

Assessing the in vivo Safety of Dendrimer-Based Formulations Used in Photodynamic Therapy

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Introduction: Photodynamic therapy (PDT) is a promising cancer treatment. However, the efficacy of photosensitizers such as rose bengal (RB) is often limited by poor delivery. Dendrimer-based nanocarriers can enhance PDT efficacy in vitro, but their in vivo safety profile remains largely uncharacterized. This study aimed to assess the systemic safety of three different dendrimer-based RB delivery systems in a healthy mouse model.

Methods: BALB/c mice were randomly divided into eight groups and received weekly intraperitoneal injections of either PBS (control), free RB, or one of three carriers (phosphorus dendrimer 1cat, dendrimersome DG2, PPI G3 dendrimer) with or without RB. Body weight was monitored weekly. Blood and urine samples were collected over four weeks for comprehensive biochemical and microscopic analysis, assessing markers for liver, kidney, and muscle function.

Results: No significant changes in body weight were observed across any of the groups. Analysis of blood biochemical parameters (including ALT, AST, urea, creatinine, and LDH) and urine profiles revealed no statistically significant differences between any treatment group and the PBS control group over the four-week study period. The observed minor fluctuations in some parameters were not dose- or time-dependent and remained within normal physiological ranges.

Conclusion: The three tested nanocarrier systems - phosphorus dendrimer 1cat, dendrimersome DG2, and PPI G3 dendrimer - and their respective rose bengal formulations are well tolerated and do not induce systemic toxicity in BALB/c mice at the tested concentrations. These findings support their safety for in vivo applications and provide a basis for future efficacy studies in tumor-bearing animal models.

Keywords: rose bengal, photosensitizer, photodynamic therapy, phosphorus dendrimer, poly(propyleneimine) dendrimer, triazine-carbosilane dendrimersomes

Introduction

Photodynamic therapy emerged as a treatment option more than 50 years ago. The results of research by Diamond et al, published in the Lancet in 1972, showed that photodynamic therapy could be an effective approach to the treatment of brain tumors and other cancers resistant to existing forms of therapy. All that is needed is a combination of porphyrins, powerful photodynamic agents that can sensitize biological preparations, causing them to be severely damaged when exposed to visible or near-ultraviolet light. The effectiveness of the method is based on photo-oxidation reactions involving the production of electronically excited metastable molecular oxygen (singlet oxygen) as a reactive and highly toxic intermediate.¹ Many subsequent years of research led to the optimization of a method based on three elements: photosensitizer, molecular oxygen and visible light. It was confirmed that a photosensitizer absorbs energy after exposure to a specific wavelength of visible light, which led to its the excitation from the ground state (S₀) to the first excited singlet

state (S1). The excited photosensitizer might lose energy through fluorescence emission, conversion to heat, or intersystem transitions, creating a long-lived excited triplet state (T1). As we have described in our previous work, a photosensitizer in the excited triplet state can react with other molecules leading to the formation of radicals and reactive oxygen species (ROS) (Type I mechanism) or highly reactive singlet oxygen (Type II mechanism), which, regardless of the type of mechanism, leads to oxidative stress and a cascade of reactions causing cell death.² Our research has also shown that the use of a variety of polymer carriers can effectively increase the efficacy of PDT therapy. In particular, dendritic molecules, such as dendrimers – highly symmetric hyperbranched polymers – and dendrons – isolated branches of dendrimers – are considered prospective carriers for transporting photosensitizers into cells or tissues.

Among dendrimer species, polycationic phosphorus dendrimer G3 (1cat)³ proved to be a promising candidate as a carrier of rose bengal (RB) molecules. The photosensitizer complexed with the dendrimer in a 5:1 ratio (RB-1cat complex) caused increased production of singlet oxygen ($^1\text{O}_2$), showed no dark toxicity, and 1cat ensured more effective cellular uptake of RB, resulting in significant enhancement of the photodynamic effect of RB against basal cell carcinoma (BCC) cells and increased production of reactive oxygen species (ROS).² Subsequent research by our team has shown that commercially available cationic PAMAM and PPI dendrimers can also serve as efficient RB carriers in photodynamic therapy. For the various reasons previously described,⁴ PPI dendrimers outperform PAMAM dendrimers, and in the case of PPI G3, significantly increase singlet oxygen production. A key role is played by the selection of appropriate drug and dendrimer concentrations to ensure uniform distribution of RB within the dendrimer structure, thus preventing photosensitizer aggregation and allowing the delivery system to maintain a positive surface charge.⁴

An alternative approach involves vesicle-like assemblies of amphiphilic dendrons (dendrimersomes) to encapsulate photosensitizer and to release it in a cell after successful endocytosis. In our hands, this approach also showed good PDT efficiency. Encapsulation of rose bengal in triazine-carbosilane dendrimersomes increased the cellular uptake, intracellular ROS production and consequently the phototoxicity of this photosensitizer.⁵

These very encouraging results obtained by our team shed new light on PDT of skin cancer. Our previous work has shown that RB primarily interacts with the cationic, 1-cat and PPI-G3 dendrimers through electrostatic and hydrophobic interactions while being physically encapsulated within DG2 dendrimer bilayers.^{5,6} Although these interactions are crucial for enhancing the efficacy of photodynamic therapy (PDT) in vitro, the in vivo safety of these systems remains unknown. Therefore, this article is dedicated to assessing the safety of using carrier-photosensitizer systems selected by in vitro tests under in vivo conditions.

Materials and Methods

Chemicals

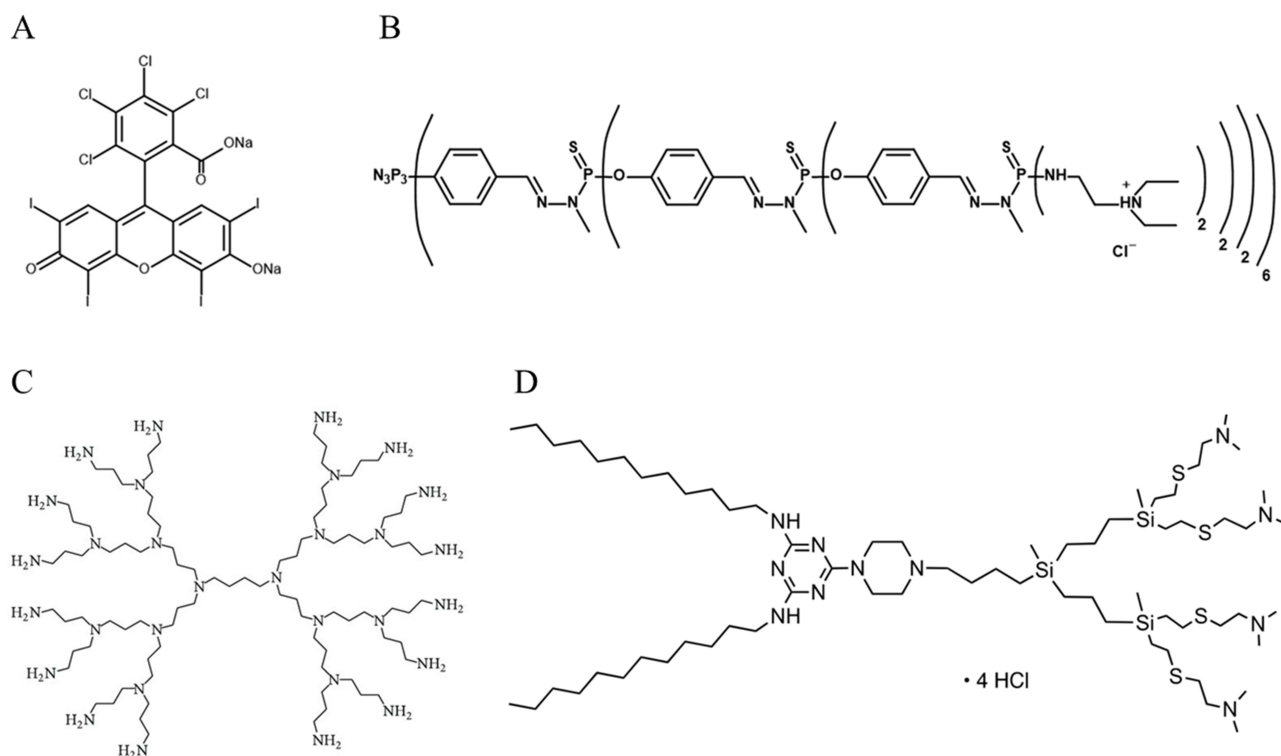
PBS, rose bengal (RB) were obtained from Sigma-Aldrich. Poly(propyleneimine) dendrimer G3 with 32 primary amino surface groups (PPI G3) was obtained from Symo-Chem (Eindhoven, the Netherlands). Phosphorus dendrimer of the third generation possessing 48 diethylammonium surface groups (referred as 1cat) was synthesized as published elsewhere.³ Dendrimer-rose bengal formulations were prepared and characterized according to protocols described elsewhere.⁴

Amphiphilic triazine-carbosilane dendron bearing branched hydrophobic moiety in the focal point and dimethylammonium surface groups (referred as DG2) was synthesized according to the previously published procedure.⁶ Both blank and rose bengal-loaded dendrimersomes were prepared and characterized as described in Sztandera et al.⁵

The structures of rose bengal dye (A), 1cat phosphorus dendrimer (B), PPI G3 dendrimer (C) and DG2 amphiphilic triazine-carbosilane dendron (D) are depicted in [Scheme 1](#).

Characterization of Complexes

This short paragraph summarizes the key physicochemical properties of nanoparticles from our previous studies. The stoichiometry of the RB-1cat complex formed by titrating the photosensitizer with a cationic dendrimer was investigated using the fluorescence properties of rose bengal. The stoichiometry of the complex was found to be 7:1 (RB:cat). However, in vitro studies have shown that a complex with a stoichiometry of 5:1 and a concentration of 0.25:0.05 μM has the most effective antitumor effect.² The formation of complexes between PPI dendrimers and RB was characterized



Scheme 1 Structures of components used in this study. Rose bengal dye (A), 1cat phosphorus dendrimer (B), PPI G3 dendrimer (C) and DG2 amphiphilic triazine-carbosilane dendron (D).

using dye fluorescence and zeta potential analysis of the nanoparticles in solution. Spectrofluorometric studies showed that adding a PPI dendrimer to an RB solution led to a significant decrease in dye fluorescence. The F564/F575 ratio was then calculated and plotted against the RB:PPI dendrimer molar ratio. Job's method was then used to approximate the stoichiometry of the binding complexes and compare it with the zeta potential measurements. The stoichiometry of the formed complex was determined based on the titration curves, with a resulting value of 21:1 for RB:PPI G3 similar to that obtained by spectrofluorometric analysis.⁴ However, *in vitro* studies have shown that a complex with a stoichiometry of 10:1 and a concentration of 2:0.2 μM has the most effective antitumor effect. The physicochemical properties of the rose bengal-loaded dendrimersomes (G2-RB) and the free dendrimersomes (G2) are presented in Table 1. The loading capacity of rose bengal in the second-generation dendrimersome structure was spectrophotometrically estimated to be approximately 22.5% and the *in vitro* antitumor efficacy at an IC₅₀ concentration of 1 μM for RB.⁵ Hence, the 1:4 stoichiometry (DG2-RB) and the RB concentration of 4 μM chosen for the *in vivo* studies.

Animals and Husbandry

Animal studies were carried out in accordance with the consent of the relevant for University of Lodz Local Ethics Committee (25/ŁB 238/2022) and conducted in accordance with the guidelines of EU legislation on animal experimentation. The BALB/c strain was chosen as it is a standard, well-characterized inbred strain widely used for toxicology and

Table 1 The Dendrimersomes second Generation Hydrodynamic Size (HS [nm]), Polydispersity Index (PDI), Zeta Potential (ZP [mV]) and Drug Loading Capacity (DLC [%]) of RB

	HS [nm]	PDI	ZP [mV]	DLC [%]
DG2	36.68 ± 7.98	0.27 ± 0.01	37.25 ± 2.60	–
DG2-RB	55.47 ± 23.97	0.38 ± 0.14	39.17 ± 3.17	>22.5

nanomedicine safety studies, providing a reliable and reproducible background for assessing potential systemic effects. 96 female BALB/c mice (it was assumed that gender would have no impact on the study results), 6–8 weeks old, were obtained and maintained in the animal house of the Faculty of Biology and Environmental Protection of the University of Lodz. Mice were housed in cages with ad libitum access to water and food, 12 h of light per day, and standard laboratory conditions (humidity between 50–60% and temperature maintained at 21–23°C).

Treatment

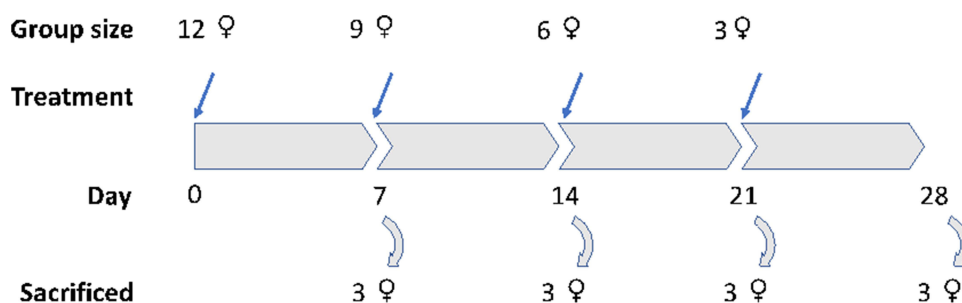
Animals were divided randomly into eight groups of 12 mice each, depending on the substance tested:

1. rose bengal (RB) at a concentration of 4 μM ,
2. cationic phosphorus dendrimer (1cat) at a concentration of 0.8 μM ,
3. rose bengal–phosphorus dendrimer complex (RB-1cat) at a concentration of 4 μM :0.8 μM ,
4. PPI dendrimer third-generation (PPI G3) at a concentration of 0.4 μM ,
5. rose bengal–PPI dendrimer complex (RB-PPI G3) at a concentration of 4 μM :0.4 μM ,
6. dendrimersome second-generation (DG2) at a concentration of 0.1 μM ,
7. rose bengal loaded dendrimersome (DG2-RB) at a concentration of 0.1 μM :4 μM ,
8. PBS as control.

The concentrations of the carriers and RB were selected based on the optimal molar ratios identified in our previous *in vitro* studies, which demonstrated the greatest photodynamic efficacy whilst minimizing dark toxicity.^{2–6} The final RB dose of 0.07 mg/kg is within the lower range of doses previously reported for *in vivo* studies, enabling a targeted evaluation of the safety of the nanocarriers. The test substances were administered intraperitoneally once a week in a volume of 30 μL . In the first week of the study, all animals in the group received the test substances; in the following weeks, three mice from each group were sacrificed to collect blood and tissue for laboratory tests, while the remainder received another dose of the compound (Scheme 2).

Body Weight, Blood, Urine Tests and Hematoxylin and Eosin Staining

Every seven days, 3 mice from each group were sacrificed to collect blood samples for laboratory analyses. To obtain an adequate blood volume, mice received anesthesia through intraperitoneal administration of a mixture of ketamine (50 mg/kg b.w) and xylazine (5 mg/kg b.w). The chests were subsequently opened, and blood was collected from the heart. Following blood collection, the abdominal wall was dissected to enable a morphological assessment of the internal organs, as well as the collection of the liver for histopathological evaluation. Despite there being no visible lesions, the liver was fixed in 4% formalin for 24 hours. Next, it was dehydrated, cleared, embedded in paraffin wax, sectioned and finally stained with haematoxylin and eosin. The following measurements were undertaken on the blood samples: alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), creatine kinase (CK), amylase, C-reactive protein (CRP), albumins, globulins, total protein. In addition, urine was collected for determination of color, transparency, pH, specific gravity, protein, glucose, urobilinogen,



Scheme 2 Schedule of administering compounds and killing animals in each of the study groups as a function of time.

bilirubin, ketones, nitrates, leukocytes, erythrocytes. All biochemical and microscopic assessments were conducted by a veterinary laboratory external to the study. Throughout the course of the experiment, the mice were closely observed for any potential adverse reactions and changes in body weight.

Data Analysis

Statistical analysis was performed using StatSoft Statistica (version 13.1). The Shapiro Wilk and the Lilliefors-corrected Kolmogorov–Smirnov tests were used to determine a normal distribution of data. Differences within and between groups were assessed using a non-parametric Kruskal–Wallis or Friedman test. The results were considered significant at $p \leq 0.05$.

Results

Body weight was measured once a week before the first and each subsequent dose of the drug (Table 2 and Figure 1A and B). The group treated with rose bengal was characterized by the lowest body weight and weight gain, but any observed differences between the groups were not statistically significant.

Once a week, urine and blood samples were collected from three mice in each group to evaluate biochemical parameters that might indicate liver and kidney function and damage (Table 3 and Table 4). The levels of the parameters studied were compared with those of the control group. Statistical analyses revealed no significant differences between the study and control groups. In addition, comparative analyses were performed between groups at different time points (weeks) and to determine changes within each group during the course of the experiment.

During the first week, a statistically significant difference ($p = 0.028$) was found in urea blood level between the RB-PPI G3 and RB groups (Figure 2A and B). In the following weeks of the experiment, no statistically significant differences were found between the animal groups for the other parameters tested.

As mentioned above, the statistical significance of the biochemical parameters was also checked between the following weeks of the experiment for each group of animals. Analysis of changes in the biochemical parameters showed a decrease in urea concentration in the group of animals receiving DG2-RB in the third week of the experiment and an unexpected increase in the following week (Figure 2, left panel). This difference was considered statistically significant ($p = 0.03$). In the group of animals receiving PPI G3, a statistically significant increase in creatinine level was observed during the first two weeks of the experiment ($p = 0.047$) (Figure 3). In the remaining groups of animals, no statistically significant changes in the levels of biochemical parameters were observed during the entire four-week experiment.

Once a week, 3 mice from each group were sacrificed for blood collection, while tissues (including liver) were collected from them for histopathological and molecular analyses. Since no significant changes were observed in the morphology and structure of the collected tissues, and the mice did not lose weight throughout the experiment, it was decided not to subject the collected tissues to molecular analysis. Figure 4 shows a comparison of liver tissue samples stained with haematoxylin and eosin from the control group (PBS) and the groups that received rose bengal and

Table 2 Average Weight Gain Expressed as a Percentage Compared to the Initial Body Weight (week 0)

	1	2	3	4
DG2	0.02	0.05	0.03	0.15
DG2-RB	0.01	0.09	0.13	0.15
PPI G3	0.05	0.09	0.14	0.19
RB-PPI G3	0.06	0.11	0.17	0.16
I cat	0.03	0.08	0.17	0.24
RB-I cat	0.03	0.07	0.14	0.17
RB	0.02	0.06	0.09	0.08
Control	0.01	0.04	0.08	0.13

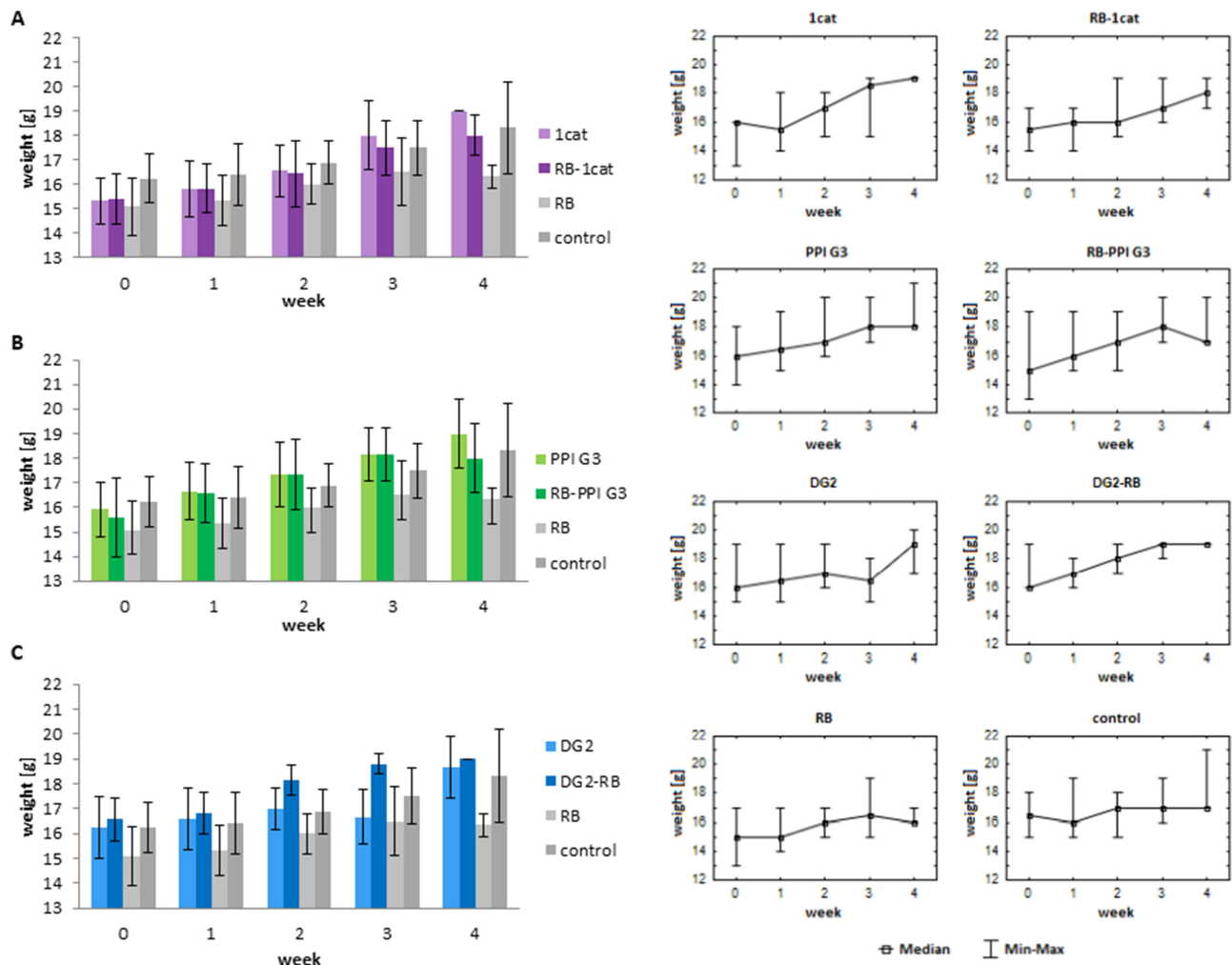


Figure 1 The impact of the tested compounds on the body weight of mice. Left panel: Mean body weight and its standard deviation of the mice treated with: (A) cationic phosphorus dendrimer (1cat), rose bengal-phosphorus dendrimer complex (RB-1cat), rose bengal (RB) and control; (B) PPI dendrimer third-generation (PPI G3), rose bengal-PPI dendrimer complex (RB-PPI G3), rose bengal (RB) and control; (C) dendrimersome second-generation (DG2), rose bengal loaded dendrimersome (DG2-RB) compared to rose bengal (RB) and control. Week 0 indicates the day of administration of the first dose of the drug. Right panel: Median body weight of mice with marked minimum and maximum values in the study groups: cationic phosphorus dendrimer (1cat), rose bengal-phosphorus dendrimer complex (RB-1cat), PPI dendrimer third-generation (PPI G3), rose bengal-PPI dendrimer complex (RB-PPI G3), dendrimersome second-generation (DG2), rose bengal loaded dendrimersome (DG2-RB), rose bengal (RB) and control. Results are presented as means $\pm$ SD (n=3).

dendrimers: cationic phosphorus (1cat), third-generation propylene imine (PPI G3) and second-generation dendrimer (DG2) complexes. Tissues from both the control and treated groups demonstrate normal tissue architecture, with no evidence of inflammation, necrosis or cellular damage.

Table 3 Median of Biochemical Parameters Determined from Blood in the Studied Groups of Animals: Rose Bengal (RB), Cationic Phosphorus Dendrimer (1cat), Rose Bengal-Phosphorus Dendrimer Complex (RB-1cat), PPI Dendrimer Third-Generation (PPI G3), Rose Bengal-PPI Dendrimer Complex (RB-PPI G3), Dendrimersome second-Generation (DG2), Rose Bengal Loaded Dendrimersome (DG2-RB) and Control

Week 1	RB	1cat	1cat-RB	PPI G3	PPI G3-RB	DG2	DG2-RB	Control
AIAT [U/l]	205.9 (258.1–523.9)	505.1	241.4	307.3	368.9	451.1	539.8	405.3
AspAT [U/l]	536.7	330.0	1077.6	625.0	389.5	922.0	853.5	452.0
Urea [mg/dl]	32.5	44.0	36.5	41.1	48.5	50.5	58.2	47.9

(Continued)

Table 3 (Continued).

Creatine [mg/dl]	0.6	0.5	0.6	0.5	0.6	0.4	0.4	0.5
LDH [U/l]	967.0	547.0	837.0	1593.0	833.0	987.0	1183.0	662.0
ALP [U/l]	145.0	86.0	155.0	125.0	135.0	246.0	130.0	173.0
CK [U/l]	3607.0	1078.0	2450.0	2035.0	1306.0	1354.0	2068.0	983.0
Amylase [U/l]	446.0	369.0	409.0	513.0	446.0	293.0	259.0	537.0
CRP [mg/l]	0.3	0.9	0.6	1.0	1.0	0.4	0.9	1.0
Albumins [g/l]	18.5	16.2	19.5	16.2	20.9	24.1	22.8	16.9
Globulins [g/l]	27.4	26.8	29.4	28.8	24.1	24.5	23.8	27.1
Total protein [g/dl]	4.6	4.3	4.7	4.0	4.5	4.9	4.7	4.5
Week 2	RB	Icat	Icat-RB	PPI G3	PPI G3-RB	DG2	DG2-RB	Control
AIAT [U/l]	217.9	384.8	302.7	152.1	406.6	565.4	253.6	459.7
AspAT [U/l]	959.7	618.5	1033.7	660.9	766.9	531.2	813.4	431.3
Urea [mg/dl]	43.2	43.0	48.2	31.6	45.6	58.1	59.2	50.0
Creatine [mg/dl]	0.5	0.6	0.5	0.7	0.6	0.5	0.6	0.6
LDH [U/l]	730.0	659.0	437.0	1481.0	942.0	1550.0	978.0	1868.0
ALP [U/l]	99.0	114.0	137.0	126.0	157.0	146.0	140.0	116.0
CK [U/l]	3096.0	2887.0	788.0	2600.0	1770.0	1621.0	3105.0	2320.0
Amylase [U/l]	353.0	419.0	405.0	353.0	190.0	457.0	398.0	464.0
CRP [mg/l]	0.4	0.7	1.0	1.0	0.6	0.7	0.9	1.0
Albumins [g/l]	21.0	17.5	18.1	24.2	22.7	19.6	20.4	23.7
Globulins [g/l]	29.0	24.5	24.3	22.0	26.3	23.4	22.6	27.3
Total protein [g/dl]	5.0	4.2	4.2	4.2	5.0	4.4	4.3	5.1
Week 3	RB	Icat	Icat-RB	PPI G3	PPI G3-RB	DG2	DG2-RB	Control
AIAT [U/l]	131.7	655.6	105.5	189.8	281.8	221.6	443.2	190.6
AspAT [U/l]	471.8	285.0	345.1	443.3	576.0	926.0	1012.0	301.2
Urea [mg/dl]	38.5	40.0	36.0	64.4	53.4	65.6	45.0	59.9
Creatine [mg/dl]	0.6	0.6	0.6	0.6	0.7	0.6	0.6	0.7
LDH [U/l]	1942.0	1862.0	1949.0	1691.0	1117.0	1294.0	1082.0	1920.0
ALP [U/l]	146.0	106.0	137.0	133.0	123.0	89.0	140.0	153.0
CK [U/l]	3063.0	1836.0	3123.0	2082.0	1866.0	509.0	646.0	2034.0
Amylase [U/l]	445.0	462.0	427.0	212.0	327.0	577.0	507.0	199.0
CRP [mg/l]	1.0	1.0	0.6	0.7	0.3	0.5	0.9	0.7
Albumins [g/l]	20.4	20.1	19.3	25.7	22.0	18.6	19.5	26.0
Globulins [g/l]	26.6	24.6	25.7	27.3	23.5	23.4	23.5	24.6
Total protein [g/dl]	4.7	4.6	4.5	5.1	4.7	4.2	4.3	4.8
Week 4	RB	Icat	Icat-RB	PPI G3	PPI G3-RB	DG2	DG2-RB	Control
AIAT [U/l]	1191.0	228.2	611.5	159.2	323.3	172.7	175.1	362.1
AspAT [U/l]	321.4	673.4	502.3	838.4	413.8	370.5	522.0	392.0
Urea [mg/dl]	57.2	59.9	52.8	79.9	79.5	64.7	67.2	94.3
Creatine [mg/dl]	0.7	0.3	0.6	0.6	0.6	0.6	0.6	0.6
LDH [U/l]	1839.0	1716.0	1512.0	1992.0	1092.0	901.0	962.5	1651.0
ALP [U/l]	142.0	142.0	108.0	133.0	120.0	140.0	133.5	141.0
CK [U/l]	2705.0	2117.0	1924.0	3361.0	2950.0	2250.0	2753.0	2488.0
Amylase [U/l]	256.0	165.0	246.0	449.0	537.0	514.0	544.0	313.0
CRP [mg/l]	0.8	1.0	0.8	0.8	0.1	0.6	0.3	1.0
Albumins [g/l]	27.8	24.1	22.1	21.3	19.0	21.1	21.7	24.0
Globulins [g/l]	30.2	26.4	28.3	24.3	23.1	24.9	26.8	27.0
Total protein [g/dl]	5.7	5.1	4.9	4.6	4.2	4.6	4.9	5.1

Abbreviations: AIAT, alanine aminotransferase; AspAT, aspartate aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; CK, creatine kinase; CRP, C-reactive protein.

Table 4 The Values Obtained from Urine Microscopy and Biochemical Analysis of Studied Groups: Rose Bengal (RB), Cationic Phosphorus Dendrimer (Icat), Rose Bengal–Phosphorus Dendrimer Complex (RB-Icat), PPI Dendrimer Third-Generation (PPI G3), Rose Bengal–PPI Dendrimer Complex (RB-PPI G3), Dendrimer some second-Generation (DG2), Rose Bengal Loaded Dendrimer some (DG2-RB) and Control

Week 1	RB	Icat	Icat-RB	PPI G3	PPI G3-RB	DG2	DG2-RB	Control
Color	Light yellow	Yellow	Light yellow	Light yellow	Light yellow-yellow	Yellow	Yellow	Light yellow
Urine transparency	Complete	Complete	Complete	Complete	Complete-incomplete	Incomplete	Incomplete	Complete
pH	6	6	6	6–6.5	6	6	6	6
Specific gravity	1.03	1.03	1.03	1.03	1.03	1.03	1.03	1.03
Protein [mg/dl]	Absent	Trace	Absent-100	Absent-trace	Trace	Trace	Trace	Absent-100
Glucose [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Urobilinogen [mg/dl]	Normal	Normal	Normal	Normal-I	Normal	Normal-I	I	Normal-I
Bilirubin [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Ketones [mg/dl]	Absent	Absent	Absent	5.2–I	Absent-5.2	Absent-5.2	5.2	Absent
Nitrates [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Leukocytes [Ieu/ul]	Absent	Absent	Absent-50	Absent-25	Absent-50	25	25	Absent-75
Erythrocytes [ery/ul]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Week 2	RB	Icat	Icat-RB	PPI G3	PPI G3-RB	DG2	DG2-RB	Control
Color	Light yellow-yellow	Light yellow	Yellow	Light yellow- yellow	Yellow	Light yellow	Light yellow	Light yellow-yellow
Urine transparency	Complete	Complete	Complete-incomplete	Complete-incomplete	Complete-incomplete	Incomplete	Complete-incomplete	Complete-incomplete
pH	6	6–6.5	6	6–6.5	6–6.5	6	5–6	6.5
Specific gravity	1.03	1.03	1.03	1.03	1.03	1.03	1.03	1.03
Protein [mg/dl]	Absent-trace	Absent-trace	Absent-100	Absent-100	Absent-trace	Trace-100	Absent-trace	Trace-100
Glucose [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Urobilinogen [mg/dl]	Normal	Normal-I	Normal	Normal-I	Normal-I	Normal-I	Normal-I	I
Bilirubin [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Ketones [mg/dl]	Absent-5.2	5.2	Absent	Absent-5.2	Absent-16	5.2	Absent-5.2	5.2
Nitrates [mg/dl]	Absent	Absent	Absent	Absent	Absent-present	Absent	Absent	Absent-present
Leukocytes [Ieu/ul]	Absent	Absent	Absent-75	Absent-250	Absent-50	25–75	Absent-25	Absent-250
Erythrocytes [ery/ul]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Week 3	RB	Icat	Icat-RB	PPI G3	PPI G3-RB	DG2	DG2-RB	Control
Color	Light yellow	Light yellow-yellow	Yellow	Yellow	Light yellow	Yellow	Yellow	Light yellow-yellow
Urine transparency	Complete-incomplete	Complete-incomplete	Incomplete	Complete-incomplete	Incomplete	Incomplete	Incomplete	Complete-incomplete
pH	6	6–6.5	6–6.5	6–6.5	6	6	6–6.5	6–6.5
Specific gravity	1.03	1.03	1.03	1.03	1.03	1.03	1.029–1.035	1.03
Protein [mg/dl]	Absent-100	Absent-100	Trace-100	Absent-100	Trace	Trace-100	Trace	Absent-trace
Glucose [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Urobilinogen [mg/dl]	Normal	Normal-I	Normal-3	Normal-I	Normal-I	Normal	Normal	Normal-I
Bilirubin [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Ketones [mg/dl]	Absent-5.2	Absent-5.2	Absent-5.2	Absent-5.2	5.2	Absent	Absent	Absent-5.2
Nitrates [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent-present	Absent	Absent
Leukocytes [Ieu/ul]	Absent-75	Absent-75	50–250	Absent-75	25–75	Absent-250	75–250	Absent-25
Erythrocytes [ery/ul]	Absent	Absent	Absent-50	Absent	Absent-75	Absent	Absent	Absent

Week 4	RB	Icat	Icat-RB	PPI G3	PPI G3-RB	DG2	DG2-RB	Control
Color	Yellow	Yellow	Yellow	Light yellow	Light yellow-yellow	Yellow	Yellow	Light yellow
Urine transparency	Complete	Incomplete	Complete-incomplete	Complete-incomplete	Complete-incomplete	Incomplete	Incomplete	Incomplete
pH	6	6	6–6.5	6	6–6.5	6.5	6–6.5	6
Specific gravity	1.03	1.03	1.03	1.03	1.03	1.03	1.030–1.039	1.03
Protein [mg/dl]	Absent	Absent-trace	Absent-trace	Absent-trace	Absent	Trace	Trace-100	Trace-100
Glucose [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Urobilinogen [mg/dl]	Normal	Normal	Normal-I	Normal	Normal	I	Normal	Normal-6
Bilirubin [mg/dl]	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Ketones [mg/dl]	Absent	Absent-5.2	Absent-5.2	Absent-5.2	Absent	5.2	Absent-5.2	Absent-5.2
Nitrates [mg/dl]	Absent	Absent	Absent	Absent	Absent-present	Absent	Absent-present	Absent
Leukocytes [leu/ul]	Absent	Absent-25	Absent-50	Absent-25	Absent	250	75–250	25–75
Erythrocytes [ery/ul]	Absent	Absent	Absent	Absent	Absent	Absent	Absent-25	Absent

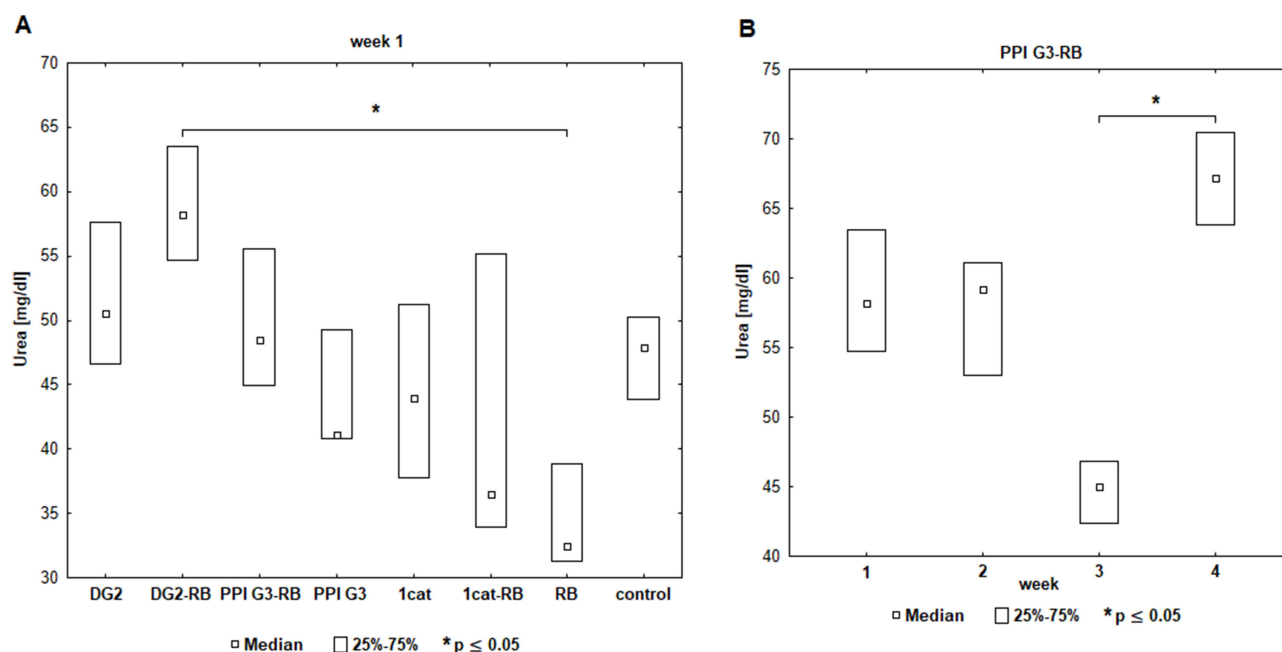


Figure 2 Changes in blood urea concentration: **(A)** During the first experimental week in study group mice: dendrimer second generation (DG2), rose bengal loaded dendrimer (DG2-RB), PPI dendrimer third-generation (PPI G3), rose bengal–PPI dendrimer complex (RB–PPI G3), cationic phosphorus dendrimer (1cat), rose bengal–phosphorus dendrimer complex (RB–1cat), rose bengal (RB) and control. **(B)** During the four-week experiment in rose bengal–PPI dendrimer complex (RB–PPI G3) group. Results are presented as median (25th percentile, 75th percentile), * $p \leq 0.05$.

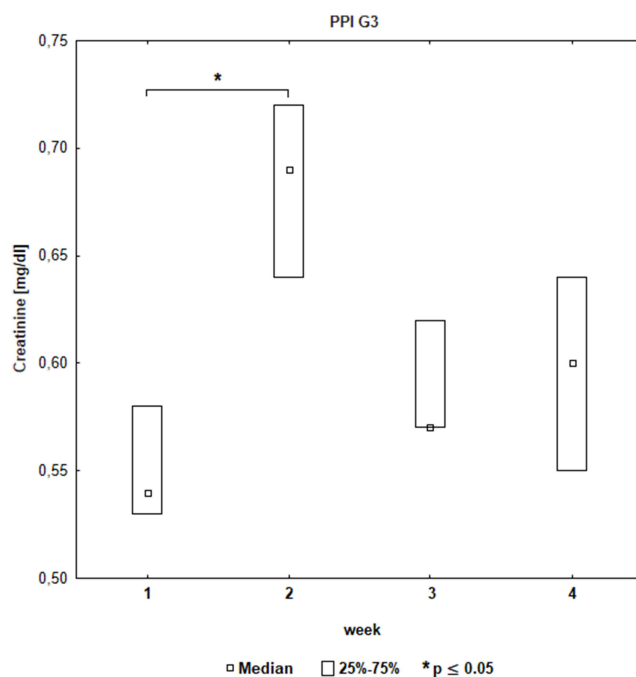


Figure 3 Blood creatinine levels during the four-week experiment in PPI dendrimer third-generation (PPI G3) group. Significant differences at * $p \leq 0.05$.

Discussion

PDT, as a method with minimal invasiveness, convenient feasibility and good efficacy, has undoubtedly contributed to the development of anticancer therapy. However, natural barriers, such as low solubility of photosensitizers, low efficiency of transport into cells, or hypoxic tumor microenvironment, may hinder the photodynamic reaction in vivo

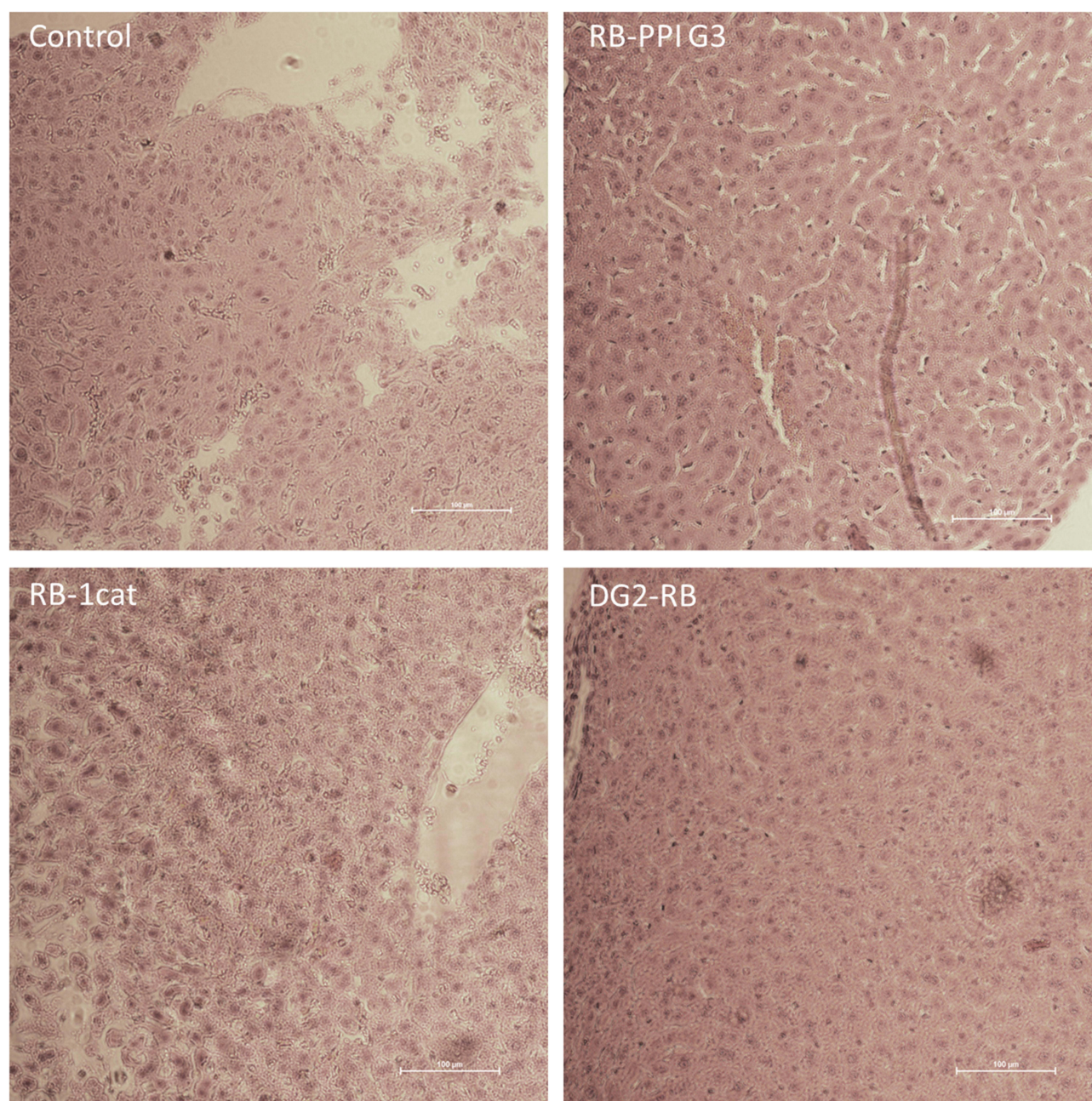


Figure 4 Representative H&E stained histological images of liver tissues from mice at week 4. Scale bar = 100 µm.

and prevent its widespread application in cancer treatment. A report by Wang et al shows that the efficacy of PDT may be reduced as a result of oxygen consumption associated with the ROS production,⁷ and according to Kimáková et al may aggravate tumor hypoxia and promote tumor progression and metastasis, increasing the risk of PDT resistance.⁸ Recently, numerous researches have been done to improve the efficacy of PDT and significant progress has been made. For example, the use of perfluorocarbons combined with a photosensitizer/drug,^{9–11} lipid-polymer bilaminar oxygen nanobubbles,¹² MnO₂ nanomaterials,¹³ or new non-reactive oxygen carriers such as hemoglobin (HB) encapsulated in liposomes have been proposed.¹⁴

Since each of these systems has its advantages but also disadvantages, eg as an oxygen donor, acellular HB has a low stability, a short circulation time¹⁵ and may cause potential side effects, ie a sudden increase in blood pressure after contraction of the vessels, which traps the by-product nitric oxide (NO),¹⁶ we decided to focus our research on various

polymer systems that can deliver a photosensitizer to the cancer cells. The use of carriers can improve the solubility of photosensitizers, the speed and efficiency of their penetration into cancer cells, prevent the removal of the photosensitizer from the cells and, like phosphorus dendrimers, enhance the production of singlet oxygen.^{2,4,5,17} An example of a photosensitizer with limited use as a therapeutic agent, but expanded thanks to carriers, is rose bengal - a dye with a hydrophilic tendency,¹⁸ a shortened half-life,¹⁹ difficult to cross cell membranes, with low cellular uptake, accumulation and poor biodistribution.

As mentioned in the introduction, of the many photosensitizer delivery systems tested by our team, the most effective and worthy of further research are the non-commercial cationic phosphorus dendrimer (1cat), the commercial cationic poly(propyleneimine) dendrimer third generation (PPI G3), and the non-commercial dendrimersome second generation built of amphiphilic triazine-carbosilane dendrimers - all have proven to be excellent *in vitro* rose bengal (RB) carriers.^{2,4,5} However, it should be remembered that improving the hydrophobicity of RB and increasing the intracellular uptake by using carriers does not ensure selectivity towards cancer cells, and the accumulation of the photosensitizer also in cells other than the target cells should be considered, which may lead to adverse effects. Furthermore, cationic dendrimers (1cat, PPI G3) are known to be toxic *in vitro* and, therefore, safe *in vivo* only at appropriate doses. When planning studies and selecting dendrimer doses, it is important to consider factors such as clearance by the reticuloendothelial system (RES) to ensure the dose is below the threshold for systemic toxicity, bearing in mind that complexation with RB may alter the charge of dendrimers. Therefore, before starting *in vivo* studies, it is necessary to evaluate the safety of the selected systems, with and without RB.

The assessment of *in vivo* biosafety of dendrimers and dendrons themselves is of particular importance even beyond the application as carriers for PDT agents. Dendritic molecules are actively used in nanomedicine both as therapeutic agents themselves and as carriers for bioactive moieties.^{20–25} For instance, therapeutic formulations based on phosphorus and carbosilane dendritic molecules - having molecular structures quite similar to those used herein - have been reported to exhibit pronounced antitumor activity *in vitro*,^{26–30} immunostimulatory effects *ex vivo*,³¹ and antibacterial activity *in vivo*.³² In addition phosphorus dendrimers were found very active against several diseases as Parkinson,³³ osteoarthritis,³⁴ tuberculosis,³⁵ cancers,³⁶ inflammation,³⁷ tumor imaging³⁸ to name as a few. At the same time, dendrimers are known to cause changes of biochemical and immunological profiles at the cellular level (see references above). Therefore, it is highly important for future development of dendrimer-based therapeutics to investigate, whether these effects observed *in vitro* will manifest at the organism level upon dendrimer administration in the treatment-imitating regime.

Interestingly, there are a number of literature reports, not only on dendrimers or dendrons, discussing the potential use of various nanoparticles in PDT therapy, highlighting their role in enhancing the efficacy of PS *in vivo*, but none strictly aimed at assessing the system-wide toxicity of these systems.^{39,40} Our research has filled this gap for a group of three carriers of the bengal rose.

For this purpose, the following preparations were administered intraperitoneally to the first group of 48 BALB/c mice (4 groups of 12 mice each): phosphorus dendrimer (1cat), second-generation dendrimersome (DG2), third-generation PPI dendrimer (PPI G3), and solvent (PBS). Then, the second group of 48 BALB/c strain mice (4 groups of 12) were administered intraperitoneally the following preparations: phosphorus dendrimer complex with RB (RB-1cat), second generation dendrimersome with encapsulated RB (DG2-RB), third generation PPI dendrimer complex of RB (PPI G3: RB) and rose bengal (RB). Intraperitoneal administration was chosen because intratumoral (directly into the tumor tissue) administration of the tested systems is planned in the future, as is the case with other RB-containing products currently in clinical trials (ClinicalTrials.gov: NCT00521053; NCT00237354; NCT02693067; NCT00219843). In addition, despite the biological half-life of excretion of RB is 30 minutes,¹⁹ it cannot be ruled out that, due to the use of systems that enhance the transport of the photosensitizer, active RB may enter the bloodstream and affect the body of the mouse. Once a week, 3 mice from each group were sacrificed to collect blood and tissues for histopathologic and molecular analyses. No significant changes were observed in the morphology and structure of the collected tissues. Blood biochemical parameters related to hepatic and renal toxicity (aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine (Cre), and urea) and morphology were evaluated. There were no statistically significant differences in biochemical parameters compared to the control group. Morphological determinations and assessment of urinary parameters also showed no differences between the control group receiving PBS and the study

groups receiving rose bengal carriers and complexes. After the first week of administration of the tested systems, a statistically insignificant decrease in urea level was observed in the RB group and an increase in the DG2-RB group compared to the control. Significance at the $p < 0.05$ level was observed only when comparing these two groups (RB and DG2-RB). Thereafter, no changes in urea levels were observed for the next three weeks. Although no significant toxicity was observed with any of the nanosystems, we considered whether the larger DG2 dendrimer assemblies, which are likely to have a different biodistribution profile to smaller dendrimer complexes or free rose bengal, may have contributed to the transient change in urea levels observed. Urea is mainly produced in the liver. The concentration of urea in the serum can be used to determine whether the kidneys are performing their excretory function (suspicion of renal failure). For the test to be accurate and reliable, it should be combined with the measurement of creatinine (the ratio of urea to creatinine in blood serum). The absence of changes in creatinine levels in the above-mentioned groups allows us to exclude renal failure caused by the administration of studied systems. The transient and statistically insignificant fluctuation in creatinine levels observed in the PPI G3 group only at week 2, which normalized thereafter without accompanying changes in urea, suggests that it was likely a biological anomaly rather than a sign of dendrimer-induced nephrotoxicity. An increase in albumin levels was also observed, which may indicate dehydration of the body or be the result of a high protein diet or the use of medications such as insulin, growth hormone, or hormonal drugs. Excluding the influence of diet or the above-mentioned drugs in the mice tested, we can hypothesize that the observed increase in albumin levels in the RB and 1cat groups after 4 weeks of the experiment could be the result of stress associated with the intraperitoneal administration of systems. This hypothesis is supported by the stable results of urinary parameters showing that renal filtration is not impaired. All described changes have statistical significance at the level of $p < 0.05$ for comparisons between subsequent weeks of the experiment within the same group. However, no results are statistically significant when compared to the control group.

We can only support our results with literature data for rose bengal, as unfortunately there are no reports on the *in vivo* safety assessment of its complexes with polymeric carriers. Klaassen studied the pharmacokinetics of RB in rats, rabbits, guinea pigs, and dogs over a dose range of 0.01–10 mg/kg and found that RB was excreted in bile without biotransformation.¹⁹ Based on the results of the *in vitro* studies, RB was used for *in vivo* studies at a dose of 4 μ M, which, based on the volume of sample administered, gives a dose of 0.07 mg/kg mouse body weight and is in the lower range of doses used by Klaassen. Depending on the type of research, mice are typically given up to 30 to 75 mg/kg of rose bengal dye dissolved in physiological saline.^{41,42} Therefore, we were not concerned about changes in key biochemical parameters in mice after administration of such a low dose of rose bengal. What we did not know was how the body would respond to the administration of cationic polymeric RB delivery systems. As our previous *in vitro* studies have shown, cationic polymers are toxic to cells and, unfortunately, their ability to form complexes and the efficiency of intracellular transport are directly proportional to the level of their toxicity. Moreover, the lack of statistical significance for the differences between the results obtained for the control group and the groups receiving free polymers and their complexes with RB is a satisfactory result that allows us to recommend all the tested complexes as safe for use *in vivo*.

When leading the discussion, it is important to highlight both the strengths and limitations of our research. The major strength of this study is that it provides the first systematic and comparative *in vivo* safety assessment of these three distinct RB-nanocarrier formulations. The longitudinal four-week design allowed for the detection of both acute and sub-chronic toxicity. We also acknowledge several limitations. Firstly, this study was conducted in healthy, immunocompetent mice, so the safety profile may differ in a tumor-bearing or immunodeficient model. Furthermore, only one dose and one administration route (intraperitoneal) were tested. In future studies, intravenous administration and a broader dose range could be considered to establish an even more comprehensive toxicological profile before clinical use.

In conclusion, this study demonstrates that the phosphorus dendrimer (1cat), PPI G3 dendrimer, and triazine-carbosilane dendrimer (DG2) are safe for *in vivo* use as carriers for the photosensitizer rose bengal at the tested concentrations. A comprehensive four-week analysis of biochemical and histological parameters in BALB/c mice revealed no signs of systemic toxicity. These results provide a critical safety foundation for advancing these promising nanocarrier-based PDT formulations into future preclinical efficacy studies in tumor-bearing animal models.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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