**Effects of albumin binding on photocytotoxicity of extracellular
photosensitization reaction using talaporfin sodium to rat
myocardial cells.**

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ABSTRACT

Background: We previously proposed a new treatment for tachyarrhythmia using an extracellular photosensitization reaction occurring in the interstitial space of myocardia shortly after the injection of talaporfin sodium. Using myocardial cells, we studied the photocytotoxicity of this extracellular photosensitization reaction between talaporfin sodium and albumin.

Methods: The albumin concentrations tested spanned the physiological range found in the interstitial space (0-15 mg/ml) while the talaporfin sodium concentration were varied from 0-40 µg/ml. The reactions were conducted in 96-well plates. To obtain the binding ratio and the amount of energy deposited into the photosensitizer, we measured the change in the absorbance spectra of talaporfin sodium solutions containing different concentrations of albumin.

Results: Photocytotoxicity to myocardial cell due to the reaction decreased when physiological concentrations of albumin were added to the reaction mix, and decreased sharply when the molar concentration ratio of albumin to talaporfin sodium was between 0.3-1.2. A monotonic binding ratio was obtained, ranging from 10-80%, at albumin concentrations of 0.1-1.0 mg/ml. We found that the lethality of the extracellular photosensitization reaction towards myocardial cells had a threshold albumin concentration, even though the energy deposited into the talaporfin sodium solution was calculated to be almost constant (4.23 ± 0.19 J/well) in the presence of 0-15 mg/ml albumin.

1 Conclusions: Based on the likely concentration of albumin in the interstitial space, we
2 conclude that the photodynamic efficacy of talaporfin, under conditions used here, will
3 markedly decrease if the albumin level exceeds 0.65 mg/ml.

4

1 INTRODUCTION

2 Radiofrequency catheter ablation is widely used for atrial tachyarrhythmia treatment since it is
3 generally superior to conventional rate and rhythm control drugs (1). Radiofrequency catheter
4 ablation causes myocardium electrical conduction blockage by thermal coagulation to shut
5 down abnormal impulse propagations that cause tachyarrhythmia (2-3). Severe complications
6 caused by an increases in the temperature of the surrounding tissues are a problem, since
7 temperature control inside tissues is difficult (4). Radiofrequency catheters with irrigation
8 cooling systems have been developed to prevent these complications (5). However, these
9 devices only reduce the surface temperature, restricting their ability to suppress complications.
10 We have proposed a new, non-thermal treatment for tachyarrhythmia using talaporfin sodium
11 in which an extracellular photosensitization reaction in the interstitial space of the myocardium
12 is initiated by laser irradiation shortly after injection of the photosensitizer (6-9). Talaporfin
13 sodium is approved in Japan for treating early stage lung cancer and brain malignancies (10-
14 11). In contrast to cancer therapy, an immediate therapeutic effect is required in
15 tachyarrhythmia treatment since electrical physiological analysis is used to judge the
16 effectiveness of blocking myocardium electrical conduction during the ablation procedure.
17 Permanent electrical conduction blockage is also required. The selective accumulation of
18 photosensitizer in tumor tissues is utilized in cancer photodynamic therapy, with specified
19 intervals between photosensitizer injection and laser irradiation (12). Photosensitizer
20 accumulation in tumor cells produces intracellular singlet oxygen that induces apoptosis (13).
21 The photosensitizer is not uptaken selectively by healthy tissues, such as myocardium, which
22 we are targeting for tachyarrhythmia treatment. We therefore aimed to block the electrical

1 conduction of myocardial tissue by an extracellular photosensitization reaction with laser
2 irradiation via a laser catheter shortly after talaporfin sodium intravenous injection, since
3 almost all talaporfin sodium is in the interstitial fluid and blood at that time (6). We found that
4 the extracellular photosensitization reaction can cause myocardial cell necrosis with Ca^{2+}
5 inflow caused by membrane damage and ion channel dysfunction (7). This cell damaging
6 mechanism, which is different from the photodynamic therapy used for cancer, would provide
7 an immediate response for tachyarrhythmia treatment. Talaporfin sodium is mainly bound to
8 albumin *in vivo* (14). In a cancer *in vitro* basic study of the intracellular photosensitization
9 reaction, Sheyhedin *et al.* reported that cancer cell lethality changes according to the fetal calf
10 serum (FCS) concentration in a solution containing talaporfin sodium. They also reported that
11 the absorbance peak of the photosensitizer solution changed with the FCS concentration (15).
12 The cell killing effect in our proposed extracellular photosensitization reaction may be affected
13 by the albumin concentration in the interstitial fluid even if the mechanism leading to cell
14 death is completely different between the intracellular and extracellular photosensitization
15 reaction. The albumin concentration may be as high as a few mg/ml in the interstitial space,
16 where our intended reaction occurs, and therefore might affect the cytotoxicity of the
17 photosensitization reaction. We investigated the effect of the binding between talaporfin
18 sodium and albumin of the extracellular photosensitization reaction on cytotoxicity towards
19 myocardial cells *in vitro*. The binding ratio between talaporfin sodium and albumin was
20 obtained by absorbance spectroscopy from the shift in the talaporfin sodium Q band.

21 MATERIALS AND METHODS

22 **Photosensitizer solution:** Saline with bovine serum albumin (BSA) was used as a solvent for

spectroscopic measurements. Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Invitrogen) was used as a culture medium and solvent for cell photocytotoxicity measurements. Talaporfin sodium (Laserphyrin®; molecular weight: 799.69; Meiji Seika Pharma Co., Ltd., Tokyo, Japan) was dissolved in each solvent in the dark. This photosensitizer is a hydrophilic chlorin family photosensitizer with high absorption in the Q band and quick egestion (10). The absorption peak is at 664 nm and has a bandwidth of 20 nm (10).

Laser irradiation system: A continuous red diode laser with a wavelength of 663 ± 2 nm was used as an excitation light for the extracellular photosensitization reaction. The laser irradiance was applied homogenously in an 8-mm diameter hole on a black irradiation table to irradiate the well, from bottom to top, using the optical setup described in Figure 1. Laser irradiance was set to 0.29 W/cm^2 . This value was chosen from preliminary experiments designed to avoid oxygen depletion by high concentrations of the photosensitizer (16).

>Figure 1<

Photocytotoxicity measurement: Rat myocardial cells (Primary Cell Co., Ltd., Hokkaido, Japan) were cultured in collagen-coated black 96 micro-well plates (Corning Inc., Corning, NY, USA) for 6-7 days in an incubator at 37°C and 5% CO_2 . Photosensitizer solution (0.1 ml) was added to each well. The solution depth was 2.8 mm. The photosensitizer concentration and albumin concentration were varied from 0-40 $\mu\text{g/ml}$ and 0-15 mg/ml , respectively. Laser irradiation was applied within 15 min of the photosensitizer solution contacting the albumin solution. Radiant exposure was varied from 0-40 J/cm^2 varying the exposure duration between 0-137 s using the constant irradiance of 290 mW/cm^2 . Cell morphology just after laser irradiation was observed by a bright field microscope (IX70; Olympus Company Ltd., Tokyo, Japan). We

studied the photocytotoxicity that is not exactly the same meaning with cell lethality. There are the reports that discuss the photocytotoxicity with percent description of viability using the criteria of the value obtained by WST or MTT assay of dead cells and alive cells (15). We adopt the cell lethality as the description of the value for photocytotoxicity in result. Cells were cultured for 2 h after laser irradiation in 0.1 ml of culture medium and 10 μ l of water-soluble tetrazolium-8 (WST-8) solution (Doujinkagaku Co., Ltd., Kumamoto, Japan). The absorbance at 450 nm was measured by a microplate absorbance reader (SunriseTM; Tecan Group Ltd., Maennedorf, Switzerland). Cell lethality was defined by setting the absorbance of wells containing undamaged viable cells at 0%, and that of wells with completely unviable cells (treated with 30 μ g/ml talaporfin sodium and 40 J/cm² radiant exposure; non-viability confirmed by bright field microscopy with a criteria of bleb formation, peeling of substances) at 100%.

Spectroscopic measurement: The talaporfin sodium absorption peak at around 650 nm shifts to a longer wavelength with increased binding of serum proteins (15). The absorbance spectra were measured following the addition of 0-20 mg/ml BSA to calculate the energy deposited to the talaporfin sodium solution. Consequently, photosensitizer solutions containing 24 μ g/ml talaporfin sodium and 0-20 mg/ml BSA were prepared. Each photosensitizer solution was placed in a cuvette with a 1 cm optical pathlength, and the absorbance spectra were measured using a spectrophotometer (UV3600; Shimadzu Co., Kyoto, Japan) between 550 and 700 nm with 0.05 nm resolution. The binding ratios between talaporfin sodium and albumin were calculated using equation (1).

$$\text{Talaporfin sodium - albumin binding ratio (\%)} = 100 \times (C - A) / (B - A) \quad (1)$$

A: Peak wavelength of the photosensitizer solution without BSA

B: Peak wavelength of the photosensitizer solution with saturated BSA

1 C: Peak wavelength of the individual spectrum

2 The energy deposited into the talaporfin sodium solution was calculated by integrating the overlap
3 between the absorbance spectrum and excitation light spectrum. This deposited energy means the
4 absorbed energy of the excitation light into talaporfin sodium. This calculation was held to
5 confirm the difference of the absorbed energy by the spectrum shift with albumin binding change
6 of talaporfin sodium.

7 **RESULTS**

8 **Photocytotoxicity of the extracellular photosensitization reaction with different** 9 **albumin concentrations**

10 We varied the albumin concentration (0-15 mg/ml), photosensitizer concentration (0-40
11 $\mu\text{g/ml}$), and radiant exposure (5-40 J/cm^2). Myocardial cell necrosis was induced after laser
12 irradiation. Figure 2 shows cell morphology changes before and after the extracellular
13 photosensitization reaction with a photosensitizer concentration of 20 $\mu\text{g/ml}$, radiant exposure
14 of 20 J/cm^2 , and albumin concentration of 0.17 mg/ml. Multiple blebs and cell detachments
15 were observed immediately after the laser irradiation.

16 >Figure 2<

17 Figure 3 shows the dependence of myocardial cell lethality on the photosensitizer
18 concentration at each radiant exposure level at two albumin concentrations. Cell lethality
19 increased with increased photosensitizer concentration and radiant exposure at both
20 concentrations of albumin. The increase in cell lethality at a given photosensitizer
21 concentration was steeper at lower albumin concentrations.

1 >Figure 3<

2 Figure 4 shows the dependence of cell lethality on albumin concentration at a
3 photosensitizer concentration of 20 $\mu\text{g/ml}$ at each radiant exposure. The cell lethality
4 decreased as the albumin concentration increased. The cell lethality changed markedly at
5 albumin concentrations between 0.65-15 mg/ml , but did not change between 0.17-0.65 mg/ml
6 albumin.

7 >Figure 4<

8 **Binding ratio between talaporfin sodium and albumin**

9 Figure 5 shows the absorbance spectra of talaporfin sodium solutions containing albumin
10 concentrations ranging from 0.1-5 mg/ml . The peak wavelength of the Q band shifted $13.0 \pm$
11 0.65 nm higher with increasing albumin concentrations up to 5 mg/ml . The peak absorbance
12 increased 1.9-fold in the presence of 5 mg/ml albumin compared to 0 mg/ml .

13 >Figure 5<

14 The calculated amount of energy deposited into the talaporfin sodium solution is shown in
15 Figure 6. The energy was almost constant, at $4.23 \pm 0.19 \text{ J/well}$, in the albumin concentration
16 range 0-15 mg/ml .

17 >Figure 6<

18 The binding ratio between talaporfin sodium and albumin was calculated using equation (1).
19 Figure 7 shows the binding ratio at each albumin concentration from 0.1-5 mg/ml on a
20 logarithmic scale. A linear relationship between the binding ratio and albumin concentration
21 is obtained below 1 mg/ml in Figure 7. The binding ratio tended toward saturation at albumin
22 concentrations of 2.1 and 5 mg/ml .

DISCUSSION

Energy efficiency at each binding ratio

We calculated the amount of energy deposited by laser irradiation into a talaporfin sodium solution containing albumin concentrations of 0-15 mg/ml in a single well. Irradiation caused the absorbance spectrum to shift as the albumin concentration changed within physiological bounds. We used this albumin concentration range since it has been reported that 65-75% of talaporfin sodium binds to albumin and 29-33% binds to high-density lipoprotein (14). The albumin concentration in blood is 38-58 mg/ml, whereas in the interstitial space it is about 1/3rd that (17). The calculated amount of energy deposited into the talaporfin sodium solution was essentially constant, at 4.23 ± 0.19 J/well between 0-15 mg/ml albumin. Therefore, we found that the factor controlling the rate of photocytotoxicity is the binding ratio between talaporfin sodium and albumin, and not the amount of energy deposited into the talaporfin sodium solution and causing a corresponding absorbance spectrum shift.

The cytotoxic effect change depends on the molar concentration ratio between talaporfin sodium and albumin

The binding ratio between talaporfin sodium and albumin changed drastically between 0.1 and 1 mg/ml albumin at a talaporfin sodium concentration of 20 μ g/ml. The photocytotoxicity induced by the extracellular photosensitization reaction changed drastically between 0.65 and 2.1 mg/ml albumin at a talaporfin sodium concentration of 20 μ g/ml. As seen in Figures 4 and 6, the photocytotoxicity was essentially unchanged between 0.17 and 0.65 mg/ml albumin,

1 even though the binding ratio changed by more than 40%. We found that the
2 photocytotoxicity of the reaction to myocardial cells had an albumin threshold molar
3 concentration ratio of 0.3-1.2, albumin to talaporfin sodium, despite the calculated energy
4 deposited into the talaporfin sodium solution being almost constant at 4.23 ± 0.19 J/well
5 between 0-15 mg/ml albumin. Because the molar concentration ratio of albumin to talaporfin
6 sodium is 0.3-1.2 in this range, talaporfin sodium and albumin bind in a one-to-one ratio may
7 be implied. Consequently, based on the likely concentration of albumin in the interstitial
8 space, we conclude that the photodynamic efficacy of taliporfin, under conditions used here,
9 will markedly decrease if the albumin level exceeds 0.65 mg/ml.

10 **LIMITATIONS**

11 Interstitial fluid contains various proteins in addition to albumin. We only investigated the
12 influence of albumin concentration on the extracellular photosensitization reaction. We used
13 bovine serum albumin, although the interaction may be different with albumin from different
14 species since the binding sites are different (18).

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FIGURE CAPTIONS

Figure 1. Experimental setup for the extracellular photosensitization reaction.

Figure 2. Cell morphological changes before and after the extracellular photosensitization reaction of which makes the cell lethality of 80%. (Photosensitizer concentration: 20 µg/ml; radiant exposure: 20 J/cm²; albumin concentration: 0.17 mg/ml).

Figure 3. Dependence of cell lethality on photosensitizer concentration and radiant exposure at two albumin concentrations: (a) 0.17 mg/ml; (b) 0.65 mg/ml (SD for 10 J/cm² is shown, N=6).

Figure 4. Dependence of cell lethality on different albumin concentrations. (Photosensitizer concentration: 20 µg/ml, SD for 40 J/cm² is shown, N=6).

Figure 5. Typical talaporfin sodium absorbance spectra with different albumin concentrations. (Photosensitizer concentration: 24 µg/ml).

Figure 6. Calculated amount of energy deposited into a talaporfin sodium solution in a single well vs. albumin concentration.

Figure 7. Relation between the calculated binding ratio between talaporfin sodium and albumin at different albumin concentrations, shown on a logarithmic scale. (Photosensitizer concentration: 20 µg/ml, N=3).

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