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Improved biodistribution, pharmacokinetics and photodynamic efficacy using a new photostable sulfonamide bacteriochlorin

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Contents of supplementary information:

1. Materials and Methods

1.1 UV/VIS absorption spectra and photodegradation experiments

UV/VIS absorption spectra were recorded in 1 cm quartz cuvettes with Shimadzu 2100 spectrophotometer. In order to check the possible photodegradation the irradiation was carried out using the Hamamatsu diode laser, type LA0873, S/N M070301, delivered ~100 mW at 746 nm. This diode laser was controlled by a ThorLabs 500 mA ACC/APC Laser Diode Controller. The laser energies of this and the other higher-energy lasers employed in this work were regularly checked with spectroradiometer IL2000 (Spectrocube).

1.2 Fluorescence quantum yield measurements

Fluorescence studies were carried out using a Luminescence Spectrometer LS 50B (Perkin–Elmer). The absorptions of both reference and sample solutions in fluorescence quantum yield measurements were matched at A=0.2 at the excitation wavelength of 515 nm, and then the solutions were diluted 10 times before collecting the fluorescence. The fluorescence quantum yields were obtained from the ratio of the fluorescence bands of the samples versus those of the 5,10,15,20- tetrakis(2,6-dichlorophenyl) bacteriochlorin (TDCPB) multiplied by the fluorescence quantum yield of TDCPB (Φ_F=0.012 in toluene)[1], after correction for the difference of refractive indices of ethanol and toluene.

1.3 Triplet state lifetime measurements

The triplet state lifetimes were measured with the Laser Flash Photolysis Spectrometer. The kinetic curves of the transient absorption of Cl_2BEt in the range 270-780 nm were registered after the laser excitation (λ=355 nm, E_{max}=100 mJ/pulse and full width at half maximum, FWHM=6 ns). At least ten kinetic runs were registered under each set of conditions and their average was recorded. The measurements were done at 20°C. All photosensitizer solutions were freshly prepared before the measurements. The stability of the samples was confirmed by comparing their absorption spectra before and after each flash photolysis experiment. The photolysis experiments run in the presence of oxygen involved the saturation of the solutions with oxygen, by flushing with O_2 and sealing the samples, or else they were air-equilibrated at 20ºC. For the experiments in the absence of oxygen, the solutions were purged with argon for 30 min until no change in the decay rate was observed, and were kept under argon during the measurements.

1.4 Quantum yield of singlet molecular oxygen formation

Singlet oxygen quantum yields in ethanol were obtained with a procedure described in details elsewhere, but using phenalenone as reference, for which Φ_Δ=0.95 in ethanol[2]. Singlet molecular oxygen phosphorescence at 1270 nm was detected at room temperature following laser excitation of ethanol solutions containing the sensitizer at the concentration necessary to produce an absorbance of 0.2 in 1 cm quartz cuvette at the excitation wavelength. The excitation of the samples at 355 nm (the third harmonic of a Nd-YAG laser Spectra-Physics Quanta-Ray GRC-130) was used as usual, but the flash photolysis equipment was modified to allow for luminescence detection in the 800-1400 nm region. The emission was filtered by a Melles Griot dielectric mirror reflecting more than 99.5% of the incident light in the 610-860 nm range, and by a Scotch RG665 filter. The infrared emission transmitted by these filters was resolved by a monochromator with a 600 lines grading mounted in the place of the usual UV/VIS grading. The wavelength of 1270 nm was selected for detection in a Hamamatsu R5509-42 photomultiplier, cooled to 193 K in a liquid nitrogen chamber.

1.5 Animals and tumour model

The animal model used in the present study was the DBA mouse bearing the Cloudman S91 melanoma. The S91 cells were cultured *in vitro* and after a subcutaneous inoculation of $1x10^6$ cells into the right flank induced tumours in 100% of mice. The tumours grew exponentially and displayed only a little size scatter between animals. Dark toxicity, biodistribution and pharmacokinetic studies were performed in mice, weighting 20-30 g. The mice come from the Animal House of the Polish Academy of Science Medical Research Center (Warsaw, Poland) and were kept on a standard laboratory diet with free access to drinking water. The use of animals for experimental studies was approved by the Jagiellonian University Committee for Ethics of Experiments on Animals (decision no. 89/2008 from 11 Dec 2008 and no. 11/2011, 23 Feb 2011).

1.6 Toxicity in the dark

The mice were divided into 6 groups of 10 animals differentiated by the following doses of ClBEt: 2 mg/kg, 5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and control group. The Cl_2BEt dissolved in Ethanol/PEG/PBS, 1:3:5 formulation (0.5 ml) was injected intraperitoneally (i.p.) and for 30 days the animals were closely observed. After 30 days the animals were euthanized using ketamine and xylazine (Biowet, Poland), their organs and tissue samples were excised, and blood morphology as well as histology of selected organs was performed.

1.7 Biodistribution and pharmacokinetics

The animals were treated three weeks from the tumour inoculation. A solution of Cl_2BEt was prepared and 10 mg/kg were administered to mice via i.p. The analyses of the bacteriochlorin tissue distribution were performed at following intervals: 2h, 6h, 12h, 24h, 48h and 72h post-injection. The animals were euthanized using ketamine and xylazine (Biowet, Poland), their organs and tissue samples were excised, weighted and then stored at –30°C until further analysis. The content of photosensitizer in the tissue samples was analysed spectrofluorometrically. In order to extract the bacteriochlorin, tissue samples were homogenized 1 min in 7 ml of ice-cold ethanol/DMSO (75:25) solution using a tissue homogenizer MPW-120 (Medical Instruments, Poland) at the speed of 10 000 rpm. The homogenate was centrifuged at 2000 g for 10 min at 4°C, the supernatant was collected and the pellet was re-extracted with 90% aqueous acetone to ensure a complete recovery. The extracts were pooled and analysed for bacteriochlorin content. The samples were excited at 517 nm and the fluorescence spectra were recorded in the range between 600 and 850 nm. The bacteriochlorin concentration in the tissues was estimated from the respective calibration curves.

1.8 Photodynamic therapy

S91 tumours were grown in DBA/2 mice as described above. The treatment was initiated when the tumour attained ca. 5-7 mm in mean diameter in each animal. The day the tumours reached the treatment size, the mice were injected i.p. with a 10 mg/kg dose of Cl_2BEt. At 24h post-injection, the mice were anesthetized with ketamine and xylazine, and restrained in plastic holders, then treated with the Hamamatsu laser described above, at a fluence rate of 80-90 mW/cm^2 for 20 minutes. The mice (5 mice/group in three groups (not treated, treated with light, treated with light and photosensitizer) were checked daily, the tumours were measured using two radicular diameters L and W and the volumes were calculated using the formula $V=LxW^2/2$.

2. *Spectroscopic properties of 5,10,15,20-tetrakis(2,6-dichloro-3-N-ethylsulfamoylphenyl)bacteriochlorin (Cl_2BEt) in comparison to ClBOH together with their octanol/water partition constants*

Table 1. Absorption and fluorescence data in ethanol together with octanol/water partition constants of chlorinated sulfonamide bacteriochlorin and sulfonated bacteriochlorin.[3-5]

Photosensitizer	λ_{max}/ nm	λ_{max} / M^{-1} cm^{-1}	λ_{em} / nm	Φ_F	log P_{OW}
ClBOH	746	23 400	748	0.04	-1.70
Cl_2BEt	745.5	97 000	749	0.008	1.83

3. *Photophysical properties of 5,10,15,20-tetrakis(2,6-dichloro-3-N-ethylsulfamoylphenyl)bacteriochlorin (Cl_2BEt) in comparison to ClBOH*

Table 2. Triplet state lifetimes in the presence and absence of oxygen determined in ethanol, with respective oxygen quenching rate constants and singlet oxygen quantum yields chlorinated sulfonamide bacteriochlorin and sulfonated bacteriochlorin.

Photosensitizer	τ_T^0 [s]	τ_{T,O_2} [s]	k_q [$M^{-1}s^{-1}$]	Φ_Δ
ClBOH	$3.9*10^{-5}$	$2.46*10^{-7}$	$1.3*10^{10}$	0.43
Cl_2BEt	$3.01*10^{-5}$	$1*10^{-7}$	$4.7*10^{9}$	0.66

4. *Concentrations of 5,10,15,20-tetrakis(2,6-dichloro-3-N-ethylsulfamoylphenyl)bacteriochlorin (Cl_2BEt) in different organs and tissues*

Table 3. Concentrations of sulfonamide photosensitizer (Cl_2BEt) in different organs and tissues extracted from DBA2 mice.

Time [h]	Concentration of Cl_2BEt in different tissue [µg/g]									
	Blood	Tumour	Skin	Muscle	Spleen	Liver	Kidneys	Intestine	Heart	Lungs
2	0.09	0.04	0.05	0.03	2.3	0.98	0.1	0.2	0.04	0.2
6	0.24	0.16	0.07	0.05	7.0	4.8	0.4	1.8	0.1	0.3
12	0.97	0.37	0.18	0.12	6.1	4.0	0.3	1.2	0.2	0.3
24	0.23	0.96	0.17	0.13	10.0	2.0	0.5	0.75	0.8	0.85
48	0.13	0.32	0.08	0.07	7.3	2.4	0.4	0.9	0.3	0.5
72	0.05	0.15	0.019	0.015	3.0	0.63	0.2	0.3	0.2	0.3

5. References:

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