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In vivo Toxicity of Water-Soluble Chlorin e_6 – Galactose Conjugates: Potential Antitumor Photosensitizers

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A series of chlorin e_6 derivatives bearing galactose fragments at the periphery of the macrocycle and exhibited good water solubility were studied for the in vivo toxicity. Preliminary results obtained on adult male white outbred mice shows that, in general, conjugates of chlorin e_6 with galactose exhibit significantly lower toxicity than chlorin e_6 itself. The most toxic of the studied compounds has an LD_{50} equal to 772 ± 11 mg/kg that is at least three times greater than that of chlorin e_6, despite the fact that all studied compounds are significantly more soluble in water and therefore more bioavailable, than chlorin e_6. The primary structural factor influencing toxicity is the number of galactose fragments on the periphery of the macrocycle: the greater the number of galactose fragments in the molecule, the higher the compound's toxicity. It can be expected that such derivatives will exhibit relatively low toxicity and thus may serve as a basis for further synthetic modifications in the development of new photosensitizers.

Keywords: Toxicity, *in vivo*, chlorin e_6 conjugates, galactose fragments.

Токсичность *in vivo* водорастворимых конъюгатов хлорина e_6 с галактозой – потенциальных противоопухолевых фотосенсибилизаторов

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Ряд производных хлорина e_6, содержащих фрагменты галактозы на периферии макроцикла, был изучен на токсичность in vivo. Предварительные результаты, полученные на самцах белых беспородных мышей, позволяют сделать вывод о значительно меньшей токсичности конъюгатов хлорина e_6 с галактозой по сравнению с собственно хлорином e_6. Самое токсичное из изученных соединений имеет $ЛД_{50}$ равную 772 ± 11 мг/кг, что как минимум в 3 раза превышает $ЛД_{50}$ хлорина e_6. Следует отметить, что все изученные соединения значительно лучше растворимы в воде (а значит и более биодоступны), чем хлорин e_6. Основным структурным фактором, влияющим на токсичность соединений, является количество фрагментов галактозы на периферии макроцикла: чем больше фрагментов галактозы содержится в молекуле, тем токсичнее соединение. Можно ожидать, что подобные производные с большой вероятностью будут иметь относительно низкую токсичность, поэтому они могут послужить основой для дальнейших синтетических превращений при синтезе новых фотосенсибилизаторов.

Ключевые слова: Токсичность, *in vivo* исследования, конъюгаты хлорина e_6, фрагменты галактозы.

Introduction

Photodynamic therapy (PDT) for oncological diseases is a rapidly developing field of medicine.[1–7] Chlorophyll *a* derivatives are effective antitumor photosensitizers (PS), some of which have been used in clinical practice for many years.[8-13] These compounds possess spectral and photochemical properties suitable for PDT and are capable of accumulating in rapidly proliferating tissues.[10,11,14–18] Chlorins derived from chlorophyll *a* generally exhibit strong photoluminescence, which, in combination with their tropism for malignant neoplasms, enables there use in the visualization of malignant tumors for diagnostic purposes.[4,6,13–16] The aforementioned advantages of chlorophyll *a* derivatives makes these compounds a promising foundation for the development of new antitumor PS. However, significant challenges associated with the use of chlorin-based PS in medicine stem from the hydrophobicity of the chlorin macrocycle, which results in low water solubility and, consequently, reduced bioavailability.[4,11,12,14–17,19] This has stimulated research into synthesis of chlorophyll *a*-based PSs with improved bioavailability, achieved through the introduction of additional substituents in the macrocycle that enhance molecule's hydration and, consequently, increase its bioavailability.[19] The introduction of carbohydrate fragments into the PS molecule significantly enhances hydrophilicity and, in many cases, results in the formation of water-soluble compounds.[20-26] It is believed that the presence of galactose residues not only increases compound bioavailability but also facilitates the active transport of PS molecules into cells.[22] Previously, we synthesized a series of chlorin e_6 derivatives bearing galactose fragments at the periphery of the macrocycle and exhibited good water solubility.[23-26] Investigation of these derivatives demonstrated low dark but high photoinduced cytotoxicity: values of $IC_{50_{ph}}$ ranges from 0.1 to 1.0 μM,[23–25] and the half-maximal inhibitory concentration under dark conditions (IC_{50_d}) exceeds the half-maximal inhibitory concentration under photoinduced exposure ($IC_{50_{ph}}$) by at least two orders of magnitude. This, combined with good water solubility, supports the consideration of these compounds as promising potential antitumor PS. Simultaneously, the introduction of galactose fragments at the periphery of the chlorin macrocycle may not only enhance the compound bioavailability and improve cellular uptake in cancer cells but also alter their toxicological profile. High toxicity is undesirable for PS applications; however, to date, the impact of galactose fragments on the toxic properties of chlorin e_6 derivatives has not been investigated. Therefore, the present study focuses on preliminary studying of the *in vivo* toxicity of water-soluble chlorin e_6 derivatives bearing galactose fragments at the macrocycle periphery (Figure 1).

Experimental

Compounds. The chlorin e_6 derivatives bearing galactose fragments were obtained according to previously published methods (**1** and **4**,[23] **2** and **3**[24]).

Animals. The experiment was conducted in March-April on adult male white outbred mice aged 3–5-month obtained from the Scientific Collection of Experimental Animals at the Institute of Biology, Komi Scientific Center, Ural Branch of the Russian Academy of Sciences (https://ckp-rf.ru/catalog/usu/471933/). Mice from the control and experimental groups, weighing 31.2 ± 6.0 g, were housed under identical conditions (+19–22 °C, 40–50% relative humidity) with free access to water and food ("Chara," Favorit, Russia). Each cage (35 × 22 × 90 cm, Tecniplast, Italy) housed five animals. The animals were maintained in accordance with the "Regulations on the Vivarium of Experimental Animals" (Protocol No. 1, January 24, 2017), with due consideration of sanitary, hygienic, and bioethical standards. The experiments with laboratory animals were carried out in strict accordance with the Russian Federation laws and ethical principles established by the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (adopted in Strasbourg on March 18, 1986 and confirmed in Strasbourg on June 15, 2006) and in accordance with the ethical standards established by the Declaration of Helsinki. All the experimental procedures were approved by the Bioethics Committee of the Institute of Biology of the Komi Scientific Center of the Ural Branch of the Russian Academy of Sciences (Protocol No. 3, February 06, 2025).

Experimental procedures. Animals were randomly divided into 18 groups, with five mice in each group, and individually marked. Mice in the experimental groups received a single intraperitoneal injection of compounds 1–4 at doses of 200, 400, 600, or 1000 mg/kg body weight during morning hours. Aliquots were prepared with an accuracy of 0.001 g (XP205, Mettler Toledo, Japan) by dissolving the compounds in isotonic saline solution (0.9% NaCl, PFK Obnovlenie, Russia), such that the total injection volume corresponded to the animal's body weight at a rate of 0.1 ml per 10 g of body weight. Animals in the control group were injected with the same volume of isotonic saline solution.

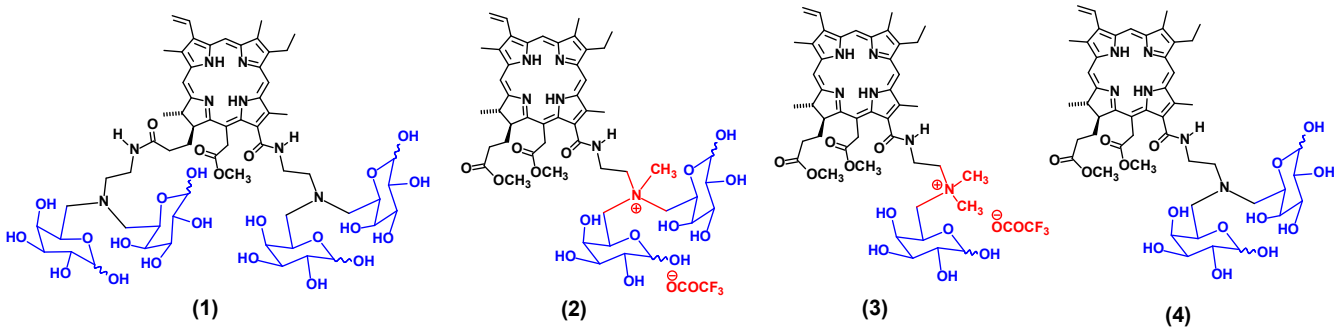

Figure 1. The structure of chlorin e_6 derivatives bearing galactose fragments at the macrocycle periphery. The numbers in brackets correspond to the compound numbers in the text.

After injection, mice were housed individually in a darkened room to avoid photoinduced effects.[27] Animal condition and survival were assessed every hour for the first six hours following injection, on the third day mice were grouped in fives by the compound concentration and examined every 12 h. Assessment of external conditions included examination of fur, skin, and motor activity. Water and food consumption were evaluated in total for each group by weighing residual food volume of water prior to animal weighting. Changes in body weight were determined with an accuracy of 0.1 g (CasBEE MW-1200, South Korea). Animals were weighed immediately before injection and on days 3, 7, 14, and 21 of the 21-day observation period.

Statistical analysis. The experimental results are presented as mean values ± standard deviation. The half-maximal lethal dose (LD_{50}), defined as the concentration at which 50% of animals survive after injection with the substance, was calculated using the drc package in the R programming environment.[28,29] Statistical analysis of the results was performed using R software.[29] Multiple pairwise comparisons were conducted using Mann-Whitney test at a significance level of 95%.

Results and Discussion

Assessment of overall mice condition

No adverse effects were observed in the control group administered saline solution. Administration of the lowest dose (200 mg/kg) of all compounds studied also resulted in no adverse effects (Table 1), except for two individuals in the group treated with compound 2, in which skin ulceration at the injection site was observed on day 14. However, by day 21, the ulcer had healed.

A decrease in motor activity during the first two days was observed in mice from groups injected with compounds **1** and **2** at a dose of 400 mg/kg (Table 1). Skin coloration and edema at the injection site were observed during the first week in animals from all four groups (Figure 2). Ulceration at the injection site, which developed on day 14, had healed by day 21. During the subsequent observation period, the general condition and behavior of animals administered compounds 1–3 remained normal. Additionally, increased aggressiveness, manifested as more frequent conflicts among animals, was observed in the compound **4** group compared with the control group and groups treated with compounds **1**–**3**.

Injection of compounds at a dose of 600 mg/kg resulted in decreased water and food consumption during the first two days, as well as reduced motor activity during the first three days, in animals from all four groups (Table 1). The injection site exhibited discoloration and swelling. In the group receiving compound **2**, a lump formed at the injection site, whereas in groups receiving compounds **3** and **4**, an ulcer developed at day 7, which healed by the end of the observation period. Intraperitoneal injection of the highest concentration (1000 mg/kg) of the studied compounds resulted in a significant decrease in motor activity, as well as reduced water and food consumption, in animals from all four groups (Table 1). All animals exhibited edema and ulceration at the injection site (Figure 3).

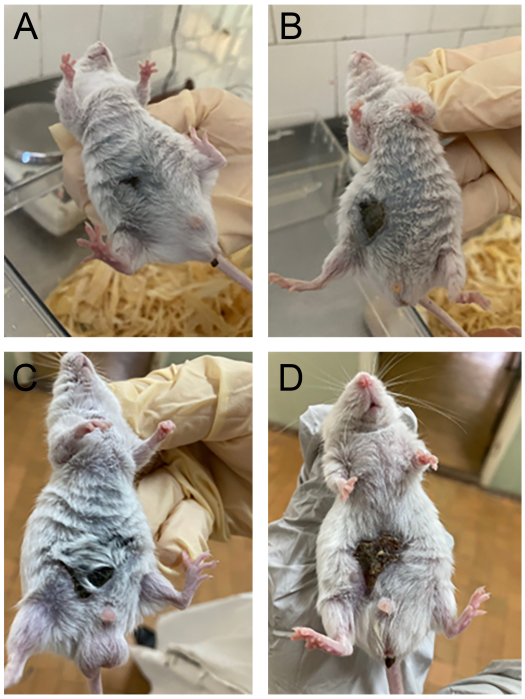

Figure 2. Appearance of the abdomen in males following intraperitoneal administration of the compound **3** at a dose of 400 mg/kg at day 3 (A), day 7 (B), day 14 (C), and day 21 (D) of observation. In the treated animals, dark green discoloration of the abdomen was observed, accompanied by skin ulceration; by the 21st day, the ulcer had healed with scarring.

Figure 3. Appearance of the abdomen in the males from control group (A) and in the male injected with the compound **2** at a dose of 1000 mg/kg on day 3 (B), day 7 (C), and day 21 (D) of observation. In the treated animal, a non-bleeding ulcer was visible on day 3 (B), which healed by day 21 (D), leaving a large scar.

Table 1. Effects observed after chlorin e_6 derivatives administration.

Effects observed	Compound **1**	Compound **2**	Compound **3**	Compound **4**
		200 mg/kg		
Motor activity	Normal	Normal	Normal	Normal
Food consumption	Normal	Normal	Normal	Normal
Water consumption	Normal	Normal	Normal	Normal
Ulcer	-	Observed on 14 day; healed on 21 day	-	-
Edema	-	-	-	-
Skin coloration	-	-	-	-
Aggressiveness	-	-	-	-
		400 mg/kg		
Motor activity	Decreased during the first 2 days	Decreased during the first 2 days	Normal	Normal
Food consumption	Normal	Normal	Normal	Normal
Water consumption	Normal	Normal	Normal	Normal
Ulcer	Observed on 14 day; healed on 21 day in only one animal from the group	Observed on 14 day; healed on 21 day	Observed on 14 day; healed on 21 day	-
Edema	-	First 7 days	First 7 days	First 7 days
Skin coloration	First 14 days	First 7 days	First 7 days	First 7 days
Aggressiveness	-	-	-	+
		600 mg/kg		
Motor activity	Decreased during the first 3 days	Decreased during the first 3 days	Decreased during the first 3 days	Decreased during the first 3 days
Food consumption	Decreased during the first 2 days	Decreased during the first 2 days	Decreased during the first 2 days	Decreased during the first 2 days
Water consumption	Decreased during the first 2 days	Decreased during the first 2 days	Decreased during the first 2 days	Decreased during the first 2 days
Ulcer	Bald spots	Lump at the injection site	Observed on 7 day; healed on 21 day	Observed on 7 day; healed on 21 day
Edema	+	+	+	+
Skin coloration	+	+	+	+
Aggressiveness	-	-	-	-
		1000 mg/kg		
Motor activity	Decreased	Decreased	Decreased	Decreased
Food consumption	Decreased during the first 3 days	Decreased during the first 3 days	Decreased during the first 3 days	Decreased during the first 3 days
Water consumption	Decreased	Decreased	Decreased	Decreased
Ulcer	+	+	+	+
Edema	+	+	+	+
Skin coloration	+	+	+	+
Aggressiveness	-	+	+	-

Mice body weight changes

The average body weight of animals administered isotonic saline solution increased significantly ($p < 0.01$) by approximately 5 g by the end of the experiment. In animals treated with the lowest concentration of chlorin e_6 derivatives, an increase in average body weight of 2–6 g was observed across all four groups (Figure 4A). However, significant changes from the initial weight are detected only in groups administered compounds **3** and **4** by the end of the experiment. Administration of compound **2** resulted in the minimal increase in body weight, which differed significantly from that of the control group. No significant increase or decrease in body weight compared to initial values was observed after injection of the studied compounds at 400 mg/kg (Figure 4B). The absence of differences in body weight relative to values in the control group was significant for all compounds by the end of the experiment.

Administration of 600 mg/kg of compounds **1** and **3** resulted in increases in body weight similar to those in the control group (Figure 4C). For compounds **2** and **4**, lower increases in body weight were significant compared to the control group but did not fall below initial values by the end of the experiment. An appreciable decrease in body weight was observed in mice treated with compound 4 by

day 14. The highest dose (1000 mg/kg) administration resulted in no increase in body weight (Figure 4D); by the end of the experiment, differences compared to the control group were significant, although no considerable decrease relative to initial weight was observed.

Mice mortality assessment

All animals survive at the end of observation in control group and groups administered 200 mg/kg of chlorin e_6 derivatives **1**-**4**. Administration of the 400 mg/kg dose resulted in death of two animals in the compound **4** group by the day 14 (Figure 5). At 600 mg/kg, death of one animal was recorded in groups administered compounds **1** and **3** by days 3 and 14 respectively. At the highest concentration (1000 mg/kg) two mice from five died in the groups administered compound **2** and **4**, and four mice died in the compound **1** group. All animals in the compound **3** group survived until the end of the observation period. Investigation of higher doses using a single injection was not possible due to limitations on maximal intraperitoneal injection volume and the solubility limits of chlorin e_6 derivatives.

Kaplan–Meier analysis revealed significant differences (p=0.0061) in mouse survival only for compound **1** administered at 1000 mg/kg. For this compound, the LD_{50} value was calculated as 772.5 ± 11.1 mg/kg (0.575±0.009 mmol/kg); LD_{16}, and LD_{84} values were 570±13 mg/kg (0.42±0.01 mmol/kg) and 1046±21 mg/kg (0.78±0.02 mmol/kg) respectively. For the other chlorin e_6 derivatives studied none of concentrations resulted in death of more than half of the animals. The death of two out of five mice after administration of compounds **2** and **4** and survival of all animals in the compound **3** group at the 1000 mg/kg dose suggests an LD_{50} for compounds **2**-**4** more than 1000 mg/kg.

Chlorin e_6 derivatives are generally low-toxicity compounds.[30] Chlorin e_6 itself is soluble in DMSO, but poorly soluble in water.[31,32] Low water solubility may limit the achievement of sufficient pharmacokinetics and biodistribution.[31] Increasing the bioavailability of this compound can be achieved through preliminary dissolution in organic solvents such as DMSO, the use of auxiliary chemical compounds like polyvinylpyrrolidone,[33] or incorporation into micellar formulations.[32] Another approach to enhancing bioavailability is chemical modification of the PS molecule itself, specifically by introducing hydrophilic fragments such as carbohydrates (including galactose),[20-25] polyamines, and some others,[19] onto the periphery of the macrocycle.

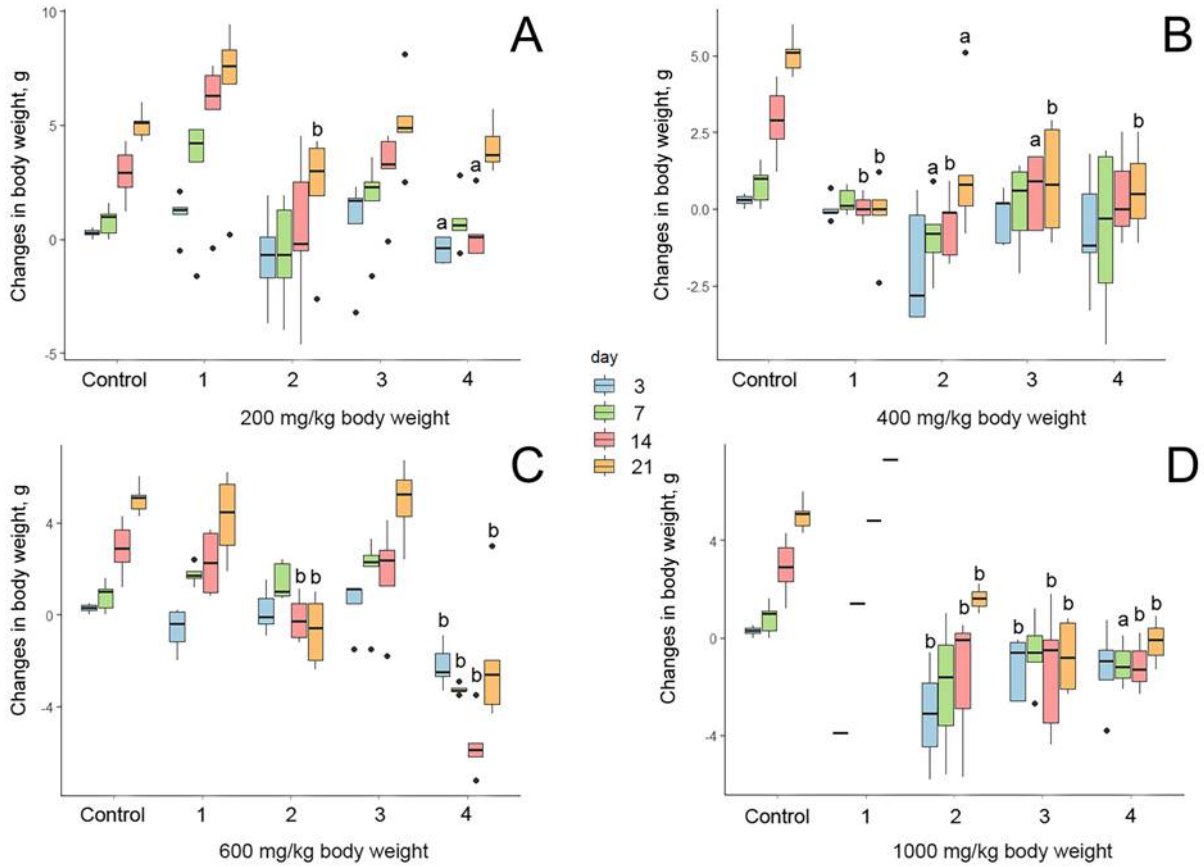

Figure 4. Changes in body weight of mice treated with chlorin e_6 derivatives at doses of 200–1000 mg/kg from day 3 to day 21, compared with the control group. Panels show concentrations of chlorin e_6 derivatives as follows: (A) 200 mg/kg, (B) 400 mg/kg, (C) 600 mg/kg, and (D) 1000 mg/kg. Control reflects body weight changes in animals administered isotonic saline solution; control data are included for easier comparison of changes. Numbers 1-4 correspond to compounds **1**-**4**. a: $p < 0.05$; b: $p < 0.01$ indicate significant differences compared to corresponding values in the control group. Differences relative to the initial weight for compounds **1**–**4** are discussed in the text.

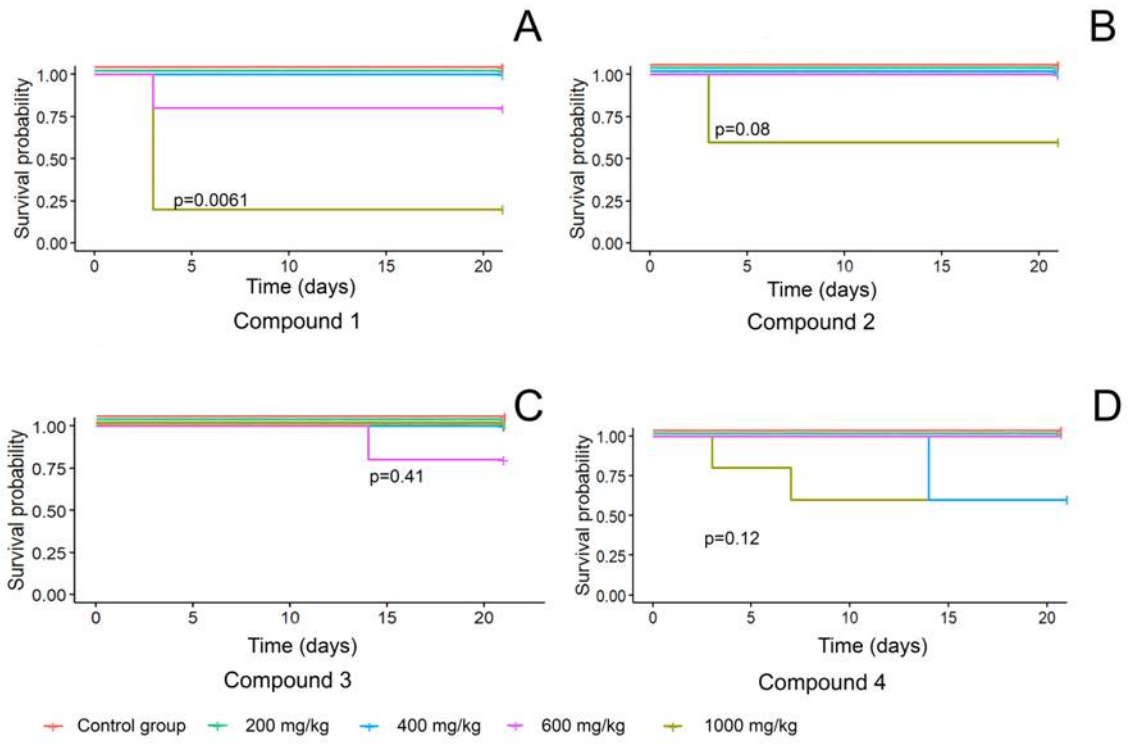

Figure 5. Kaplan-Meier analysis of survival of the mice administered with chlorin e_6 derivatives. Panels show compounds as follows: A - **1**, B - **2**, C - **3**, and D - **4**.

Increased bioavailability may lead to a significant rise in the phototoxicity of chlorin e_6 derivatives, while maintaining low dark toxicity[31,32] in experiments on cell cultures. Incorporation of the cationic group[34] increased water solubility but toxicity in rats was comparable to that of chlorin e_6 itself (~100 mg/kg for the monocationic chlorin derivative[34] *vs.* 113±18 mg/kg for the chlorin e_6 itself[35]). Thus, changes in a compound's toxicity profile cannot be reliably predicted solely based on structural modifications. Moreover, results from cell culture studies do not necessarily translate to *in vivo* behavior, including nonspecific toxicity toward normal tissues. Nevertheless, information on compound toxicity is critical for determining safe therapeutic doses.

Water-soluble galactose derivatives of chlorin e_6 exhibit high photodynamic index values (IC_{50d}/IC_{50ph}) while maintaining low dark toxicity, as demonstrated in experiments on several cell lines.[23,24] The selected compounds differ in the number of galactose fragments, the presence or absence of a cationic group, and the size of the cationic group, which is determined by the number of galactose substituents at the quaternized nitrogen atom, where applicable. Investigation of these compounds in laboratory mice revealed low *in vivo* toxicity. The LD_{50}, calculated for the most toxic compound **1** was more that three times lower than that of chlorin e_6. For compounds **2**-**4**, administration of 1 g/kg resulted in survival of more than half animals per group. Use of higher concentrations was precluded due solubility limits and maximal permissible intraperitoneal injection volume per mouse. Addition of a cationic group did increase compound toxicity, and an enhanced specific charge of the cationic group also did not lead to higher toxicity. Compound **2**, structurally similar to compound **4** but contains a cationic group, exhibited similar toxicity. Compound **3**, which has a smaller cationic group volume than compound **2** (and correspondingly higher specific charge), is supposed to have lower toxicity than compound **2**.

Thus, chlorin e_6 derivative **1** can be classified as slightly toxic substance, while compounds **2**, **3** and **4** are practically non-toxic.[30] The result seems to be interesting, particularly when compared to other attempts to develop water-soluble chlorin e_6 derivatives.[34]

Conclusions

These results preliminarily indicate that conjugates of chlorin e_6 with galactose exhibit significantly lower toxicity than chlorin e_6 itself. The most toxic studied compound (compound **1**) has an LD_{50} at least three times greater [35,36] than that of chlorin e_6, despite all studied compounds (**1**–**4**) are significantly more soluble in water and therefore more bioavailable than chlorin e_6, for the preparation of aqueous solutions of which auxiliary compounds are required. The primary structural factor influencing toxicity is the number of galactose fragments on the periphery of the macrocycle: the greater the number of galactose fragments in the molecule, the higher the compound's toxicity. These derivatives are expected to exhibit relatively low toxicity and may serve as a basis for further synthetic modifications in the development of new PS.

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