SYNTHESIS OF A WATER-SOLUBLE FLUORESCENT ACTIVE COMPOUND AND ITS POTENTIAL USE FOR LABELING OF CANCEROUS UROTHELIAL BLADDER CELLS

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Abstract

Bladder cancer is the ninth most common cancerous disease in the world, whose incidence in Slovenia is expected to increase by 45% in men and 105% in women by the year 2020. Therefore, new methods of early diagnosis of bladder cancer are essential. Fluorescent dyes represent an appropriate tool because they emit cold light and may be used for labelling the cells in tumours.

Our research was aimed at differentiating between healthy (normal) and cancerous bladder urothelial cells. We developed a water-soluble fluorescence derivative of fluorescein, sodium disulfonate (SUF), which is an organic compound and emits intense fluorescence. Next, we tested its influence on the viability of normal and cancerous urothelial cells and tried to distinguish between the normal and cancerous urothelial cells using a fluorescence microscope. We analysed if SUF can be used in cancer cell diagnostic and examined its potential for targeted therapy as an alternative to the currently used fluorescent dyes, which are more expensive and harder to obtain, and with this approach make such research more accessible.

The results of the study have shown that SUF is not toxic to urothelial cells and that it may be used to distinguish between healthy and cancerous urothelial cells, based on the way of labelling. SUF was proven to be a useful fluorescent marker. Importantly, its structure also allows other functional groups or anti-cancerous substances to be added through advanced synthesis strategies.

1. Introduction

Bladder cancer is the ninth most common cancer in the world. Its detection is difficult, as the clinical symptoms can be very similar to other not so severe bladder diseases. Often it is detected too late, and can consequently prove fatal. In addition to the latter, bladder cancer cells appear multifocally, i.e. in many separate spots in the bladder, so it is difficult to completely remove them by operation. The described fact indicates a high probability of a further recurrence of the disease (1).

In order to improve the detection and treatment of bladder cancer, different diagnostic methods have been developed. A very interesting and important direction of research is based on fluorescent active dyes that emit cold light (light emitted at low temperatures from a source that is not incandescent). These dyes can be used for labeling cells. The applicative potential of this kind of labeling material has already been described in the literature (2-5). Such labeling is intended for medical applications, in particular in monitoring the development of various degenerative diseases (6-8).

One of the most important and widely used dyes is the green fluorescent protein (GFP). In the field of cell biology research, GFP has an advantage, since it is extremely small, water-soluble, emits a large amount of light and is not toxic (9). However, large amount of this protein is inaccessible due to a high price, which indicates development of new forms of fluorescent dyes. The idea in this field is to prepare an active substance (probe), which can be quickly elaborated in a simple and low cost procedure following synthetic preparation strategies, and will have equivalent or improved properties comparing to the previous preparations of GFP.

The aim of this study was therefore to prepare the fluorescence probe with the above properties and to study some of its chemical parameters (characterization data and solubility data). The prepared ingredients were also tested for toxicity and usefulness in the field of cell biology, in particular for detection of healthy and cancerous urothelial cells of the urinary bladder.

2. The urinary bladder and bladder cancer

The urinary bladder is a part of the urinary system. Its task in the body is temporary storage of urine, which is constantly produced by the kidneys and enters the bladder through the ureters (10).

The organ has the shape of a hollow ball, which is divided into three parts: the central part (*Corpus vesicae*), the upper vault (*Apex vesicae*) and the lower vault (*Fundus vesicae*). The ureter enters the vault posteriorly and the urethra emerge from it anteriorly (10). The

bladder consists of four distinct layers: urothelium, submucosa, detrusor muscle and adventitia (11).

In the urinary bladder, urea is only temporarily stored until being conveniently excreted. Since its composition should not change during rest, the bladder has a specifically built epithelium – urothelium. Urothelium covers most of the lower part of the urinary tract, i.e. proximal urethra, urinary bladder, ureter and kidney bladder (12). Its task is to form a blood-urine barrier that prevents the returning of toxins, water, ammonia and ions from the urine into the blood (13).

Bladder cancer is a common malignant disease. Due to differences in the urinary system in men and women, the specificity of the disease is highly dependent on the gender of the patient (14). In the period from 2010 and 2014, bladder cancer was the 9th most common cancer in Slovenia in men, while in women, its detection was less often (15th most common). Currently, according to the data from Cancer Registry of the Republic of Slovenia, bladder cancer is the 8th most common cancer in men and the 13th most common when both genders are considered. Indicators of cancer burden (especially its incidence) should be compared carefully in each country since registration of bladder cancer is not uniform worldwide. Some cases include in situ and non-invasive papillary carcinoma (the Ta and Tis stages). The Register of Cancer of the Republic of Slovenia (RRRS) for all years follows a rule that T1-T4 bladder cancers are included in the incidence of cancer, especially in situ cases with non-invasive papillary carcinoma (1).

3. Urothelium

The urothelium is the most impermeable epithelium in the human body and surrounds the inside of the renal pelvis, ureter, urinary bladder and urethra. It consists of several layers of cells. They are interconnected by tight junctions that prevent the transfer of molecules into the tissue below and thus provide the blood-urine barrier. In the apical plasma membrane of superficial urothelial cells there are transmembrane proteins called uroplakins, which form urothelial plaques (15-17).

Urothelium functions as a blood-urine barrier by means of three mechanisms. 1) prevention of passive paracellular diffusion by tight intercellular contacts, 2) by presenting a specialized apical plasma of urothelial superficial cells built by uroplakin proteins, 3) by very low exocytosis/endocytosis activity, thereby reducing transcellular transport (18-21). The degree of permeability of urothelial cells depends on their degree of differentiation. Poorly differentiated cells have a higher endocytosis/exocytosis activity than terminally differentiated urothelial cells (19). Despite extremely low permeability, some substances pass through the urothelium, since they must be connected to their environment for the

proper functioning of the cells and their survival (viability). Substances can reach individual cells via two pathways, the paracellular pathway (between cells) and the transcellular pathway (through cells) (19).

4. Objective, purpose and hypothesis

The aim of this study was to synthesize the fluorescent active substance SUF (sodium 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-4',5'-disulfonate (based on reference (22)) and test its applicative use in medicine. The purpose of the work is to find new methods and reagents, which could replace expensive and sophisticated procedures for obtaining fluorescent dyes in the field of cell biology. According to the innovative synthetic approach, SUF is suitable for preparation on a larger scale, since the preparation path is quick, simple and highly efficient (high yield). We believe that the proposed method could improve efficiency in tumor detection and advance cell biology in general.

We hypothesised that with the help of the compound SUF, the cancerous urothelial cells could be distinguished from the healthy (normal) ones by differences in the fluorescence intensity and/or distribution.

Research work with this approach requires knowledge of *in vitro* cell culture techniques, the use of microscopy techniques with an inverted and fluorescence microscope and work in a chemical laboratory.

5. Material and Methods

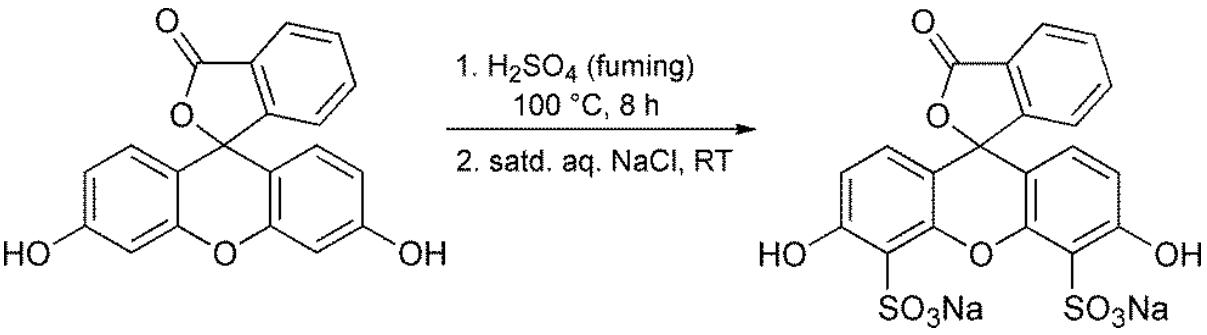

Figure 1: Scheme of SUF synthesis.

5.1. Synthesis and characterisation of fluorescence activity compound, sodium 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-4',5'-disulfonate (SUF)

In a 250 mL flask 10.00 g of fluorescein (15.09 mmol, 1 eq) and 20 mL of oleum were mixed under an inert atmosphere. The reaction mixture was heated in an oil bath at 100° C for 8 h. Then, the resulting reaction mixture was cooled to the room temperature and stirred overnight at room temperature. The mixture was poured into a 140 mL mixture of ice/water in small portions. 200 mL of saturated NaCl solution was added to the mixture. The resulting product was then filtered and washed with ice-cold water (3 × 50 mL). The crude product was recrystallized (purificated) from water (90 mL). Further elaboration by filtration, washing and drying in an evacuated chamber gives 15.34 g of orange-brown product (95.0 % yield).

The compound was characterized by analytical methods and its chemical parameters and characteristics were determined. Knowing these properties improves understanding of the function of the molecule, its binding ability and uses. Preliminary tests of solubility in polar Fluorescence dye SUF was caracterised with fluorescence wavelength and intensity, ^{1}H and ^{13}C-NMR, UV/Vis and TLC (R_f). In addition, the fluorescence analysis under UV light of different wavelengths (366 and 254 nm) was performed. We found that SUF dissolve in both, polar and non-polar liquids (therefore it can be dissolved in an aqueous medium and can pass through the lipid bilayer) and better fluoresces at lower concentrations (0.1 mol/L) at UV light of 366 nm.

We measured: ^{1}H-NMR (300 MHz, DMSO-d_6): δ 12.17 (s, 2H), 8.00 (d, J = 7.5 Hz, 1H), 7.89 – 7.78(m, 1H), 7.73 (t, J = 7.4 Hz, 1H), 7.32 (d, J = 7.6 Hz, 1H), 6.62 (app q, J = 8.8 Hz, 4H). ^{13}C-NMR (300 MHz, DMSO-d_6): δ 168.53, 156.72, 152.22, 149.13, 135.88, 130.40, 130.18, 126.44, 124.90, 124.16, 116.86, 114.51, 109.28. λ_{max} (UV/Vis, 10^{-4} M in EtOH) = 485 nm, fluorescence at 504 nm and TLC (CH_2Cl_2 / MeOH, 9:1), R_f = 0.72. Here, ^{1}H-NMR (300 MHz, DMSO-d_6) is proton nuclear magnetic resonance, ^{13}C-NMR (300 MHz, DMSO-d_6) is carbon NMR (nuclear magnetic resonance), measured at 300 MHz in deuterated solvent dimethyl sulfoxide (DMSO), δ is chemical shift (for the resonance) of nucleus of element X (positive when the sample resonates to high frequency of the reference), J is indirect coupling tensor, s is singled, d is doublet, t is triplet, appq is apparent quartet, m is multiplet, λ is wavelength, TLC is thin layer chromatography, R_f is retardation factor which is equal to the distance migrated over the total distance covered by the solvent, CH_2Cl_2 is dichlorometane and EtOH is Ethanol.

5.2. Biological applications

The biological part of the study consists of the preparation of culture media for the cell cultures, seeding, and maintaining cell cultures in appropriate *in vitro* conditions as

described previously (23-25). In our case, the research involved testing the viability of urothelial cells in a 0.1 mol/L SUF solution.

Cell cultures of normal porcine urothelial (NPU) cells were established from normal porcine urinary bladders. The experiments were approved by the Veterinary Administration of the Slovenian Ministry of Agriculture and Forestry in compliance with the Animal Health Protection Act and the Instructions for Granting Permits for Animal Experimentation for Scientific Purposes. Urinary bladders were cut into 5 cm long and 2 cm wide strips and urothelial cells were gently scraped from the urothelium with a scalpel and collected in UroM medium as described previously (26,27). After collection of urothelial cells, the cells were centrifuged at 200 *× g* for 5 min, washed in UroM medium and filtered through a 40 µm Cell strainer (BD FalconTM, BD Biosciences, New Jersey, USA) to obtain a single-cell suspension. Primary and subsequent subcultures were plated with a seeding density of 2×10^5 cells/cm^2. They were grown in UroM with 0.9 mM calcium and 2.5 % fetal bovine serum (FBS) (Gibco) until confluence and were then transferred to UroM with 2.7 mM calcium and without FBS. For experiments in this study, urothelial cells from the V-XII passages were used. To obtain highly differentiated urothelial cells, the cells were cultured for 2 months (15).

T24 cell line originated from human invasive urothelial neoplasm (ATTC, Manassas, VA, USA). They were cultured in Advanced-Dulbecco's modified essential medium and medium F12 (1:1), 5 % FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin as described in 27 28. The seeding densities of cancer T24 urothelial cells were 5×10^4 cells/cm^2. All cells were grown on plastic dishes at 37 °C in a humidified atmosphere and 5 % CO_2.

5.3. Cell viability

Due to the multilayer nature of urothelial models, Trypan blue viability assay was used to determine the viability of urothelial cells after SUF exposure. Cells were grown in plastic dishes and then incubated with 100 µg/mL of SUF for a week. After one week of incubation, cells were washed to remove non-bound SUF, trypsinized until all cells were detached and immediately stained with Trypan blue dye (following manufacturer's instructions), which labels only dead cells. Live and dead cells were then counted manually under inverted light microscope (Leica DM IL).

The percentage of viable cells (% Viability) in a given sample was determined as the ratio between the number of viable cells in the sample (NS) and the number of all cells in the non-treated control (*N0*) for each cell model: *% Viability = 100 × (NS/N0)*. Two independent experiments were conducted, each in three technical repeats for each urothelial model.

5.4. Fluorescence microscopy

We established normal and cancer urothelial cell models in 4-well chamber slides and treated them with 100 µg/mL of SUF dye for 2 days. Then, the medium was removed with a vacuum pump and cells were washed with fresh medium several times in order to rinse all unbound SUF. After washing, a 4 % formaldehyde fixative was added and left therein for 15 min at room temperature. After 2 minutes, cells were washed with PBS solution for 10 times. Vectashield (a fluorescence-preserving compound) and DAPI (for staining DNA) were added. The samples were then observed under fluorescence microscope (Nikon Eclipse TE 300).

6. Results and discussion

6.1. Chemical part

Synthesis of the fluorescence active substance SUF was carried out as expected, with a high yield of the prepared product. The prepared product was successfully assessed by the characterization parameters that indicated a mechanism of its formation. With preliminary solubility tests of the fluorescent dye SUF in nutrient (culture) media, it has been shown that the SUF dye is very soluble in all the culture media used. Since nutrients are prepared on a water basis (polar agent), it has been shown that the active substance is very soluble in polar solvent systems. This kind of property was also used to measure the spectroscopic and separation properties of the active ingredient.
If we examine the entire structure from the point of view of substituents bound to the basic fluorescein skeleton, polar and nonpolar structural fragments are observed. It was assumed that the SUF could also dissolve in non-polar solvents, so hexane was used for the model solvent. An attempt to dissolve the SUF in hexane has shown that SUF is also partially soluble in non-polar solvents. From this, we concluded below that it will be able to pass by diffusion through a nonpolar phospholipid bilayer of the cell membrane.
An experiment with UV light has shown that SUF in polar solvents intensively fluoresces, while in nonpolar ones, it does not fluoresce. A preliminary study showed that the supplements in culture media are fluoresce better at lower concentrations (100 µg/mL) than at higher doses, which is of key importance for further studies on cell cultures. It is more difficult for cells to withstand higher levels of dye concentration (29).
The fluorescence of the SUF in a culture medium was qualitatively tested by excitation light of wavelengths 366 and 254 nm. It was found that under the light of the wavelength of 254 nm the fluorescence strength was negligible (barely noticeable). Under the light of the wavelength of 366 nm, however, the solution fluoresced the most. The culture medium did not dampen the fluorescence, the latter being more dependent on the wavelength of UV light.

6.2. Biological part

Viability

Cells were counted using a hemocytometer and their viability and number in each sample were calculated as described in the Methods. The obtained results are presented in the form of tables and graphs. Also we calculated averages of viability parameters.

We repeated counting and analysis sixty times, as NPU cells grow slowly and are more demanding for cultivation. Due to relatively small number of cells, the deviation of the results can be very large, which in our case was solved by calculating the averages of several repetitions.

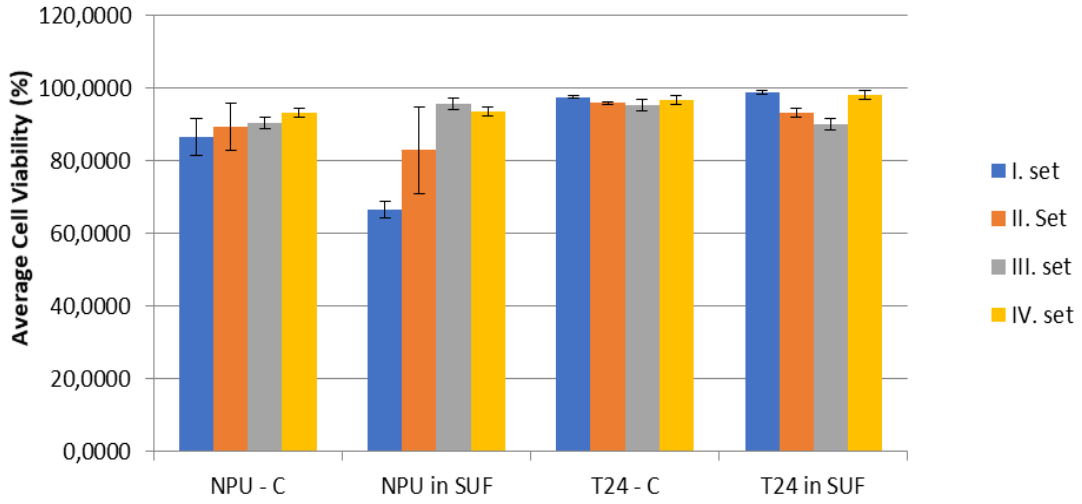

Figure 1. Comparison of the viability of SUF-treated normal cells (NPU in SUF), SUF-treated cancer cells (T24 in SUF), control normal cells (NPU-C) and control cancer cells (T24-C) . Each set represents one independent experiment.

Table 1: Average viability of NPU cells. From the average values of all four cell viability analyses, it was found that the SUF dye with a concentration of 100 µg/mL did not affect the viability of urothelial cells and could be suitable for use in further *in vitro* and *in vivo* studies.

Set	Average viability			
	NPU K	NPU and SUF	T24 K	T24 K and SUF
I	86.6667	66.6667	97.6667	98.8667
II	89.4600	83.0000	96.0000	93.4333
III	90.5100	95.8767	95.3567	90.0533
IV	93.3200	93.5717	96.9700	98.1483

5.3. Microscope visualisation

We observed samples under the fluorescence microscope. In the first sample, T24 p26 cancer cells with a setting density of 5×10^4 cells/cm^2 were fixed. In the second sample, the NPU P5U12a (the code represent the biological sample P5 and 12 represents the passage) cells with a density 2×10^5 cells/cm^2 and the T24 33p cells with a density of 5×10^4 cells/cm^2 were fixed.

The samples were examined and photographed under the green and blue light, and the subsequent photographs of the same field of vision were merged. The analysis of the photographs gave us an insight into the functioning and binding of the fluorescent dye SUF to the urothelial cells.

We observed, in particular, the sites that were more fluorescing, meaning that more fluorescent dye had passed into the cells. The source of this effect could be damaged or dead cells (17). To find the decisive answer, we determined also the state of the nuclei. Analysis of the images (Figure 2) revealed that a certain number of nuclei of NPU and T24 cells were fragmented (Figure 2A, yellow thin arrows). SUF dyes accumulated in the vicinity of the damaged nuclei, resulting in a more intense fluorescence in these areas (Figure 2B, red thin arrow). Further, we assumed that the fluorescent dye accumulated in the intracellular membrane compartments (Figure 2B, red thin arrows) or in the plasma membrane (Figure 2C and E, white arrows), which was perceived as a stronger fluorescence. We can not say with certainty that the fragmentation of the nuclei was due to the accumulation of the SUF dye, as the results of the cell viability analysis contradict the described ones. In addition to this, many cells with intact nucleus exhibited SUF in their internal membrane compartments.

In the sample, there were differences in the degree of differentiation between cells. We found that the SUF staining intensity was influenced by the degree of cell differentiation; less differentiated cells were more strongly labelled since they were not yet able to perform urothelial barrier tasks.

It is also evident from the fluorescence images that the labelling with SUF dye is non-specific. For comparison; the DAPI dye selectively binds to DNA molecules and consequently labels only the nucleus of the cell. The SUF dye is located around the nucleus and differs strongly in the fluorescence labelling of the intracellular membrane compartments in cancer cells.

The last analysis included also differentiated normal urothelial cells. The analysis was carried out in order to show that *in vitro* the distinction between differentiated and cancerous cells can be achieved.

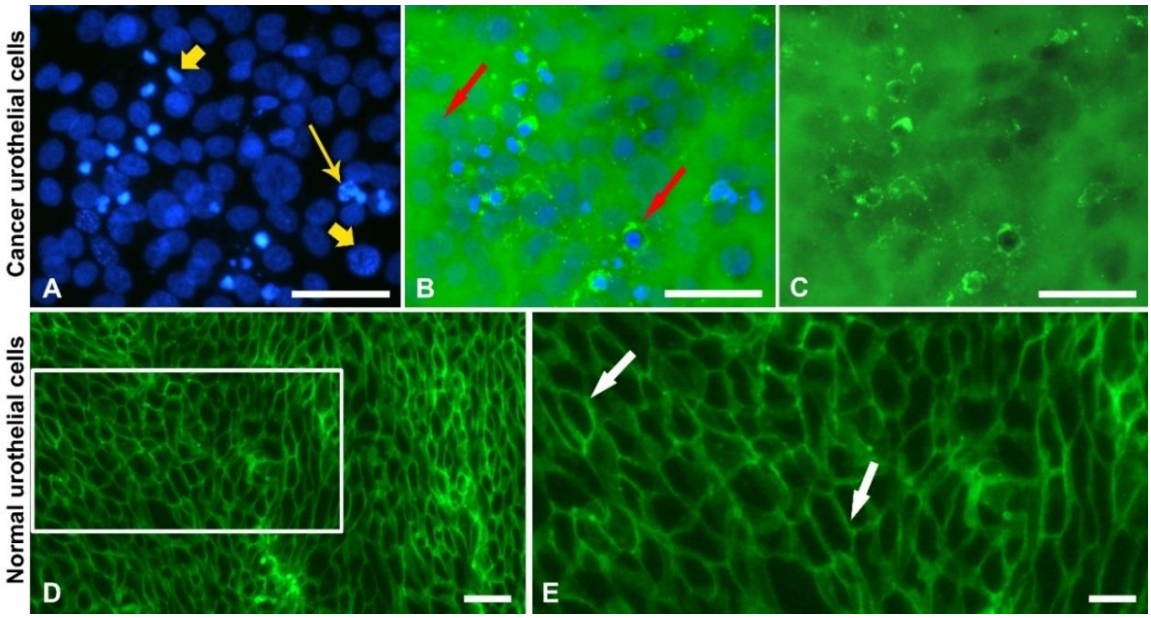

Figure 2: Cancer and normal urothelial cells labelled with SUF. A: cells labelled with DAPI (blue nuclei), fragmented nuclei (yellow thin arrows; chromosomes of cells in mitosis (yellow thick arrows) B: cells labelled with DAPI (blue nuclei) and SUF (green). Cancer urothelial cells with fragmented nuclei (yellow thin arrows) and membrane compartments with the compound SUF (red arrows), a cancer urothelial cell with a normal nucleus and diffuse labeling with SUF (orange thin arrow). C) The same field of view as in A and B, however only SUF is seen. D and E: Differentiated normal urothelial cells labeled with SUF (green) In E, the area inside the white box is enlarged. White arrows show SUF labelling of the apico-lateral part of the cells. Scale bars 50 µm in A, B, C and E, and 100 µm in D.

The results showed that SUF-labelled differentiated normal urothelial cells appear different from SUF-labelled cancer cells. Differentiated normal urothelial cells have special proteins on their membranes – uroplakins that act as a transport barrier. Because of this, SUF almost never entered the cells, but a lot of it accumulated in the areas between the apical and the basolateral cell membranes, resulting in the contrast in fluorescence light emitted by the cell inside and its outside. Since SUF does not fluoresce in non-polar media, we have ruled out the possibility of its binding to the lateral cell membranes. In cancer cells we assumed that the dye passes into their interior by endocytosis and induces labelling of endocytotic compartments. From the above we concluded that in *in vitro* conditions it is possible to distinguish between cancerous and normal urothelial cells.

Conclusions

The main aim of this research work was i) to synthetize the flurescein derivative, which is chemically referred to as sodium 3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene] -4',5'-disulfonate – SUF, and ii) to test SUF on normal urinary bladder cells and cancerous urothelial cells.

The compound demonstrated application potential in the field of medical applications. Based on its chemical structure, it was indicated that it is not toxic and that it can be used as a cellular marker. We showed experimentally that the active ingredient did not harm the cells and could show a difference between the normal and cancerous urothelial cells.

Moreover, from the viability analyses it can be seen that the SUF does not affect urothelial cells growth and viability. The results of viability analyses showed no differences between controls (cells grown without SUF) and samples (cells grown in the presence of SUF). In some cases, cell viability was even greater in the SUF dye medium. These results were also supported by the examination of cells with a fluorescence microscope, where cells were detected in different stages of cell division, which confirms that SUF does not inhibit cell division. Using the results obtained, we expect that the compound SUF is not cytotoxic. But further studies are needed to give a decisive answer.

Acknowledgements

This research work was supported by the Slovenian Research Agency (ARRS) (Grants J3-9262, J1-9162, J2-8166, J2-8169, J1-7302, J3-7494, P3-0388 and P3-0108), MRIC UL IP-0510 Infrastructure program and Grant H2020 VES4US – financed by EU Commission.

We express gratitude to Sanja Čabraja, Sabina Železnik and Taja Železnik Ramuta for their professional and technical assistance.

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