Porphyrin–bile acid conjugates: from saccharide recognition in the solution to the selective cancer cell fluorescence detection†

Jarmila Králová,*[a] Juha Koivukorpi,[b] Zdeněk Kejík,[c] Pavla Poučková,[d] Elina Sievänen,[b] Erkki Kolehmainen[b] and Vladimír Král*[c,e]

Received 13th November 2007, Accepted 1st February 2008
First published as an Advance Article on the web 12th March 2008
DOI: 10.1039/b717528k

This paper describes the preparation and use of conjugates of porphyrins and bile acids as ligands to bind to tumor expressed saccharides. Bile acid–porphyrin conjugates were tested for recognition of saccharides that are typically present on malignant tumor cells. Fluorescence microscopy, *in vitro* PDT cell killing, and PDT of subcutaneous 4T1 mouse tumors is reported. High selectivity for saccharide cancer markers and cancer cells was observed. This *in vivo* and *in vitro* study demonstrated high potential use for these compounds in targeted photodynamic therapy.

Introduction

Recently, a slight decline in the incidence of cancer has been achieved worldwide, but still long-term mortality rates remain high. For successful therapy, early diagnosis of cancer plays the key role.[1] For decades, the microscopy of biopsy samples has represented the principal diagnostic method. However, this method suffers from subjectivity and limited ability to detect the early events of cancer.[2] To fulfil the demand for earliest possible diagnostics, new modern tools have to be found and applied. It is well known that when a tumor is detected, certain changes at the molecular level have already occurred. The main goal of the new diagnostic approaches is to recognize these changes as early as possible. This recognition can be based on a specific interaction of diagnostic agents with suitable molecular partners; cancer biomarkers. Biomarkers (e.g., proteins, polysaccharides, or nucleic acids) are important molecular signatures of cell phenotype, and they can be used for specific detection and recognition of particular cell types. Since oncogenic transformations are accompanied by morphological changes of the cells and the related expression of genes and proteins, the cell signature changes during cancer development as well. By reading these changes accurately, we can improve the early detection and diagnosis of a specific form of cancer. Optimal recognition for diagnostic or therapeutic agents preferably uses biomarkers (targets), which are overexpressed on all tumor cells but not present on the normal cells, and are required for cell survival or critical functions. The recognition component of a diagnostic agent can also be used for the effective enhancement of drug delivery systems. At present, many biomarkers of cancers have been identified.[1,3] High levels of glycosylation of glycoproteins and glycolipids are one of many molecular changes that accompany malignant transformations.[4] This phenomenon includes overexpression of the cell surface polysaccharides (heparan sulfate,[5] polysialic acid,[6] hyaluronic acid[7]), oligosaccharides, and modification of the surface receptors (e.g., sialylation of glycolipids).[4,8] These changes are characteristic for cancer cells and can protect them from immune surveillance and chemotherapeutic agents, and enhance their metastatic capacity.[9] Many studies have shown a correlation between higher glycosylation degrees and poor prognosis of cancer. For instance, a general increase in sialylation[10] can lead to the decline of clinical state, metastasis and poor prognosis. On the other hand, desialylation[11] of tumor cells decreases tumor malignancy and inhibits tumor growth. Suppression of glucan synthesis targets the decrease of tumor growth.[4] For detection of saccharides various probes have been successfully utilized.[12] Changes in glycosylation of specific glycoproteins associated with tumor appearance can be monitored using a specific recognition of cancer cells by a monoclonal antibody. Changes in the localization and relative abundance of carbohydrate species on cell surfaces can be monitored with the aid of specific carbohydrate binding proteins, such as lectins.[13] Lectin histochemistry has been utilized to identify modulation of the expression of sialic acid on human cervical carcinomas.[12]

Chemical probes for specific saccharide recognition are under continuous development. They are based on a wide variety of chemical structures; in particular boronic acids[14,15] have been extensively studied.

Chemoprobes designed for recognition of cancer saccharide receptors have been described. Yang *et al.* prepared fluorescent diboronic acid probes for specific determination of cancer cells with overexpressed sialyl Lewis X carbohydrate.[16,17] Lectins and lectin-based probes[18,19] use a hydrophobic cavity for saccharide recognition.

In our experience,[20–26] an effective probe designed for saccharide sensing in aqueous solutions should have a hydrophobic binding domain, which in our design has been created by the array of conjugated aromatic groups. This domain plays an important

[a]*Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083, 14220 Prague 4, Czech Republic. E-mail: kralova@img.cas.cz; Fax: +420-241 063 586*
[b]*Department of Chemistry, P.O. Box 35, FIN-40014 University of Jyväskylä, Finland. E-mail: jkkorpi@cc.jyu.fi; Fax: +358-14-260-2501*
[c]*Institute of Chemical Technology, Technická 5, 16628 Prague 6, Czech Republic. E-mail: vladimir.kral@vscht.cz; Fax: +420-224-310-859*
[d]*Institute of Biophysics, 1st Medical Faculty of Charles University, Ovocný trh 3 Prague 1, Czech Republic. E-mail: pouckova@volny.cz*
[e]*Zentiva R & D, U Kabelovny 130, 10237 Prague 10, Czech Republic*

† Electronic supplementary information (ESI) available: Dependence of absorption on the concentration for tested probes **1**–**4** in water, and mixed solvents. See DOI: 10.1039/b717528k

part in the interaction, especially if the recognition of oligo- or polysaccharides is required, as these compounds can have hydrophobic parts.[12] Our strategy in the construction of the saccharide probes is based on a hydrophobic cavity, represented by a porphyrin core, decorated with the saccharide binding groups, steroidal macrocyclic substituents.

We reported earlier several saccharide binding ligands. The general design is based on multiple binding modes generated by introduction of recognition groups on porphyrin scaffold, which serves as chromophoric sensing unit.

Our designed ligands are based on a wide variety of chemical structures, *e.g.* binaphthols,[20–22] phosphonates,[23,24] steroids,[25] boronic acids[14,15] and porphyrin-cryptand systems.[26] Thus anionic porphyrin phosphonates (bis and tetrakis) were reported for mono- and oligosaccharide binding in water;[23,24] also a corresponding porphyrin sulfonated for polysaccharide binding was reported by our group.[27] Another set of interesting ligands was created with 1,1′-binaphthyl peripheral substitution of a porphyrin macrocycle, and mono- and oligosaccharide binding in aqueous environment was reported with selectivity for oligosaccharides.[20–22]

Novel steroid meso-substituted porphyrin derivative was prepared; synthetic strategy was based on the application of novel steroid aldehyde precursors for the synthesis of porphyrin skeleton. The ligand combines advantages of steroids, which are responsible for saccharide binding, and of the porphyrin moiety acting as a signaling component of the probe, due to changes in UV-visible electronic spectra. Selectivity for complexation of saccharides was reported.[25]

We have reported bisporphyrin ligands with multiple binding sites for oligosaccharides.[26] Two macrocyclic porphyrin sandwich systems have been prepared and examined as saccharide receptors. The cyclic porphyrin-cryptand conjugates bind saccharides efficiently in highly competitive media with a preference for trisaccharides, probably due to a complementary topology of hydrophobic and hydrophilic solvating regimes with respect to the sugar guests.[26]

Inspired by natural ligands for selective saccharide complexation, we have designed and prepared simple water-soluble lanthanum and europium complexes, which proved to be effective for detection of neutral sugars as well as glycolipids and phospholipids. At physiologically relevant pH the fluorescent lanthanum complex binds neutral sugars with apparent binding constants comparable to those of arylboronic acids.[28] Detection of sialic acid was achieved.[29]

We have described the interactions of two water-soluble metallotexaphyrins, containing coordinated lutetium(III) and gadolinium(III) cations, with uronic acids (D-galacturonic and D-glucuronic acids), and neutral (amylose, galactan) and anionic (pectate, alginate) polysaccharides studied using UV-VIS titrations.[30]

Recently, we have reported porphyrin–bile acid conjugates, where, for water solubility reasons, quaternary ammonium linkers have been embedded into their structures.[31] We reported a binding study for simple mono- and disaccharides.[31]

Here we describe the binding study of porphyrin–bile acid conjugates **1–4** (Fig. 1) with biologically important saccharides with possible application for cell surface recognition. This represents a novel alternative to classical approach based on a well-known principle using specific monoclonal antibodies (MABs) for cancer cell recognition.

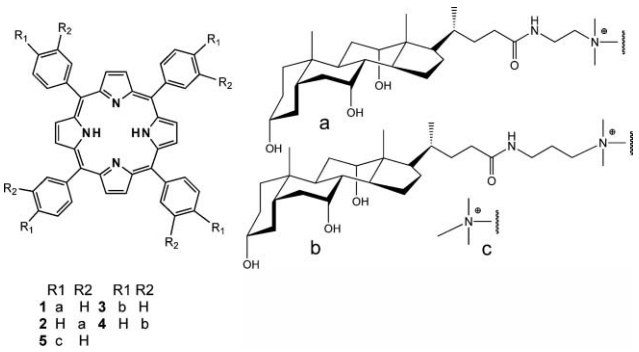

Fig. 1 Synthetic ligands **1–5**

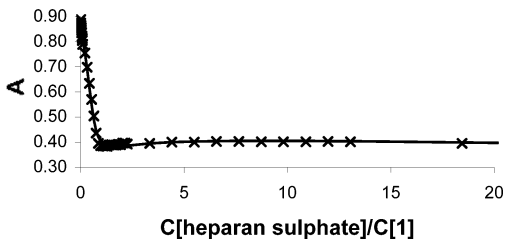

Fig. 2 Titration curve of **1** with heparan sulfate. Plots denote measured data points. The solid line is a calculated curve. Concentration of porhyrin **1** is 1.6 μM.

Results and discussion

Specificity of reported porphyrin probes for biologically relevant oligosaccharides was tested in an environment, in which our probes do not aggregate. The binding results are reported for mixed solvent: 30% MeOH–water (Table 1, Fig. 2).

We have also tested the behaviour of our porphyrins under physiological conditions, namely in the presence of glucose. The binding study clearly showed that we could selectively detect cancer markers in the presence of monosaccharides, namely glucose (Fig. 3 and 4), while we have not seen any interaction with proteins, namely HSA.

The outcome of binding studies showed that the probes have a high potential to recognize various oligosaccharides under physiological conditions. Here, we demonstrate that they can be used for selective fluorescence detection of transformed cells expressing highly glycosylated markers.

Table 1 Log K_s-values and stoichiometry (porphyrin : saccharides) of complexes of **1–5** with saccharides (errors < 20)

Saccharide	Stoichiometry of complex	**1**	**2**	**3**	**4**	**5**
Glucose	1 : 1	5.7	5.7	5.7	5.8	2
Sialic acid	1 : 1	5.7	5.8	5.8	5.8	4.3
Hyaluronic acid	1 : 1	5.4	7.0	6.6	5.1	5.7
	1 : 2	11	12	11	11	12
	1 : 1	7.0	7.7	8.3	9	7.0
Heparan sulfate	1 : 2	13.1	14.4	7.4	16	13
	2 : 3	26	29	30	33	—

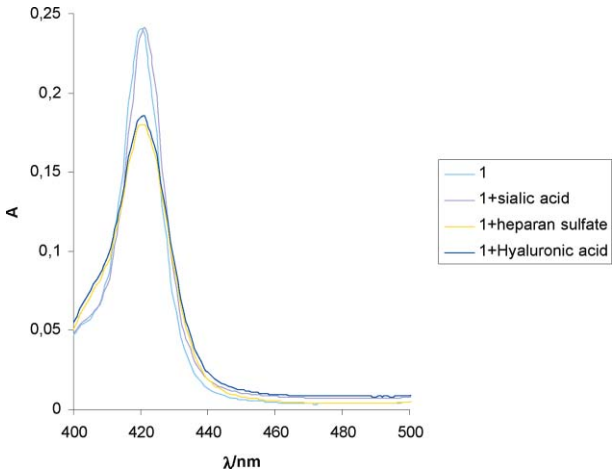

Fig. 3 UV-VIS spectra of porphyrin–bile acid conjugate **1** in the presence of 1 mM glucose, pH = 7.4. Saccharide cancer markers were supplemented 24 h after dissolving of **1**. Concentration of porphyrin is 1.6 μM. Concentration of markers is 0.1 mM.

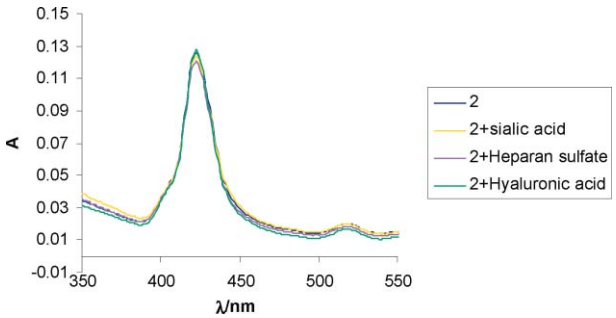

Fig. 4 UV-VIS spectra of porphyrin–bile acid conjugate **2** in the presence of 1 mM glucose, pH = 7.4. Saccharide cancer markers were supplemented 24 h after dissolving of **2**. Concentration of porphyrin is 1.6 μM. Concentration of markers is 0.1 mM.

We used compound **5** as a control molecule for recognition of difference in binding and therapeutic properties of a simple cationic porphyrin and of tested cholic acid porphyrin conjugates. Binding affinities and selectivities of **1–4** have been studied with three saccharides (heparan sulfate, hyaluronic acid, and sialic acid), which are expressed on tumor cells. Because the tested markers are negatively charged and our ligands are cationic compounds, control binding experiments (ruling out simple coulombic interaction) using parental cationic porphyrin without cholic acid substituents (compound **5**) were included. For control porphyrin **5** we have not observed oligosaccharide binding selectivity.

All saccharides, especially heparan sulfate, showed high binding affinities for **1–4** as can be seen from Table 1 (where log K_s is the binding constant) in contrast to control compound **5**. The particularly high binding affinity of heparan can be explained by the high negative charge of the heparan sulfate unit.

After the *in vitro* binding studies of saccharides with probes **1–5** were determined, the selectivity of the probes for transformed cells was tested. Initial experiments were undertaken to assess their ability to recognize the surface of tumor cells. Various cell lines when incubated with 0.5–1.0 μM bile acid–porphyrin conjugates (**1–4**) showed distinct intracellular fluorescence (Fig. 5A), while no fluorescence was detected with control compound **5** (Fig. 5C). The potential of bile acid-porphyrin probes to selectively recognize and accumulate in cells with transformed phenotype is demonstrated using ligand **2** as an example. The transformed cells (PRRSBL, CEF/RSV, and SW480) display a bright cytoplasmic fluorescence, whereas their untransformed counterparts (murine and chicken embryo fibroblasts, 3T3 and CEF respectively, and normal colon epithelial cells, FHC), treated under identical conditions show minimal fluorescence (Fig 5B). At studied concentration range compounds **1** and **2** were not toxic to the cells incubated in darkness, but after irradiation the transformed cells exhibited high level of cell death (Fig 6).

The type of cell death was concentration and light dose dependent. At low doses (0.5 μM, 4.3 J/cm^2) cells died mainly *via* apoptosis, while at higher doses necrosis also took place. In contrast, untransformed cells treated under identical conditions remained viable.

Importantly, the intracellular uptake of these porphyrin derivatives is an active process requiring energy since it can be inhibited by temperature switch to 0 °C. Prevalent lysosomal localization of porphyrins suggests that the uptake is most likely mediated by endocytosis pathway(s). The involvement of receptor-mediated endocytosis is currently under investigation.

Next, the photodynamic therapy (PDT) efficacy was tested *in vivo* using BALB/c mice with mammary carcinoma. Various time intervals between porphyrin conjugate application (intravenous injection) and light irradiation (100 J/cm^2) were tested (Fig. 7, and Table 2). The highest efficiency of PDT was found at a 2 h interval. Control porphyrin **5** was very toxic to mice therefore these results are not shown.

Conclusions

Analysis of DNA fragmentation and other apoptotic markers revealed, that mechanism of PDT using porphyrin-cholic acid conjugates **1–4** is a combination of apoptosis and necrosis.

In conclusion, this paper combines results from oligosaccharide binding studies, cancer cell line selective recognition and in vivo PDT examination. This work clearly proves that cholic acid-porphyrin conjugates are preferentially taken up by transformed cells and can be used for selective ablation of tumors by PDT. High efficiencies of cholic acid-porphyrin conjugates in *in vivo* and *in vitro* targeted PDT were observed.

Experimental section

Synthesis of 1–5

Compounds **1–5** were prepared as we described previously.[31,32]

Determination of binding constants of 1–5 with saccharides

The association of **1–5** with saccharides was studied using UV-Vis spectroscopy according to the method reported previously.[31] Binding constants (K_s) were calculated from absorbance changes of the porphyrin using Soret band maximum (ΔA) by nonlinear regression using the program Letagroup spefo 2005. Because polymer chains have various lengths, the K_s values of **1–5** with polysaccharides were calculated using a polysaccharide concentration defined by the concentration of each repeated disaccharide unit.

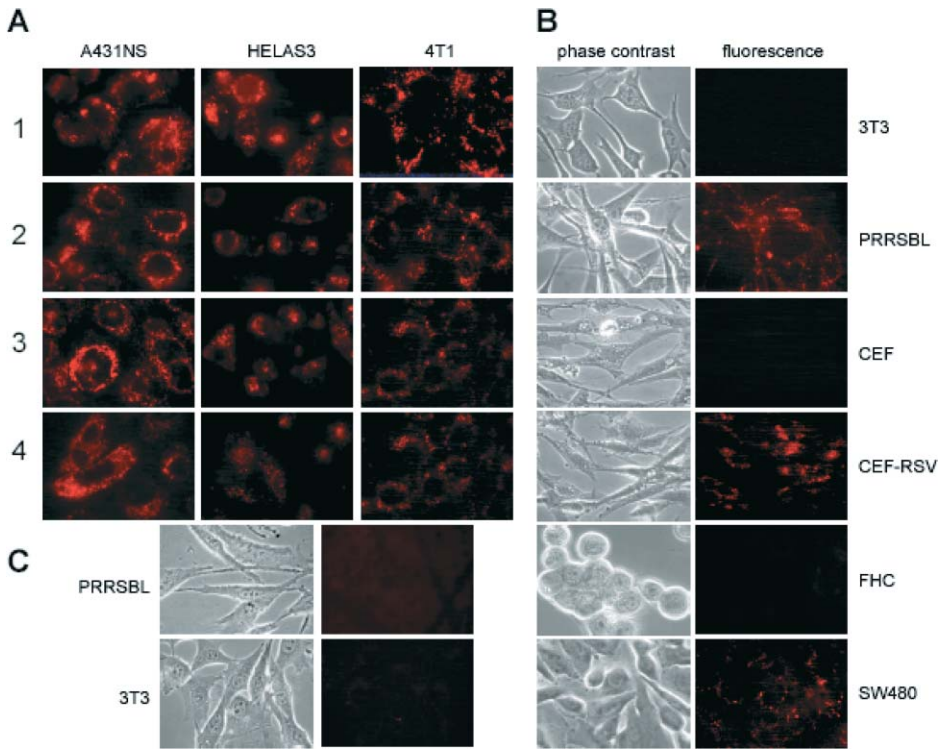

Fig. 5 Cellular localization pattern. (A) Fluorescence images of cancer cells (A431NS, HeLaS3, 4T1) treated with **1**, **2**, **3**, and **4**. (B) Comparison of transformed (tumor) cells (PRRSBL, CEF-RSV, SW480) stained by **2** and their untransformed counterparts (3T3, CEF, FHC). (C) Fluorescence and phase contrast images of cells treated with **5**.

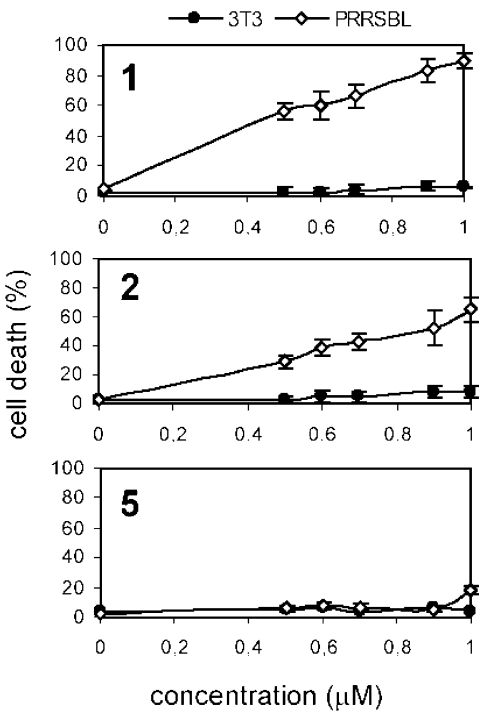

Fig. 6 Phototoxicity of porphyrin conjugates **1**, **2** and **5**.

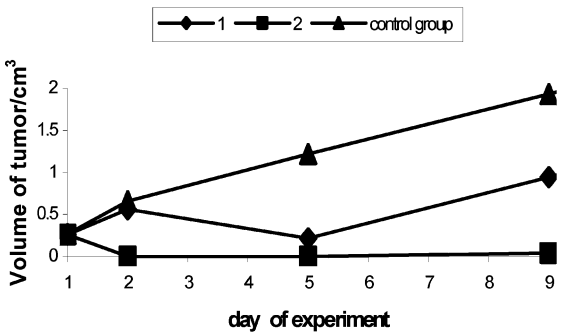

Fig. 7 Effect of porphyrin **1**- and **2**-mediated PDT on the growth of subcutaneous mouse mammary carcinoma 4T1 at 2 h time interval between the drug administration (5 mg/kg) and irradiation (100 J/cm^2).

Cell lines

Cell lines, 4T1 (mouse mammary carcinoma), HeLaS3 (human cervical carcinoma), A431NS (human epidermoid carcinoma), and FHC (normal colon epithelial cells), used in this study were obtained from American Type Culture Collection (Manassas, VA) and were propagated in the recommended media (Sigma), with 10% fetal calf sera (PAA Laboratories, Linz, Austria) and other suggested additives at 37 °C in a humidified 5% CO_2 atmosphere. PRRSBL (murine sarcoma cell line induced[33] by RSV), SW480 (human colorectal adenocarcinoma), and NIH3T3 (murine embryo fibroblast) were obtained from Dr E. Sloncová

Table 2 PDT reduction of tumour volumes by cholic acid–porphyrin conjugates at various time intervals between porphyrin application and irradiation

Time of dwell	**1**	**2**
2 h	0.51	0.98
8 h	0.85	0.38
24 h	0.63	0.43

(IMG, Czech Rep.). CEFs (chick embryo fibroblasts) were prepared from 11-day-old embryos and maintained in culture in Dulbecco's Modified Eagle's Medium supplemented with 5% FCS and 2% chick serum (Sigma). To make CEF-RSV cells, cultures of fibroblasts were transformed by the Prague C (Pr–C) strain of Rous sarcoma virus (RSV) carrying v-src oncogene and used for experiments 10 days after infection.

Microscopic studies

Cells grown on coverslips in 35-mm Petri dishes were incubated with 0.5–1 μM bile acid-porphyrin conjugates **1–4** in the complete culture medium at 37 °C overnight. To remove the loosely bound sensitizer, the cells were rinsed with PBS, re-fed with fresh medium without phenol red, and incubated for 1 h at 37 °C. Bile acid-porphyrin stock solutions were freshly prepared in DMSO for each experiment and the final DMSO concentration in culture did not exceed 0.1%. Fluorescence was monitored live under a fluorescence microscope (Leitz DM IRB, Leica) equipped with digital camera DFC 480 (Leica) using a 100× oil immersion objective and Leica filter cube N2.1 (excitation filter BP 515–560 nm and long pass filter LP 590 nm for emission).

In vitro photosensitization and cell death determination

Cells 3T3 and PRRSBL (4×10^5) seeded in 35 mm dishes were grown overnight at 37°C, 5%CO_2. Bile acid-porphyrin derivatives **1** and **2**, and control **5** were added to the cells at a final concentration of 0–1 μM, and then the cells were incubated for 16 h before irradiation. Cells were rinsed with PBS, re-fed with fresh medium without phenol red for 1 h and then illuminated by a 75 W halogen lamp with a band pass filter (Andover, Salem, NH, USA) with resulting wavelength 500–510 nm. The fluence rate at the level of cell monolayer was 0.7 mW cm^{-2} and total light dose was 4.3 J cm^{-2}. Following irradiation, the viability of post-PDT cultures was determined next day by the Trypan blue exclusion method. Control 'dark' experiments (without illumination) were performed in parallel.

In vivo experiments

BALB/c mice were subcutaneously transplanted with 4T1 mammary carcinoma cells as described before.[34] When the tumor mass reached a volume of 200–300 mm^3 (about 7–10 days after transplantation) mice were injected with porphyrin conjugates (5 mg kg^{-1}, intravenous injection) in a volume of 0.1 mL per 20 g mice, and 2, 8 or 24 hours later the tumor area (2 cm^2) was irradiated by 500–700 nm xenon lamp ONL 051 (maximum at 635 nm, Preciosa Crytur, Turnov, Czech Republic) with a total impact energy of 100 J cm^{-2} and fluence rate of 200 mW cm^{-2}. Control group represented mice without drug application. Each experimental group consisted of five mice. All aspects of the animal experiment and husbandry were carried out in compliance with national and European regulations and were approved by the institutional committee.

Acknowledgements

This work was funded by grant from the Grant Agency of the Czech Republic (Grant No. 203/06/1038) and supported in part by project LC06077 and 512 awarded by the Ministry of Education of the Czech Republic, by project AV0Z50520514 awarded by the Academy of Sciences of the Czech Republic to J. Králová and by project MSM6046137307.

References

1 R. S. Negm, M. Verma and S. Srivastava, *Trends Modern Medicine*, 2002, **8**, 288–293.
2 K. M. Fong, S. Srivastava and R. Gopal-Srivastva, *Molecular, genetic basis for early cancer detection and susceptilibity*, IOS Press, Amsterdam, edn 1, 1999, pp. 13–26.
3 L. Novellino, C. Castelli and G. Parmiani, *Cancer Immunol. Immunother.*, 2005, **54**, 187–207.
4 J. W. Dennis, M. Granovsky and C. E. Waren, *Biochim. Biophys. Acta*, 1999, **1473**, 21–34.
5 D. L. M Beauvais and A. C. Rapraeger, *Reprod. Biol. Endocrin.*, 2004, **2**, 1–12.
6 K. Kameda, H. Shimada, T. Ishikawa, A. Takimoto, N. Momiyama, S. Hasegawa, K. Misuta, A. Nakano, Y. Nagashima and Y. Ichikawa, *Cancer Lett.*, 1999, **137**, 201–207.
7 V. B. Lokeshwar, D. Rubinowicz, L. S. Schroeder, E. Forgacsi, J. D. Minnai, N. L. Block, M. Nadji and B. L. J. Lokeshwar, *J. Biol. Chem.*, 2001, **276**, 11922–11932.
8 K. B. Rajpura, P. S. Patel, J. G. Chawda and R. M. Shah, *J. Oral Pathol. Med.*, 2005, **34**, 263–267.
9 K. Hobarth, U. Maier and M. Marberger, *Eur. Urol.*, 1992, **21**, 206–210.
10 Y. J. Kim and A. Varki, *Glycoconj. J*, 1997, **14**, 569–576.
11 T. Miyagi, T. Wada, K. Yamaguchi and K. Hata, *Glycoconj. J.*, 2004, **20**, 189–198.
12 R. Jelinek and S. Kolushevá, *Chem. Rev.*, 2004, **104**, 5987–6015.
13 S. Banerjee, P. Robson, W. P. Soutter and C. S. Foster, *Hum. Pathol.*, 1995, **26**, 1005–1013.
14 S. Jiang, J. O. Escobedo, K. K. Kim, O. Alptürk, G. K. Samoei, S. O. Fakayode, I. M. Warner, O. Rusin and R. M. Strongin, *J. Am. Chem. Soc.*, 2006, **128**, 12221–12228.
15 O. Rusin, O. Alptürk, M. He, J. O. Escobedo, J. Shan, D. Fareed, L. Kun, M. E. McCarroll, I. M. Warner and R. M. Strongin, *J. Fluoresc.*, 2004, **14**, 611–615.
16 W. Yang, S. Gao, X. Gao, V. V. R Karnati, W. Ni, B. Wang, W. B. Hooks, J. Carson and B. Weston, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2175–2177.
17 W. Yang, H. Fan, X. Gao, S. Gao, V. V. R. Karnati, W. Ni, W. B. Hooks, J. Carson, B. Weston and B. Wang, *Chem. Biol.*, 2004, **11**, 439–448.
18 J. W. Becker, G. N. Reeke, J. R. J. L. Wang, B A. Cunningham and G. M. Edelman, *J. Biol. Chem.*, 1975, **250**, 1513–1524.
19 O. Rusin, V. Král, J. O. Escobedo and R. M. Strongin, *Org. Lett.*, 2004, **6**, 1373–1376.
20 O. Rusin, K. Lang and V. Král, *Chem. Eur. J.*, 2002, **8**, 655–663.
21 O. Rusin and V. Král, *Sens. Act. B*, 2001, **76**, 331–335.
22 O. Rusin and V. Král, *Chem. Commun.*, 1999, 2367–2368.
23 O. Rusin, M. Hub and V. Král, *Mat. Sci. Eng. C.*, 2001, **18**, 135–140.
24 V. Král, O. Rusin, J. Charvátová, P. Anzenbacher and J. Fogl, *Tetrahedron Lett.*, 2000, **41**, 10147.
25 M. Dukh, D. Šaman, K. Lang, V. Pouzar, I. Černý, P. Drašar and V. Král, *Org. Biomol. Chem.*, 2003, **1**, 3458–3463.
26 V. Král, O. Rusin and F. P. Schmidtchen, *Org. Lett.*, 2001, **3**, 873–876.
27 A. Synytsya, A. Synytsya, P. Blafková, K. Volka and V. Král, *Spectrochimica Acta Part A.*, 2007, **66**, 225–235.
28 O. Alpturk, O. Rusin, S. O. Fakayode, W. Wang, J. O. Escobedo, I. M. Warner, W. E. Crowe, V. Král, J. M. Pruet and R. M. Strongin, *PNAS*, 2006, **103**, 9756–9760.
29 Y. Yang, P. T. Lewis, J. O. Escobedo, N. N. Luce, W. D. Treleaven, R. L. Cook and R. M. Strongin, *Collect. Czech Chem. Commun.*, 2004, **69**, 1282–1291.
30 A. Synytsya, A. Synytsya, V. Král, K. Volka, J. Čopíková and J. L. Sessler, *J. Chem. Soc. Perkin Trans. 2.*, 2000, **9**, 1876–1884.
31 J. Koivukorpi, E. Sievänen, E. Kolehmainen and V. Král, *Molecules*, 2007, **12**, 13–24.
32 V. Král, S. Pataridis, V. Setnička, K. Záruba, M. Urbanová and K. Volka, *Tetrahedron*, 2005, **61**, 5499–5506.
33 V. Sovova, H. Fidlerova, D. Trnka and E. Sloncova, *Folia Biol. (Praha)*, 1994, **40**, 291–299.
34 J. Kralova, A. Synytsya, P. Pouckova, M. Koc, M. Dvorak and V. Kral, *Photochem. Photobiol.*, 2006, **82**, 432–438.

