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Abbreviations:

PVP: Polyvinylpyrrolidone

HAL: Hexaminolevulinate

PpIX: protoporphyrin IX

DiO: 3,3'-dioctadecyloxacarbocyanine perchlorate

H&E: Hematoxylin and eosin

NMIBC: non-muscle invasive bladder cancer

Abstract

Purpose

In this preclinical study we examined the biodistribution of hypericin formulated as its water-soluble PVP-hypericin complex in the different layers (urothelium, submucosa, muscle) of a normal rat bladder and a rat bladder bearing a malignant urothelium composed of syngeneic AY-27 tumor cells. The results were compared with the biodistribution of hexaminolevulinate (HAL) induced protoporphyrin IX (PpIX).

Methods

Freshly prepared PVP-hypericin and HAL solutions were instilled in both normal as well as tumor bearing rat bladders. Following instillation, bladders were removed and snap frozen in liquid nitrogen. Fluorescence of PVP-hypericin or PpIX induced HAL was measured in the bladder layers and quantified using image analysis software.

Results

The results of these experiments show that PVP-hypericin (30 µM) accumulated about 3.5-fold more in malignant urothelial tissue as compared to normal urothelium, whereas PpIX accumulated to the same extent in malignant and normal urothelium, both after intra-bladder instillation of 8 or 16 mM HAL. Besides, PVP-hypericin and PpIX accumulated selectively in the urothelium with a tumor-to-muscle ratio of 30.6 for PVP-hypericin and 3.7 to 8.3 for 16 and 8 mM HAL, respectively.

Conclusions

This study shows that PVP-hypericin appears to have great potential as a photodynamic agent against non-muscle invasive bladder cancers after intravesical administration, with a limited risk of affecting the deeper layers of the bladder.

Keywords: Bladder cancer, orthotopic animal model, PVP-hypericin, HAL

Introduction

Urinary bladder cancer is the fifth most frequent type of cancer in western world [1]. When first diagnosed, 75-85% of these bladder tumors are staged as non-muscle-invasive bladder cancers (NMIBC), which are generally treated by transurethral resection with or without adjuvant intravesical chemotherapy or immunotherapy [2,3].

During the last decade several compounds have been tested for their capacity to photo-diagnose efficiently malignant bladder lesions, and by doing so to reduce the amount of recurrences after transurethral resection of the bladder [4,5]. One of the compounds used is hypericin, a fluorescent photosensitizer that belongs to the chemical class of hydroxylated phenantroperylenequinones [6,7]. After intrabladder instillation in patients suspected of having bladder cancer, hypericin showed a very good sensitivity and specificity for detecting NMIBC, including carcinoma *in situ* (CIS) [8-10]. The finding that hypericin is selectively taken up by urothelial tumor cells and not by normal cells is also supported by clinical studies using fluorescence cytology [11,12].

In water hypericin forms aggregates, thereby loosing its spectroscopic and tumor-selective properties [13]. In previous studies, this problem was overcome by including plasma proteins like human serum albumin (HSA) into the formulation to which hypericin adsorbs [8,9]. More recently, with the intention to avoid the presence of proteins in the bladder instillation fluid, a novel formulation was developed based on a water-soluble complex formed between hypericin and polyvinylpyrrolidone (PVP) [14,15].

5-Aminolevulinic acid (5-ALA) is another compound that is being used for fluorescence cystoscopy of the bladder. Its use in urology was first described by Leveckis *et al.* [16]. Exogenous administration of 5-ALA induces a transient rise in the cellular concentration of the fluorescent PpIX, which is more pronounced in neoplastic cells than in normal cells [17,18]. An ester precursor of 5-ALA, i.e. hexaminolevulinate (HAL) provides an even better selectivity and brighter fluorescence as compared to the parent compound. Using fluorescence cystoscopy in combination with intrabladder HAL, it was shown that 28% more patients with CIS were identified than with the standard cystoscopy method [19]. Hexaminolevulinate (Hexvix®, Photocure ASA) has recently obtained a marketing authorization in 27 European countries, including Norway and Iceland, for the improved detection of superficial bladder cancer [20].

Hypericin and ALA-induced PpIX do not only exhibit red fluorescence upon blue light irradiation, both compounds are also potent photosensitizers and hence could be useful in the photodynamic therapy (PDT) of NMIBC after intrabladder instillation [21-23]. PDT for cancer involves the local or systemic administration of a photosensitizer followed by local irradiation of

the tumor tissue with non-thermal visible light of a specific wavelength. The local generation of ROS (reactive oxygen species) by the irradiated photosensitizer results in tumor destruction [7,24].

Anticipating the clinical use of PVP-hypericin in the therapeutic management of NMIBC, it is essential to understand the selectivity of the accumulation of the photosensitizer into urothelial malignant lesions. In order to avoid any damage, it is critical that the compound does not accumulate substantially into the deeper layers of the bladder wall, especially in the detrusor muscle.

This preclinical study examined the biodistribution of hypericin formulated as its water-soluble PVP-hypericin complex (hereafter called PVP-hypericin) in the different layers (urothelium, submucosa, muscle) of a normal rat bladder and a rat bladder bearing a malignant urothelium composed of syngeneic AY-27 tumor cells [25]. The results were compared with the outcome using HAL in similar *in vivo* conditions. As there is a very limited penetration into the deeper layers of the bladder, we anticipate that PVP-hypericin is a safe compound when it will be used for intravesical instillation followed by whole bladder wall photodynamic therapy

Materials and methods

Animals

For all experiments female Fisher rats (F-344), weighing a minimum of 160 g, were used (Charles River Laboratories, Lyon, France). Rats were provided with purine chow and water *ad libitum.* All animal procedures were performed in compliance with national and European regulations and were approved by the animal care and ethics committee of the Katholieke Universiteit Leuven.

Preparation of cells for tumor implantation

AY-27 cells, originally developed by Dr. S. Selman and Dr. J. Hampton (Ohio Medical College, USA), were kindly provided by Dr. D. Notter (Université henri Poincare, Nancy, France). Cells were cultured in 175 cm^2 tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air in Minimum Essential Medium (MEM) with Earle's salts containing 2 mM L-glutamine, 1% antibiotic/antimycotic solution, 1% non-essential amino acids, tylosine (60 µg/ml) (Eli Lilly, Brussels, Belgium) and 10% foetal calf serum (FCS). All cell culture medium compounds were purchased from Invitrogen (Merlebeke, Belgium).

AY-27 cells were cultured until confluency and then incubated for 30 min with 20 µM green fluorescent cell tracker 3,3'-dioctadecyloxacarbocyanine (DiO). DiO was purchased from Molecular Probes Inc. (Eugene, OR, USA) and stored as a 20 mM stock solution in DMSO at -20°C. Just before use, the stock solution was diluted in MEM to obtain a concentration of 20 µM. Afterwards, cells were washed twice with PBS (pH 7.4), trypsinized and collected after centrifugation (2000 rpm, 5 min, Jouan B3.11; St. Nazaire, France). Cells were counted using a Coulter Z1 particle counter (Coulter Electronic, Luton, UK) and resuspended in cell culture medium to obtain a concentration of $4x10^6$ cells/0.3 ml.

Tumor model

The technique used for tumor implantation was described previously ([26] with modification). Animals were anesthetized with an intraperitoneal injection of 45 mg/kg sodium pentobarbital (Nembutal) and placed on supine position on an animal board kept at 25°C. Bladders were catheterized via the urethra using a plastic 18 gauge intravenous catheter. Subsequently, the bladder mucosa was mildly disrupted using a wash with 0.3 ml of 0.1N HCl for 20 seconds, followed by neutralization with the same quantity and strength of NaOH for 20 seconds. The bladder was then washed several times thoroughly with PBS. AY-27 cells ($4x10^6$ cells in 0.3 ml of medium) loaded with DiO were instilled into the bladder via the catheter and maintained in the bladder for at least 1 h. Rats were turned by 45° every 15 min to allow equal exposure of tumor cells to the entire bladder wall. Thereafter, the cannula was removed and the rats were allowed to

void spontaneously. Animals were used for bladder wall distribution studies of the photosensitizers three days later.

Intrabladder instillation of PVP-hypericin and HAL

Rats were anesthetized with sodium pentobarbital (Nembutal, 45mg/kg, i.p.) and after catheterization, 0.3 ml of a freshly prepared PVP-hypericin instillation fluid (30 µM in 0.9% NaCl) (Provided by Sanochemia Pharmazeutika AG) or HAL (8 and 16 mM, prepared in phosphate buffered saline according to [27]) (GE-Healthcare) were instilled in normal and in tumor bearing bladders for 2 h (n=6 per condition). Control animals (n=3) were instilled with saline only. Afterwards bladders were removed, cut open and immediately transferred into Tissue Tek embedding medium (Miles Inc., Elkhart, IN, USA) and immersed in liquid nitrogen. Cryostat microtomy was performed to cut 5 µm sections.

Imaging and quantification of fluorescence

Imaging and quantification of fluorescence in sections of normal bladder and tumor bearing bladders were performed by the use of a fluorescence microscope equipped with image analysis software. The microscope consisted of an Axioskop 2 plus fluorescence microscope (Carl Zeiss, Göttingen, Germany) illuminated by a 100W mercury lamp. Fluorescence images were acquired using a light-sensitive charge-coupled device (CCD) digital camera (AxioCam HR, Carl Zeiss, Göttingen, Germany). For fluorescence quantification, a KS imaging software system (Carl Zeiss, Vision GmbH, Hallbergmoos, Germany) was used. Throughout the study parameters such as objective lenses, excitation and emission filters and exposure time were kept constant to maintain uniformity.

The presence of tumor tissue was confirmed through the fluorescence detection of DiO, using a filter set consisting of a 450-490 nm band-pass excitation filter and a 515-565 nm band-pass emission filter. For fluorescence imaging of PVP-hypericin a filter set with a 510-560 nm band-pass excitation filter and a 590 nm long-pass emission filter was used. The filter set used for PpIX fluorescence imaging consisted of a 395-440 nm band-pass excitation filter and a 590 nm long-pass emission filter. Rapid observation and electronic image storage avoided significant photobleaching of the fluorescence signal. In each tissue section, regions of interest were delineated and within that region the fluorescence was quantified. Corrections were made for autofluorescence, by subtracting autofluorescence levels of each tissue layer (urothelium, submucosa, muscle) obtained from control animals. After examination of the fluorescence, bladder sections were stained with standard hematoxylin and eosin (H&E) staining for histological examination.

Results

In the present study the biodistribution of both PVP-hypericin and HAL induced PpIX was compared in normal and tumor bearing rat bladders. After instillation, the induced fluorescence was measured in the different layers of the bladder (urothelium, submucosa, muscle) and further quantified using image analysis software.

Figures 1 and 2 show typical fluorescence micrographs of 5 µm bladder sections after instilling PVP-hypericin (Fig. 1a, c) or HAL (Fig. 2a, c) in normal (Fig. 1a; Fig 2a) or tumor bearing bladders (Fig. 1c; Fig 2c). AY-27 cells could easily be visualized by pre-incubating them *in vitro* with the green fluorescent carbocyanine dye DiO, and interestingly, a good correlation between areas consisting of malignant urothelium loaded with the green fluorescent DiO and the localization of PVP-hypericin- or PpIX-related red fluorescence was found (Fig 1e, f; Fig 2e, f).

Overall, PVP-hypericin related fluorescence was strong, whereas accumulated PpIX showed a weaker fluorescence signal. Because of this difference, a lower gain of sensitivity (gain 2) and a higher gain of sensitivity (gain 3) was used for PVP-hypericin and PpIX imaging, respectively.

In comparison to the epithelial cell layers visualized by H&E staining (Fig. 1b) it can be seen that, PVP-hypericin penetrated the normal urothelium only very limitedly. Only the first two epithelial cell layers show bright fluorescence, whereas the deeper layers virtually do not exhibit any fluorescence signal. On the other hand PVP-hypericin migrated well throughout all cell layers of the malignant urothelium, resulting in a uniform distribution of the compound in all cell layers of the epithelium (Fig. 1d). Conversely, HAL-induced PpIX displayed a homogeneous distribution in both, malignant and normal urothelium (Fig. 2a, c).

Quantification of the fluorescence signal in sections of normal and tumor bearing bladders after PVP hypericin or HAL treatment revealed that PVP-hypericin (Fig. 3a) accumulated on average about 3.5-fold more in malignant urothelial tissue as compared to normal urothelium, whereas PpIX accumulated to the same extent in malignant and normal urothelium, both after intra-bladder instillation of 8 or 16 mM HAL (Fig. 3b, c). In order to assess the selectivity of accumulation of PVP-hypericin and PpIX in the urothelium of normal bladders and tumor bladders, (i) the ratio of the relative fluorescence present in the urothelium ($\mathbf{F_U}$) to the one present in the submucosa ($\mathbf{F_{SM}}$), and (ii) the ratio of the relative fluorescence present in the urothelium ($\mathbf{F_U}$) to the one present in the muscle ($\mathbf{F_M}$) were calculated. The results are presented in Table 1 and show that both PVP-hypericin and PpIX accumulated selectively in the urothelium with a tumor-to-muscle ratio of 30.6 for PVP-hypericin and 3.7 to 8.3 for 16 and 8 mM HAL, respectively.

Discussion

In the present *in vivo* study the biodistribution of PVP-hypericin and HAL-induced PpIX in both healthy rat bladders and in an orthotopic rat bladder tumor model was compared. For that purpose we instilled rats intravesically with PVP-hypericin (30 µM) or HAL (8, 16 mM) three days after tumor inoculation with concentrations that correspond to the ones typically used in the urological clinic [5,8,9,28-30]. The results show that administration of either of the compounds resulted in an increased uptake (PVP-hypericin) or production (PpIX) of the photosensitizers in normal or malignant urothelium as compared with the deeper tissue layers, i.e. submucosa and muscle layer.

The uptake of 5-ALA or its derivative HAL and subsequent conversion to the photoactive compound PpIX, depends on a broad range of metabolic and tissue specific factors [18], but also on the administration route, concentration and instillation time [31]. In this study a similar procedure was applied that was used before by another group investigating the biodistribution of HAL-induced PpIX in the orthotopic rat bladder tumor model for the first time [27]. Our results are in line with the published data with regard to the similar PpIX accumulation in normal and tumor urothelium that are seen when 8 or 16 mM HAL is instilled [27]. In contrast, the tumor-to-muscle ratio was previously found equal at both concentrations, whereas in our study the tumor-to-muscle ratio is twice as high when using 8 mM HAL as compared to 16 mM HAL.

A similar PpIX fluorescence in malignant urothelium after 8 and 16 mM HAL administration, does however not necessarily guarantee a similar PDT efficacy. For instance, PDT after 16 mM HAL resulted mostly in bladder wall necrosis without tumor eradication, whereas with 8 mM HAL and the same light conditions used, there was effective tumor damage without any effect on the bladder wall [27].

Of interest, a follow-up study by the same group using a gross tissue extraction method, indicated that after 8 mM HAL the PpIX concentration in tumor bladders was significantly higher than with 16 mM HAL [32], and it was concluded that PpIX-fluorescence as measured by fluorescence microscopy does not always mirror linearly drug concentrations. Besides, also the subcellular localization of PpIX critically depends on the concentration of HAL instilled. Importantly, this localization affects the damage to specific cellular organelles and therefore the PDT efficacy [32]. Hence, predicting the photodynamic therapeutic outcome based exclusively on the HAL-induced PpIX fluorescence observed appears to be difficult. This is especially problematic when it comes to potentially photo-active amounts of photosensitizer present in the detrusor muscle resulting in fibrotic and contracted bladder with upper tract obstruction [33,34]. For obvious reasons, these adverse effects should be thoroughly avoided.

The biodistribution of PVP-hypericin in the different layers of the orthotopic tumor bearing rat urinary bladder correlates well with the one observed in similar conditions when hypericin is formulated in the presence of human serum albumin (HSA) [35]. The fluorescence intensity ratios of the bladder urothelial tumor to both submucosa and muscle are comparable in both studies. As a consequence it can be expected that the outcome of whole bladder wall PDT in an *in vivo* model will largely coincide with the results previously obtained using HSA-hypericin [36]. In contrast with HAL, when using PVP-hypericin there seems to exist a good correlation between the fluorescence present in the different layers and the local damage photodynamically induced.

An interesting observation is the fact that HSA-hypericin was not selectively taken up by malignant as compared to normal urothelium [35], whereas PVP-hypericin in this study is taken up about a 3- to 4-fold more in malignant tissue than in benign urothelium. Presently, we cannot account for this difference, but definitely the present preclinical findings with PVP-hypericin support very well the clinical results obtained with both HSA- and PVP-hypericin that detect NMIBC with a high sensitivity and good specificity [8-10,28]. For example, in case of PVP-hypericin used for the fluorescence-based diagnosis of bladder cancer in patients, a sensitivity ranging from 85% (dysplasia) to 100% (CIS, T1 and T2) and a specificity of 53% was found [28].

Whether the difference in accumulation of PVP-hypericin between malignant tissue and benign urothelium will also result in a differential PDT effect, is yet unknown. However, even if not entirely the case, it is known that PDT induced urothelial damage regenerates in a short time period without significant alterations to the bladder wall [37,38].

In conclusion, we have shown that after instillation of a normal and an orthotopic tumor bearing rat urinary bladder, PVP-hypericin and HAL-induced PpIX are selectively present in the bladder urothelial tumors. Overall, the accumulation in the urothelium *vs.* the underlying tissues is much better for PVP-hypericin than for PpIX. The restrictive distribution in the urothelial tumor suggests that by using appropriate light doses, PDT with PVP-hypericin should produce a selective urothelial tumor destruction without causing damage to the muscle layer. Besides being an effective diagnostic tool, PVP-hypericin therefore appears to have great potential as a photodynamic agent against non-muscle-invasive bladder cancers.

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Disclosures

None

Tables

Table 1: Selectivity of the accumulation of PVP-hypericin or HAL-induced PpIX, expressed as (i) the ratio of the relative fluorescence present in the urothelium ($\mathbf{F_U}$) to the one present in the submucosa ($\mathbf{F_{SM}}$), and (ii) as the ratio of the relative fluorescence present in the urothelium ($\mathbf{F_U}$) to the one present in the muscle ($\mathbf{F_M}$).

	PVP-hypericin		**HAL induced PpIX (16mM)**		**HAL-induce PpIX (8mM)**	
	NB[a]	**TB**[b]	**NB**	**TB**	**NB**	**TB**
F_u/F_{sm}	34.2	12.8	3.8	2.8	6.6	3.6
F_u/F_m	47.5	30.6	8.6	3.7	7.6	8.3

[a] normal bladder; [b] tumor bladder

Figure Legends

Fig.1: Localization of PVP-hypericin in normal urothelium and urothelial tumors. Fluorescence photomicrographs of PVP-hypericin (30 µM, 2h) 3 days after tumor inoculation in normal (a) and tumor bearing rat bladders (c), and corresponding H&E stainings (b, d) of 5 µm frozen tissue sections. Visualization of the malignant epithelium by DiO (e) and overlay of DiO fluorescence and PVP-hypericin fluorescence (f). (a, c) gain=2, exposure=200, original magnification = 200X

Fig.2: Localization of PpIX in normal urothelium and urothelial tumors. Fluorescence photomicrographs of HAL induced PpIX (8 mM, 2h) 3 days after tumor inoculation in normal (a) and tumor bearing rat bladders (c), and corresponding H&E stainings (b, d) of 5 µm frozen tissue sections. Visualization of the malignant epithelium by DiO (e) and overlay of DiO fluorescence and PpIX fluorescence (f). (a, c) gain=3, exposure=200, original magnification = 200X

Fig.3: Quantification of (a) PVP-hypericin (30 µM) and (b, c) HAL (8, 16 mM) induced PpIX fluorescence in different layers of the bladder. In each graph the fluorescence intensity in normal and tumor bearing bladders is compared. Means ± SD are shown

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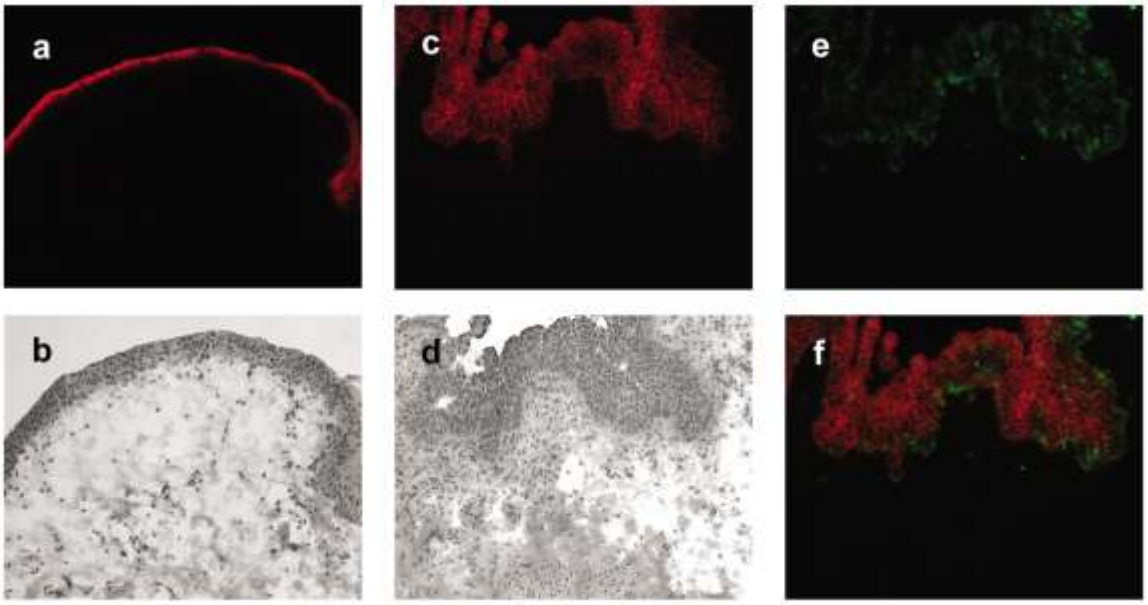

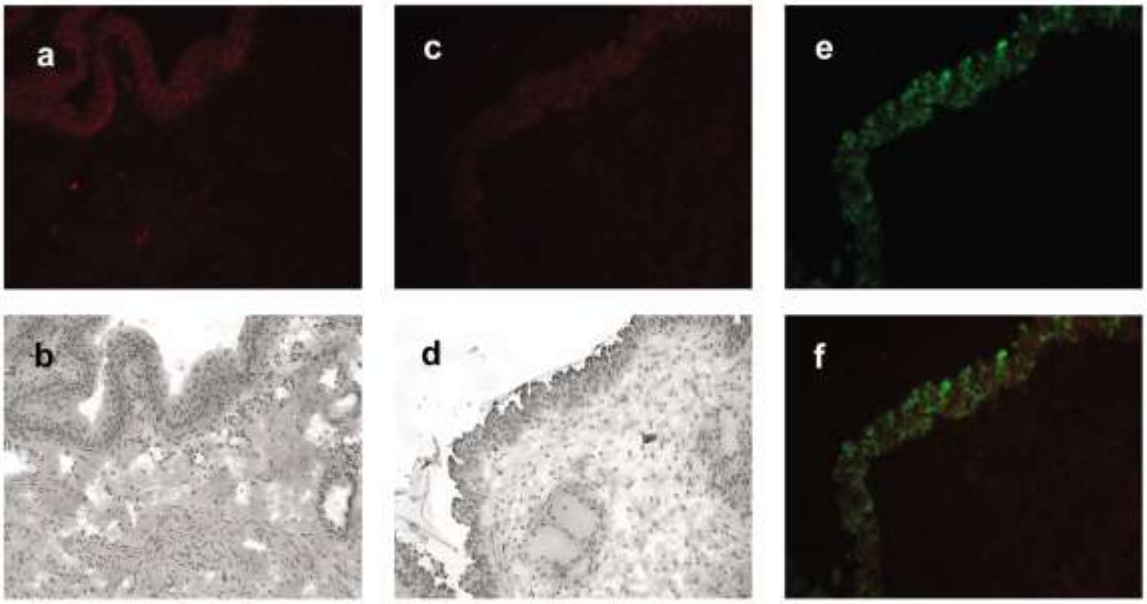
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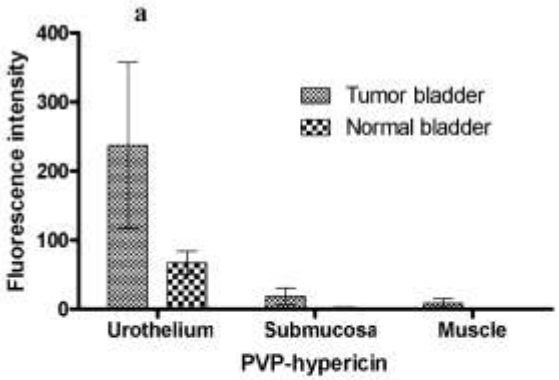
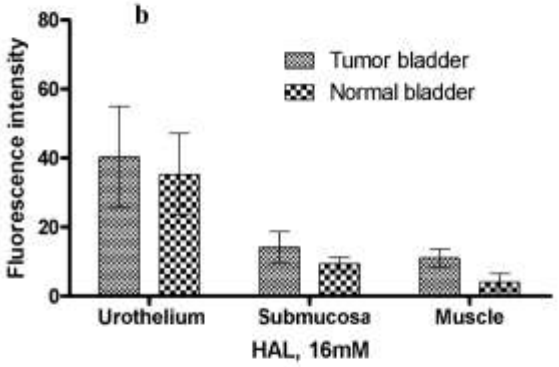
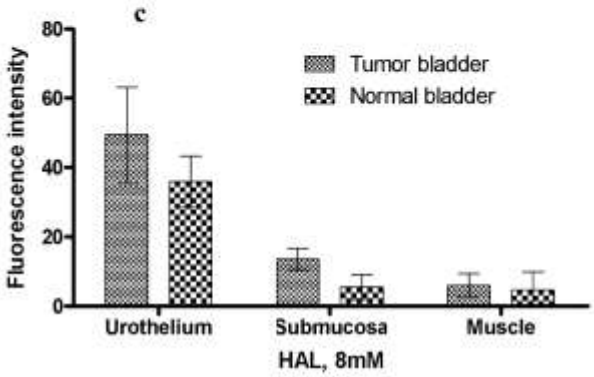

a
Fluorescence intensity
400
300
200
100
0
Tumor bladder
Normal bladder
Urothelium
Submucosa
Muscle
PVP-hypericin


b
Fluorescence intensity
80
60
40
20
0
Tumor bladder
Normal bladder
Urothelium
Submucosa
Muscle
HAL, 16mM


c
Fluorescence intensity
80
60
40
20
0
Tumor bladder
Normal bladder
Urothelium
Submucosa
Muscle
HAL, 8mM