibit selective binding to cancer and normal urothelium in rats and mice (Zupancic et al. 2014). Here, human biopsy samples from normal urothelium and bladder cancer (Ta and T1) were used to assess lectin binding *ex vivo* and compare with lectin binding to NPU, RT4 and T24 cells *in vitro*. We analysed the correlation between the binding of Jacalin, ACA and DSA and the differentiation status of superficial cells based on UPs labelling.

Jacalin (Fig. 2a), ACA (Fig. 2d) and DSA (Fig. 2g) were bound to the apical plasma membrane of superficial cells expressing UPs in normal human urothelium (Fig. 2b, e, h). In Ta tumours, all three lectins were found to bind to UP-positive and UP-negative superficial cells (Fig. 2j-s). In T1 tumours, only Jacalin was bound (Fig. 2t), while the binding of ACA and DSA was negative. Furthermore, four types of superficial urothelial cells were observed in the *ex vivo* samples: (i) UPs positive cells with lectin binding

(Fig. 2c, f, i, l, s, v); (ii) UPs negative cells without lectin binding (Fig. 2l, s); (iii) UPs positive cells without lectin binding (Fig. 2c, o, s); and iv) UPs negative cells with lectin binding (Fig. 2f, i, l, o, s, v). However, Jacalin showed widespread labelling and was bound to normal urothelium, Ta and T1 tumours.

In vitro models showed that Jacalin, ACA and DSA were bound to the apical plasma membranes of all RT4 and T24 cells, but not to all NPU cells (Fig. 3). The differences in lectin binding between cancer (RT4, T24) and normal (NPU) urothelial cells were confirmed by measuring the fluorescence intensities of the bound lectins (Fig. 3j). Jacalin and DSA showed significantly higher binding to NPU cells compared to both cancer cells (Fig. 3j). When comparing RT4 and T24 cells, the binding of Jacalin to T24 cells was significantly higher than to RT4 cells. ACA had the highest binding to RT4 cells, while binding to T24 and NPU cells was similar. Of the three lectins tested, binding of Jacalin was highest in T24 cells and binding of ACA was highest in RT4 cells. Of the three lectins tested, DSA showed the lowest binding to T24 and moderate binding to RT4 and NPU cells (Fig. 3j). Correlation of lectins with UPs showed four types of NPU cells (Fig. 4a-c): (i) UPs positive cells with lectin binding; (ii) UPs negative cells without lectin labelling; (iii) UPs positive cells without lectin labelling; and (iv) cells without UPs labelling and with lectin binding. Since RT4 and T24 cells are weakly positive or negative for UPs, respectively, the correlations of lectins with UPs were not performed.

Taken together, the results of the *ex vivo* and *in vitro* experiments showed that ACA and DSA selectively bind to normal urothelium and low-grade cancers (Ta tumours and RT4 cells) and not to high-grade cancers (T1 tumours and T24 cells). Compared to *in vitro* cancer cells, Jacalin bound more strongly to high-grade T24 cancer cells than to low-grade RT4 cells and ACA bound more strongly to low-grade RT4 cells than to high-grade T24 cells. In normal urothelial cells, four cell types were observed *ex vivo* and *in vitro* with respect to UPs and lectin labelling, demonstrating the comparability of the *in vitro* models with the *ex vivo* biopsy samples.

Endocytosis of lectins is found in cancer cells in vitro **and tumours** ex vivo, **but not in normal urothelial cells**

It is known that endocytosis is diminished in highly differentiated urothelial cells (Kreft et al. 2009b; Tratnjek et al. 2017; Lojk et al. 2018). To follow endocytosis of lectins in NPU cells, RT4 and T24 cells, live cells were incubated with FITC-conjugated lectins. In both RT4 and T24 cells, Jacalin (Fig. 5a-c), ACA (Fig. 5d-f) and DSA (Fig. 5g-i) were found in the cytoplasm of the cells as fluorescent dots, confirming endocytosis. In NPU cells, lectins were present at the apical plasma membrane but not in cytoplasm (Fig. 5g, h, i).

Next, gold-conjugated lectins were used to detect ultrastructural features of endocytosis in RT4 and T24 cells and in tumour biopsies of bladder cancer *ex vivo*. Binding of lectins to the apical plasma membrane ruffles, which are typical features of macropinocytosis (Kerr and Teasdale 2009), were also detected *in vitro* and *ex vivo* via bound gold-conjugated lectins (Fig. 5m, r). Neither coated nor uncoated pits were observed to be filled with gold-conjugated lectins. We observed the lectins in macropinosomes,

endosome-like structures and multivesicular bodies (MVBs) (Fig. 5j-s). This suggest that macropinocytosis of lectins is the major endocytotic mechanism in *in vitro* and *ex vivo* models.

Macropinocytosis is the main mechanism of lectin endocytosis in cancer urothelial cells

Till now, different mechanisms of endocytosis into cancer cells were described (Xiao et al. 2021; Hussein et al. 2021). To test the mechanism of lectin endocytosis in cancer urothelial cells, we performed immunofluorescence using antibodies against the major endocytotic markers caveolin, clathrin and flotillin, which showed no colocalisation of the individual endocytotic markers with the individual lectins (Supplementary Fig. 1–3).

To confirm that lectins are endocytosed into cancer urothelial cells *via* macropinocytosis, dextran-TRITC was used together with lectins-FITC. In the cytoplasm of RT4 and T24 cancer cells (Fig. 6a-f), we observed colocalisation of dextran and Jacalin (Fig. 6a, d), dextran and ACA (Fig. 6b, e) and dextran and DSA (Fig. 6c, f).

Additionally, we investigated the effects of inhibition of macropinocytosis on cellular endocytosis of lectins. We used EIPA (5-[*N*-ethyl-*N*-isopropyl] amiloride), the inhibitor of Na^+/H^+ exchange that specifically inhibits macropinosome formation, with no detectable effects on the other endocytic pathways studied (West et al. 1989; Koivusalo et al. 2010). EIPA caused a 1.1-fold and 1.8-fold decrease in endocytosis of Jacalin in T24 and RT4 cells, respectively (Fig. 6g). Endocytosis of ACA was decreased 1.6-fold after inhibition with EIPA in T24 cells. The 1.2-fold decrease in DSA endocytosis by EIPA was observed in RT4 but not in T24 cells (Fig. 6g).

To stimulate macropinocytosis, we used EGF (Nakase et al. 2015). The results showed that endocytosis of Jacalin and ACA was increased in T24 and RT4 cells, while endocytosis of DSA was increased only in RT4 cells (Fig. 6g). The efficiency of cellular endocytosis of Jacalin was increased 1.4-fold in T24 cells and 1.5-fold in RT4 cells. Meanwhile, EGF stimulation increased the endocytosis of ACA 2.2-fold in T24 cells and 3.4-fold in RT4 cells (Fig. 6g). EGF was not used in NPU cells because they have minimal endocytosis.

To inhibit clathrin-mediated endocytosis, we used Dyngo (McCluskey et al. 2013). This inhibition caused a 1.3-fold decrease in endocytosis of DSA in RT4 cells and no significant decrease in endocytosis of DSA in T24 cells (Fig. 6g). Furthermore, Dyngo did not alter the endocytosis of Jacalin and ACA in either RT4 or T24 cells (Fig. 6g).

Taken together, these results suggest that macropinocytosis is the main mechanism for endocytosis of the selected lectins in RT4 and T24 cells.

Discussion

We present here a way to selectively target cancer urothelial cells using lectins based on changes in the expression of sugar residues during urothelial cell dedifferentiation and cancer progression. Furthermore, we revealed that the main mechanism of lectin endocytosis in cancer urothelial cells is macropinocytosis.

Normal, terminally differentiated urothelial cells *in vivo* and highly differentiated cells *in vitro* express UPs and form urothelial plaques at the apical plasma membrane (Hudoklin et al. 2011; Romih et al. 2002; Wu et al. 1994b; Kreft and Robenek 2012). Here, we show that the expression of UPs is altered during transformation of urothelial cancer cells, as only some superficial urothelial cells of Ta, T1 and T2 tumours strongly express UPs, while the majority express UPs weakly or not at all. In addition, only single low-grade RT4 cells and none of the high-grade T24 cells express UPs (Fig. 1). Another feature of urothelial cancer transformation is the alteration of sugar residues exposed on the luminal surface (Ohyama 2008). However, it is not yet known whether lectins allow selective targeting in RT4 and T24 cells and in urothelial tumours. The later could be exploited for selective targeting of cancer urothelial cells by lectins.

Selective binding of the lectins Jacalin, ACA and DSA is observed in cancer cells from biopsy samples of tumours and in RT4 and T24 cells when compared to normal urothelium and NPU cells, respectively (Figs. 2, 3). Jacalin has the most widespread binding capacity *ex vivo* and shows binding to the apical plasma membrane of superficial urothelial cells in normal and all tumour biopsies. Binding of ACA and DSA is present in normal and Ta but not T1 biopsy samples. These results are consistent with our previous study in the mouse bladder cancer model, where DSA binding decreased with cancer progression (Zupancic et al. 2014). Jacalin and DSA show similar binding capacity *in vitro*. Both lectins have significantly higher binding capacity in normal NPU cells, then in high-grade T24 cells and the lowest in low-grade RT4 cells, similar to the binding capacity of *Aleuria aurantia* (AAL) lectin (Ezeabikwa et al. 2021). However, Jacalin shows significantly stronger affinity than DSA regardless of cell type. We hypothesise that this is because Jacalin is a polyspecific lectin that interacts with mannose, mannose-containing glycans, glucose, *N*-acetylneuraminic acid (NANA) and *N*-acetylmuraminic acid in addition to galactose (Bourne et al. 2002). Taken together, the patterns of Jacalin and DSA binding *in vitro* distinguish between normal and cancer cells. In addition, RT4 and T24 cells could be distinguished on the basis of higher Jacalin and ACA binding, as the binding capacity of Jacalin and ACA differs significantly between RT4 and T24 cells. This finding helps to identify a low-grade or high-grade tumour stage. However, this is not the case with biopsy samples. Here, the ability of Jacalin to distinguish between normal, Ta and T1 tumours is undetectable, presumably due to the differences between patients and within the tumour (Kang et al. 2020). Furthermore, T24 and RT4 cells differ from NPU cells in that they bind significantly less DSA. Our results of lectin binding *ex vivo* suggest that DSA could be used as a tool to distinguish DSA-negative T1 tumours from DSA-positive Ta tumours and normal urothelium. Whether these patterns of selective lectin binding have an even higher sensitivity could be investigated by using the extremely sensitive and accurate lectin microarray technology (Nishijima et al. 2012).

An important fact that could increase the selectivity of lectin targeting is the correlation between urothelial cell differentiation status and lectin binding. Similar to rat and mouse bladder cancer models

(Zupancic et al. 2014), we show here in human urothelial tumours and urothelial cancer cell lines that there is no correlation between UP labelling and lectin binding. This is consistent with the fact that the glycan profile changes during cell differentiation (Park et al. 2015; McMorran et al. 2016; Higashi et al. 2014). The greatest biological significance in bladder cancer is the changes in protein N-glycans, which include β1–6 branching of N-glycans due to overexpression of the enzyme GlcNAcT-V (GnT-V) and the addition of bisecting GlcNAc branches by GlcNAcT-III (GnT-III) glycosyltransferases (Takahashi et al. 2006). Alterations in the O-glycosylation pathways are also a common feature of malignant changes in the bladder, particularly the overexpression of simple mucin-type O-glycans and their sialylated counterparts, T-, sialyl-T- (ST), Tn- and sialyl-Tn- (STn) antigens. Presumably, the T and ST antigens that bind Jacalin and ACA are recognised, but these two lectins also bind to normal urothelium (Ferreira et al. 2013). The important difference is the binding capacity, which is higher in NPU cells than in RT4 and T24 cells for Jacalin and lower in NPU cells than in RT4 for ACA. Altered expression of the glycan end structures is also a common feature of bladder tumours. In particular, abnormally low or absent expression of ABO(H)-blood group termini is commonly found in high-grade and invasive bladder cancer (Bergman and Javadpour 1978), while low-grade bladder cancer cells express high levels of fucosylated Lewis X antigen (Ezeabikwa et al. 2021; Cordoncardo et al. 1988). Carbohydrate-terminal Lewis antigens are significantly under-expressed in normal urothelium compared to urothelial tumours (Cordoncardo et al. 1988; Ezeabikwa et al. 2021). Despite great progress in the glycobiology of bladder cancer, there is still not enough knowledge about the selective targeting of lectins and their endocytosis.

Selective targeting of lectins prompted us to investigate the mechanism of lectin endocytosis in RT4 and T24 urothelial cells and tumours. Lectins attach to the plasma membrane of urothelial cells, endocytose and localise in different endosomal compartments. For example, the lectin WGA is found in lysosomes and the trans-Golgi network in human prostate cancer cells (Allen et al. 1989) or only in lysosomes in CaCo-2 cells (Wirth et al. 2002). TEM analysis shows that Jacalin, ACA and DSA are endocytosed and observed in macropinosomes, endosome-like vesicles, MVBs and amfisomes (Fig. 5). However, double labelling of lectins and clathrin, caveolin and flotillin shows no colocalisation. We therefore assume that lectin endocytosis is not associated with clathrin-, caveolin-, and flotillin-mediated endocytosis, or at least to a small extent that is not detected by light microscopy. Recently, the glycolipid-lectin hypothesis has been presented, according to which glycolipids and lectins work together to promote the formation of tubular endocytic pits without the aid of cytosolic coats (Johannes et al. 2016; Renard and Boucrot 2021). However, the plant toxin ricin is endocytosed in HeLa cells *via* a macropinocytosis-like mechanism (Iversen et al. 2012). Moreover, there is ample evidence that cancer cells of various tumours use macropinocytosis mainly for nutrient uptake (Song et al. 2020; Stow et al. 2020). We therefore investigated the endocytotic pathway of lectins in RT4 and T24 cancer cells.

Macropinocytosis has a dual effect on cancer cells (Song et al. 2020). On the one hand, cells expressing RAS genes (such as *K-RAS*, *H-RAS*) under the stress of nutrient deficiency can spontaneously generate constitutive macropinocytosis to promote cancer cell growth. On the other hand, abnormal expression of RAS genes and drug treatment can trigger a novel cell death associated with hyperactivated macropinocytosis, termed methuosis (Song et al. 2020). Because of this dual effect, there is immense

potential for the development of cancer therapies that target macropinocytosis in cancer cells. Furthermore, the molecular pathways and mechanisms of macropinocytosis contribute to the impressive adaptability of cancer cells. Different molecules such as EGFR, PTEN, V-ATPase, syndecan 1 and galectin-3 play distinct roles in the metabolic regulation of RAS-mediated macropinocytosis in cancer (Stow et al. 2020). Functional studies using the macropinocytotic and clathrin-mediated endocytosis inhibitors EIPA and Dyngo, respectively, as well as the macropinocytotic stimulator EGF and dextran co-localisation show that macropinocytosis is the main mechanism for endocytosis of Jacalin and ACA in T24 and RT4 cells (Fig. 6). However, both macropinocytosis and clathrin-mediated endocytosis are possible mechanisms for the endocytosis of DSA in RT4 cells. On the other hand, these results are not confirmed by double labelling of clathrin and DSA, as no colocalisation can be detected here. The stimulation and inhibition of endocytosis supports the TEM studies showing macropinocytotic structures (Fig. 5). The absence of lectin endocytosis in normal urothelial cells is not surprising and is consistent with our previous findings that normal urothelial cells have very low endocytotic activity (Kreft et al. 2009b; Lojk et al. 2018). In conclusion, we hypothesise that anticancer drug-conjugated lectins are only endocytosed by urothelial cancer cells and could therefore represent a highly selective tool for the development of an effective intravesical targeted platform for the treatment of bladder cancer with minimal side effects on normal urothelial cells.

Conclusion

Our study investigated the selective targeting and endocytosis of the lectins Jacalin, ACA and DSA in bladder cancer tumours from biopsies *ex vivo* and *in vitro* models of normal and cancer urothelial cells. The results show that discrimination between normal and cancer urothelial cells and between low-grade and high-grade cancer cells is possible by lectin binding. Furthermore, we confirm that macropinocytosis is the main mechanism of lectin endocytosis in cancer urothelial cells. Therefore, we propose that lectins should be used for innovative targeted drug delivery systems due to their enhanced uptake into urothelial cancer cells through macropinocytosis.

Declarations

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Conflicts of Interest

The authors declare no conflict of interest.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Nataša Resnik, Tanja Višnjar, Tomaž Smrkolj and Daša Zupančič. The first draft of the manuscript was written by Nataša Resnik and Daša Zupančič all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Supplementary Figures

Supplementary Figures 1-3 are not available with this version.

Figures

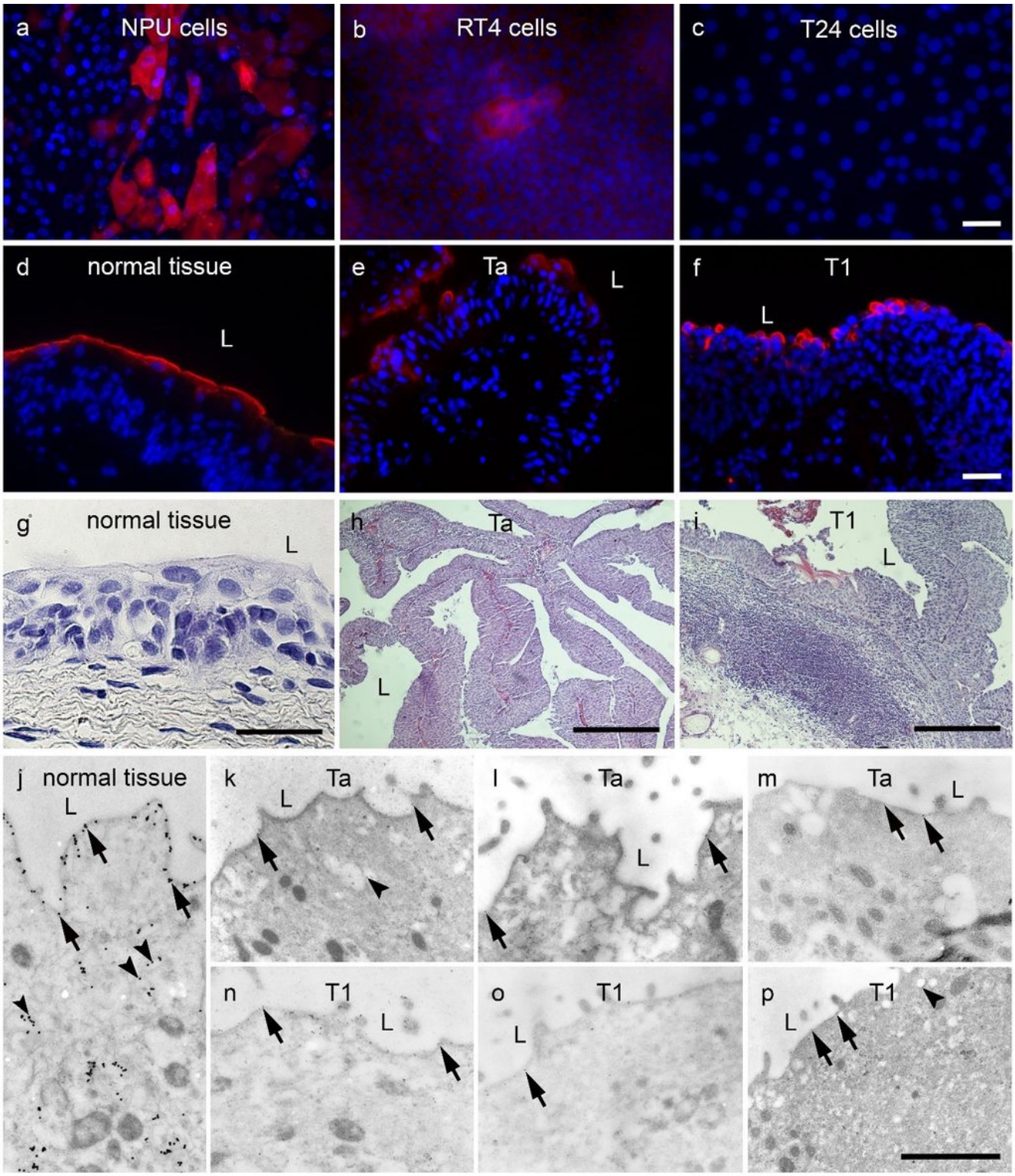

Figure 1

UPs expression in normal and cancer cells in *in vitro* and *ex vivo* models. UPs expression (red) is highest in NPU cells (a) and in normal tissue (d, j). UPs expression is restricted to individual RT4 cells (b) and to individual superficial cells of Ta (e, k-m) and T1 tissue samples (f, n-p). T24 cells do not express UPs (f). Staining with haematoxylin and eosin shows that the morphology of the urothelium is altered in Ta (h) and T1 (i) tumours compared to normal urothelium (g). Immunoelectron microscopy shows abundant

UPs in the apical plasma membrane (arrows) and in discoidal vesicles (arrowheads) in superficial cells of normal urothelium (j). In the superficial cells of Ta (k-m) and T1 (n-p) tumours, fewer UPs are labelled in the apical plasma membrane (arrows) and in round vesicles (arrowheads). L – lumen. Scale bar: a-g 50 µm, h-i 100 µm, j-p 1 µm.

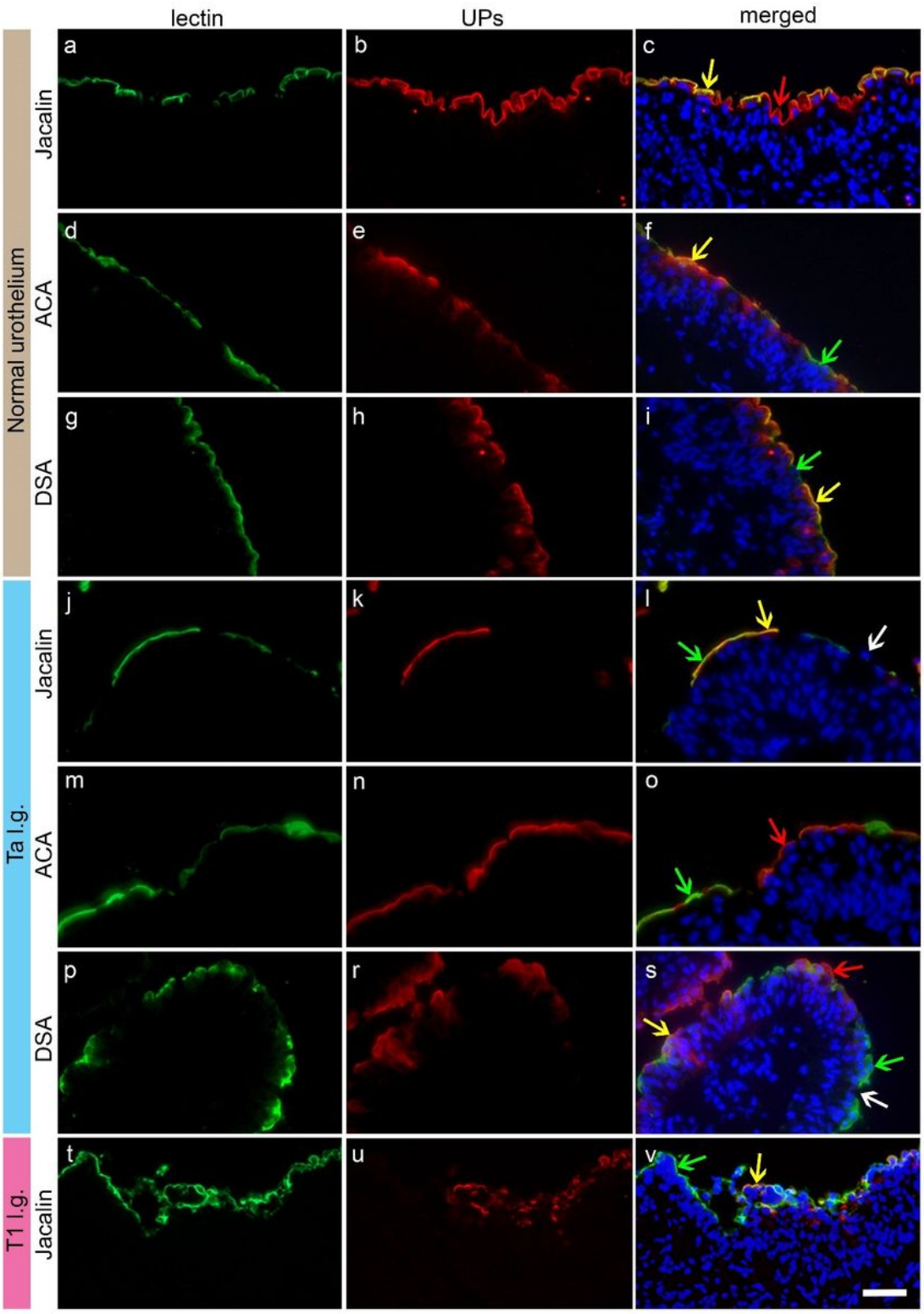

Figure 2

Lectins bind selectively to superficial urothelial cells in normal human urothelium and in cancer biopsy samples *ex vivo*. The apical plasma membrane of normal urothelial cells (a-i) and cells in Ta tumours (j-s) is labelled with Jacalin, ACA and DSA (green). The apical plasma membrane of urothelial cells in T1 tumours (t-v) is labelled with Jacalin only. Differentiated urothelial cells in normal urothelium (b, e, h), Ta l.g. (k, n, r) and T1 l.g. (u) express UPs (red). In merged images colocalisation or no colocalisation between lectins and UPs is clearly observed: UPs positive cells with lectin labelling – colocalisation (c, f, i, l, s, v; yellow arrows); UPs negative cells without lectin labelling – no colocalisation (l, s; white arrows); UPs positive cells without lectin labelling – no colocalisation (c, o, s; red arrows); and (iv) UPs negative cells with lectin labelling – no colocalisation (f, i, l, o, s, v; green arrows). Scale bar for all images: 50 μm.

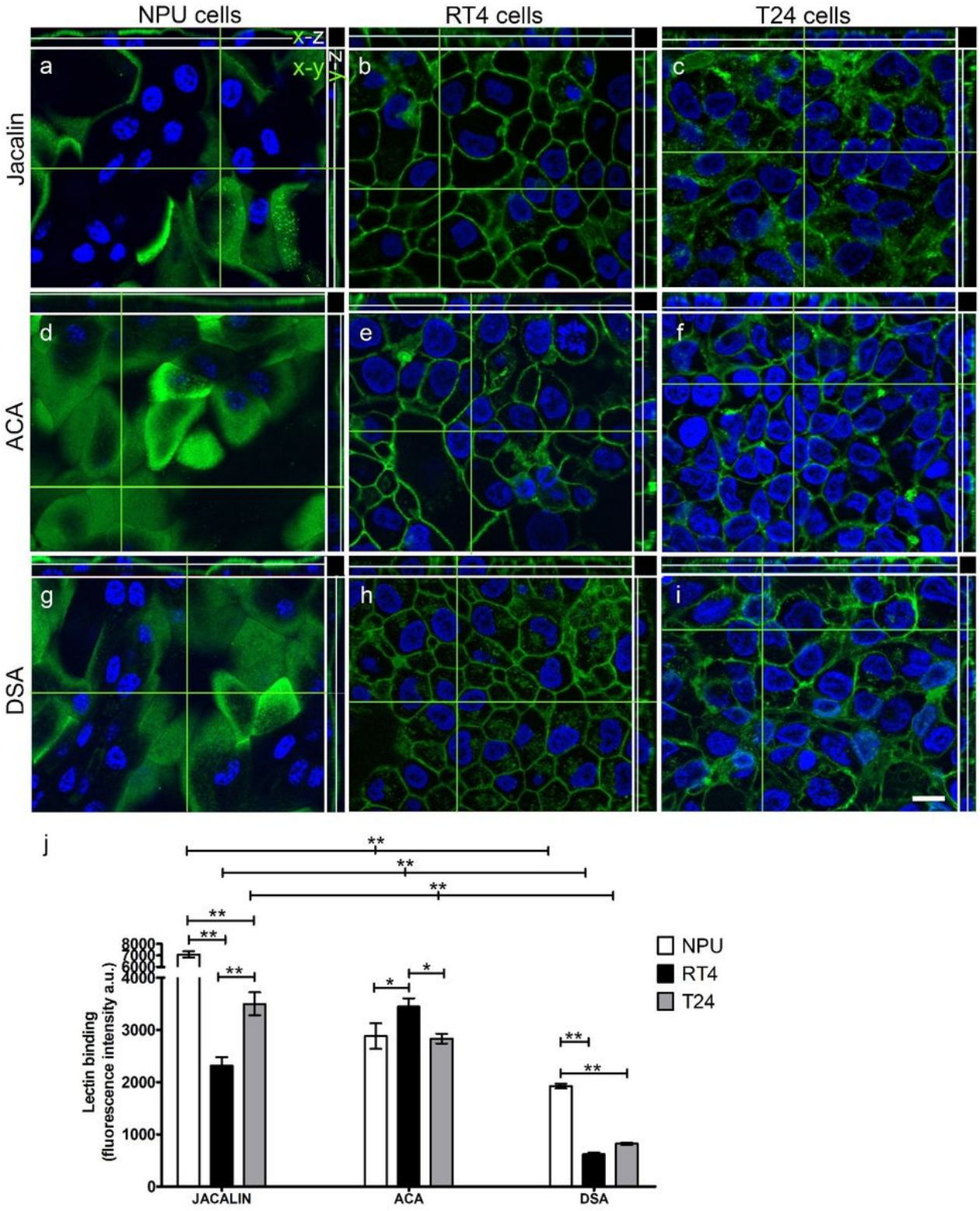

Figure 3

Differential lectin binding to normal and cancer urothelial cells *in vitro*. FITC-conjugated lectins Jacalin (a, b, c), ACA (d, e, f) and DSA (g, h, i) (green) bind to the plasma membrane of NPU, RT4 and T24 cells. The plasma membranes of all T24 and RT4 cells are uniformly labelled with Jacalin, ACA and DSA, as shown on the x-y, x-z and y-z views of optical sections. Not all NPU cells are labelled with Jacalin, ACA and DSA.

(j) Lectin binding to NPU, RT4 and T24 cells calculated from absolute values of fluorescence intensities of FITC. Shown are averages ± SE; *p<0.05, **p<0.001. Scale bar: 10 µm.

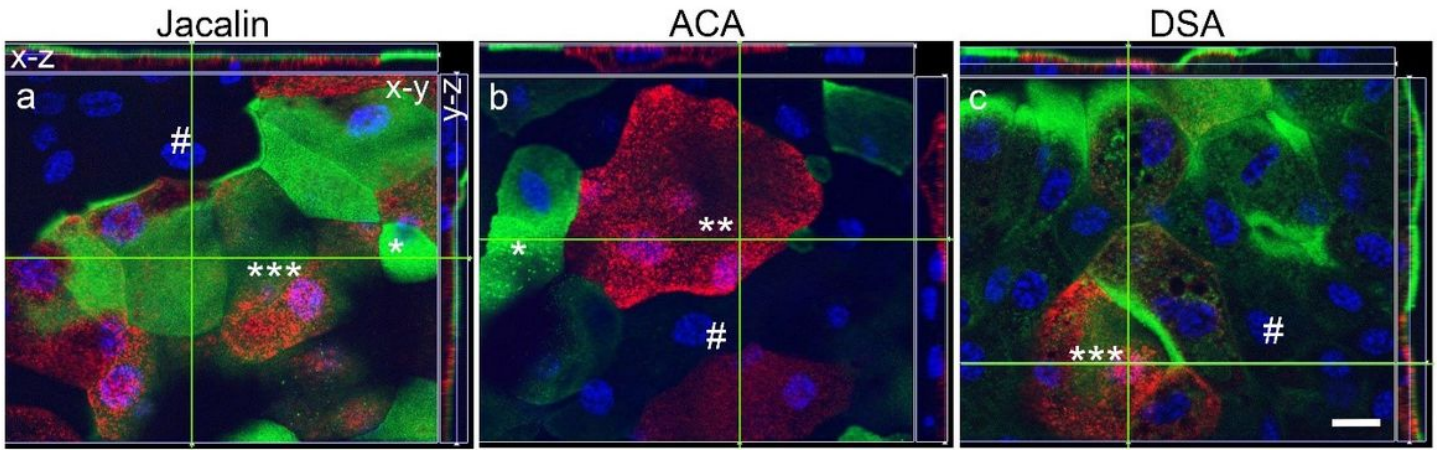

Figure 4

Correlation of lectin binding (green) and UP labelling (red) in NPU cells. UP-positive cells with lectins (a, c; three asterisks), UP-positive cells without lectins (b; two asterisks), UP-negative cells with lectins (a, b; one asterisk), UP-negative cells without ACA (a, b, c; hash). Shown are x-y, x-z and y-z views of optical sections. Scale bar: 10 µm.

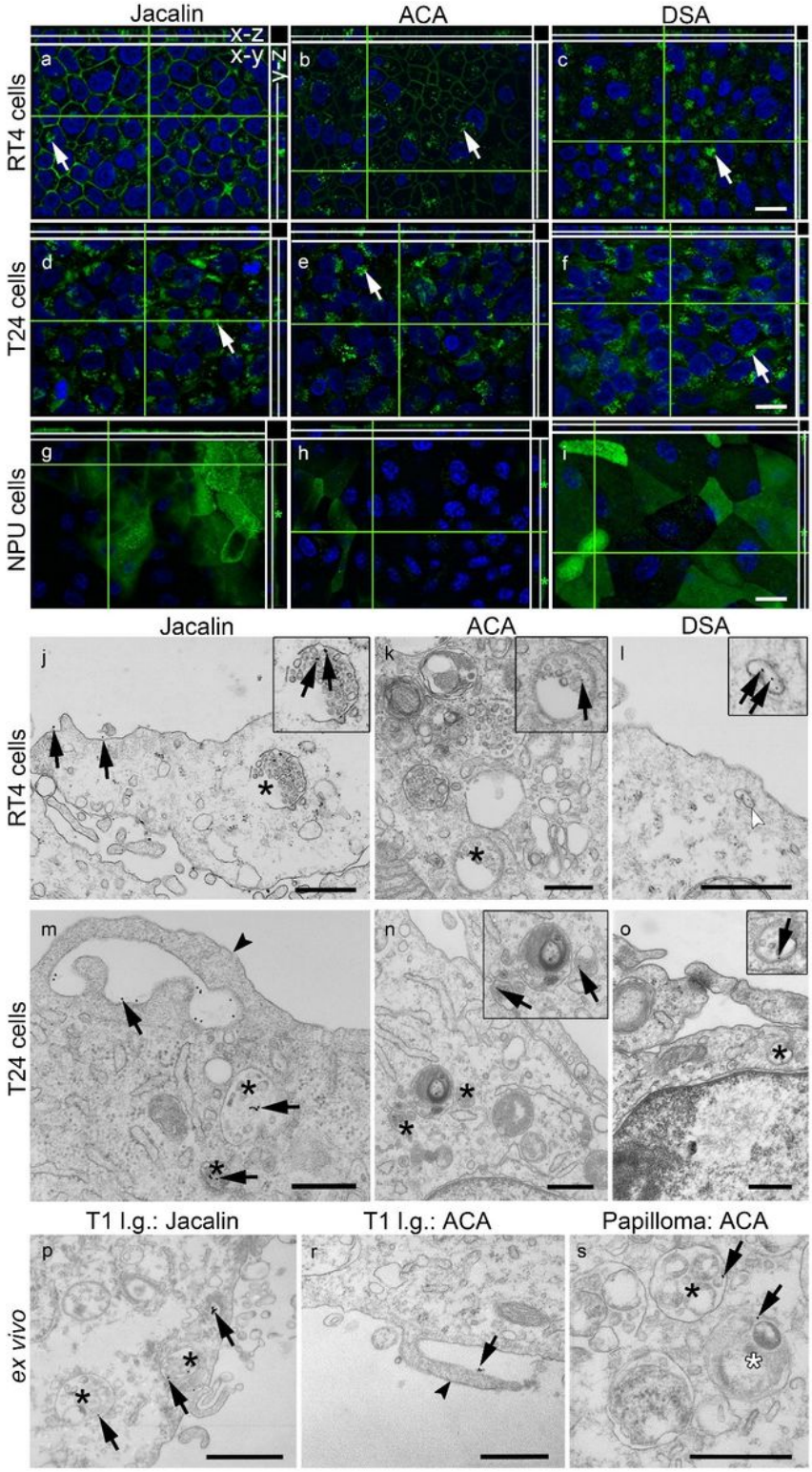

Figure 5

Endocytosis of lectins in urothelial cells *in vitro* and *ex vivo*. Jacalin (a, d), ACA (b, e), and DSA (c, f) were found in the cytoplasm of T24 and RT4 cells (white arrows) whereas in NPU cells Jacalin (g), ACA (h) and DSA (i) were not internalised, but bound to the plasma membrane (green asterisks). Shown are x-y, x-z and y-z views of optical sections. In the TEM images, the lectins are indicated by black arrows. Jacalin is surrounded by membrane ruffles (m; arrowhead), which are an ultrastructural feature of

macropinocytosis. After endocytosis, Jacalin (j, m; arrows), ACA (k, n; arrows) and DSA (l, o; arrows) are observed in MVBs (j, k, m, n, o; asterisk) and endosome-like vesicles (l; white arrowhead). The framed images are enlargements of the MVBs and vesicles with lectins. In the tumour biopsy samples *ex vivo*, the lectins (arrows) bind to the apical plasma membrane (r) and are associated with the plasma membrane ruffles (r, arrowhead). In the cytoplasm, lectins are gathered in multivesicular bodies (MVBs; p, s; black asterisk) and amfisome (s; white asterisk). Scale bar: a-i 20 µm, j-s 500 nm.

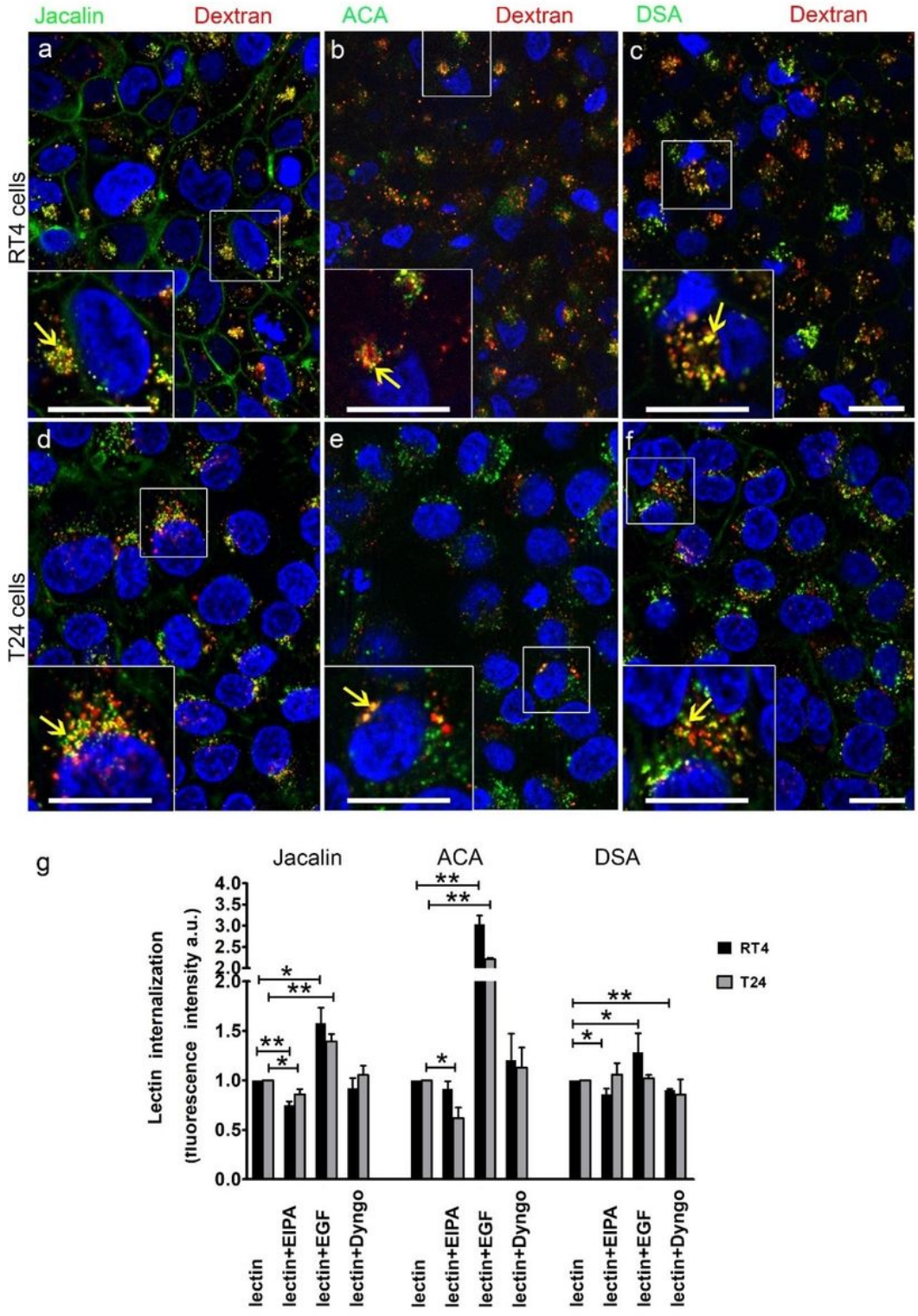

Figure 6

Macropinocytosis of the lectins Jacalin, ACA and DSA in cancer urothelial cells. (a) Dextran-TRITC (red) and lectin-FITC (green) Jacalin (a, d), ACA (b, e) and DSA (c, f) colocalise in T24 and RT4 cells (yellow). Details of dextran and lectin colocalisation (yellow arrows) are shown enlarged in the white frames. (g) Quantification of lectin endocytosis with and without the macropinocytosis inhibitor EIPA (lectin + EIPA), the stimulator of macropinocytosis EGF (lectin + EGF) and the inhibitor of clathrin-mediated endocytosis Dyngo (lectin + Dyngo). The intensities of the fluorescence were normalised to the endocytosis of the single lectin without inhibitors or stimulator (value 1). Shown are averages ± SE; *p<0.05, **p<0.001. Scale bar: 20 µm.