**Comparison of myocardial cell survival 2 hours and 24 hours after
extracellular talaporfin sodium-induced photodynamic reaction**

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1 **ABSTRACT**

2 **Background:**

3 We have proposed an application of photodynamic reaction for less-heated
4 myocardial ablation which employs talaporfin sodium. Intracellular photodynamic reactions
5 with ongoing uptake have the ability to induce apoptosis over time, raising the possibility of
6 extending the lesion depth. The objective of this study was to understand how, in myocardial
7 cells, the late cell survival levels change by incubation time with talaporfin sodium, and what
8 dependence talaporfin sodium uptake has on the duration of incubation with talaporfin
9 sodium *in vitro*.

10 **Methods:**

11 Rat myocardial cells were incubated with talaporfin sodium for 5–360 minutes and
12 intracellular concentrations measured using a fluorescence micro-plate reader after wash. Cell
13 survival was measured using a water-soluble tetrazolium assay at 2 and 24 h after
14 a photodynamic reaction using a red diode laser of 660 nm, following 15–180 minutes of
15 incubation with talaporfin sodium. Cells were stained with Hoechst 33342 to observe nuclear
16 changes.

17 **Results:**

18 Intracellular talaporfin sodium concentration increased with incubation time, with a marked
19 increase between 0 and 60 minutes. Cell survival at 24 hours increased by 20% when the
20 duration of incubation with talaporfin sodium was extended from 15 to 30 min.
21 Following incubation time of 30–180 minutes with talaporfin sodium, cell survival was
22 increased by approximately 30% between measurements at 2 h and 24 h. The intracellular
23 talaporfin sodium concentration that induced higher levels of late cell death with cell nuclei
24 fragmentation in these cells was approximately 0.2 µg/mL.

25 **Conclusion:**

26 We obtained the characteristics of late cell death occurrence and talaporfin sodium uptake to
27 myocardial cell with various incubation time with talaporfin sodium.

1 **KEY WORDS**

2 Talaporfin sodium; photocytotoxicity; myocardial cell; extracellular photodynamic reaction

3 **INTRODUCTION**

4 We have proposed an application of photodynamic reaction for less-heated myocardial ablation
5 using fluence rate without any thermal coagulation occurrence (1). This method employs
6 talaporfin sodium photodynamic reactions to solve the thermal complications encountered
7 when using radio-frequency catheter ablation (2–7). We have previously demonstrated
8 blockage of electrical conduction using talaporfin sodium in canine and porcine models (5,7).
9 Oxidation by singlet oxygen causes myocardial necrosis. A permanent electrical conduction
10 block can be realized by non-thermal induction of myocardial necrosis following oxidation
11 (4).

12 Talaporfin sodium has been used in the selective treatment of early-stage lung
13 cancer and malignant glioma in Japan (8,9). In cancer therapy, slow apoptosis occurs when
14 accumulated deposits of talaporfin sodium in cancer cell lysosomes with contact of 4–6 h are
15 subjected to photodynamic therapy (10,11). Here, we aimed to induce extracellular
16 photodynamic reactions with short drug–light intervals to achieve immediate cell death by
17 necrosis.

18 Extracellular singlet oxygen production induces an immediate electrical conduction
19 blockage by promoting ion channel dysfunction and/or cell membrane damage. This
20 immediate electrophysiological damage is an essential element of tachyarrhythmia treatment.
21 Singlet oxygen production also induces a permanent electrical conduction block by causing
22 cellular necrosis (6). However, if the photodynamic procedure exceeds several hours duration,
23 the intracellular concentration of talaporfin sodium concentration increases and late cell death
24 such as apoptosis or autophagy follows.

25 We have therefore examined whether the lesion depth of our proposed ablation
26 would increase with longer procedure times. Here, late cell death of myocardial cells would be
27 induced by the talaporfin sodium intracellular photodynamic reaction. We investigated

whether increasing the concentration of talaporfin sodium present in the photodynamic reaction could induce a late cell death effect, which would allow evaluation of the risk of the lesion size extension. We studied changes to the intracellular talaporfin sodium concentration and compared cell survival at 2 and 24 h time points following the extracellular talaporfin sodium-induced photodynamic reaction. We examined uptake of talaporfin sodium *in vitro* at varying incubation times under various fluences.

<Fig. 1>

MATERIALS AND METHODS

Myocardial cell culture: Primary rat myocardial cells (Cosmo Bio Co. Ltd., Tokyo, Japan) were seeded in a collagen-coated black 96-microwell plate (Corning Inc., NY, USA) at a density of 2×10^4 cells/well. Culture medium was Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (D-MEM/F-12; Invitrogen, Carlsbad, USA) with 10% fetal bovine serum (FBS; Invitrogen). Cells were cultured for 6–7 days at 37°C in a 5% CO₂ atmosphere. Culture medium was changed every other day.

Talaporfin sodium solution preparation: Talaporfin sodium is a chlorin photosensitizer approved for the clinical treatment of early stage lung cancer and malignant glioma in Japan (8,9). Talaporfin sodium has a Q band absorbance peak at a wavelength of 664 ± 10 nm (12). Talaporfin sodium (molecular weight 799.69 Da; Meiji Seika Pharma Co. Ltd., Tokyo, Japan) was dissolved in culture medium to give a final concentration of 20 µg/mL.

Cellular uptake of talaporfin sodium: Talaporfin sodium solution (100 µL) was added to each well containing myocardial cells. Cells were then incubated in this solution for 5–360 min. Wells were washed five times with culture medium before addition of 100 µL culture medium. We have confirmed that the fluorescence intensity returns to basal levels following five washes (data not shown). Following excitation at 405 ± 20 nm, emission fluorescence intensity was measured at 720 ± 40 nm using a fluorescence microplate reader (Infinite F200 Pro; Tecan Group Ltd., Mannedorf, Switzerland). A calibration curve of the relative fluorescence count number relative to talaporfin sodium concentrations of 0.001–0.1 µg/mL was produced. The intracellular talaporfin sodium concentration of the myocardial cells was

then calculated from the calibration curve.

Measurement of late cell survival dependence on incubation time with talaporfin

sodium: Cells were incubated with 20 $\mu\text{g/mL}$ talaporfin sodium for 15–180 min as specified. Continuous laser irradiation (red diode laser, wavelength 663 ± 2 nm) from beneath the well was used to excite the talaporfin sodium. Laser irradiance at the well bottom was 0.29 W/cm^2 . The photodynamic reaction was performed using this optical setup, both with and without the culture medium wash before the laser irradiation. Figure 1 depicts our definitions of the photodynamic reactions in our *in vitro* study. A high concentration of talaporfin sodium was present outside the cell in our system. Any extracellular photosensitizer may be taken up into the myocardial cells as the duration of incubation with talaporfin sodium with cells increases. Therefore, the ratio of intracellular photodynamic reaction to extracellular photodynamic reaction increases as the incubation time with talaporfin sodium changes. We defined this complex photodynamic situation as an extracellular photodynamic reaction because the extracellular damage was always present, even though the number of intracellular talaporfin sodium molecules increased as well. The fluence varied from 0–20 J/cm^2 during incubation with talaporfin sodium. The water-soluble tetrazolium (WST) assay was performed at 2 and 24 h time points following the photodynamic reaction, using WST-8 solution (Dojinkagaku Co. Ltd., Kumamoto, Japan). WST solution (0.1 μL) was added to 0.1 mL culture medium 2 h before the measurement was to occur. A micro-plate absorbance reader (SunriseTM; Tecan Group Ltd.) was used to measure absorbance at 450 nm. We evaluated the WST assay photocytotoxicity values as a measure of cell survival. Absorbance values from wells containing undamaged cells or completely unviable cells by photodynamic reaction with 30 $\mu\text{g/mL}$ in talaporfin sodium concentration with less than 15 min in incubation time and 40 J/cm^2 in fluence were used to define cell survival rates of 100% and 0%, respectively. Brightfield microscopy was used to confirm cell viability.

Morphological assessment of cell nuclei by fluorescent staining: A morphological assessment of cell nuclei was performed following staining with Hoechst 33342 (Cell stain[®]-Hoechst 33342 solution; Dojinkagaku Co. Ltd.) to observe late cell death. The talaporfin sodium concentration was 20 $\mu\text{g/mL}$, incubation time with talaporfin sodium was

1 15 or 60 min, fluence was 20 J/cm^2 , and the time following the photodynamic reaction at
2 which the measurement was made was 2 or 24 h. After the photodynamic reaction, cells were
3 placed into contact with a $5 \mu\text{g/mL}$ Hoechst 33342 solution for 5 min at 37°C , and
4 fluorescence images were obtained by fluorescence microscopy (IX70; Olympus, Tokyo,
5 Japan).

6 **RESULTS**

7 **Intracellular talaporfin sodium concentration increases with longer incubation time** 8 **with talaporfin sodium**

9 Uptake of talaporfin sodium by myocardial cells was time-dependent (Figure 2). The
10 intracellular talaporfin sodium concentration was increased over time, with a marked
11 increase in the initial 60 min of incubation with talaporfin sodium.

12 <Fig. 2>

13 **Cell survival dependence on incubation time with talaporfin sodium, at various** 14 **fluences**

15 Figure 3 reveals the association of cell survival with the duration of incubation with talaporfin
16 sodium at fluences of 0 and 10 J/cm^2 , measured at both 2 and 24 hours after the photodynamic
17 reaction. Cell survival changed significantly between 15 and 30 min of incubation with
18 talaporfin sodium at a fluence of 10 J/cm^2 . The difference in cell survival between 15 and 30
19 min of incubation with talaporfin sodium was approximately 20% after 2 h of photodynamic
20 reaction, and approximately 60% after 24 h. There was no significant difference in the
21 survival of cells subjected to incubation times of 30–180 min with talaporfin sodium at either
22 the 2 or 24 h measurement. For incubation times of 30–180 min with talaporfin sodium, a
23 decrease of approximately 20% was observed when comparing cell survival at 24 h
24 post-reaction with that at 2 h.

25 The influence of intracellular talaporfin sodium photodynamic reactions (i.e.,
26 without the presence of extracellular talaporfin sodium) upon cell survival is indicated
27 by triangles in Figure 3. These values were measured following culture medium wash prior to
28 laser irradiation, after 15 or 60 min of incubation with talaporfin sodium.

29 Figure 4 reveals how cell survival varied with different talaporfin sodium incubation

times and fluence 24 h after the photodynamic reaction. Cell survival decreased with increasing fluence. Negative values for cell survival may indicate a homeostatic effect under weak photodynamic reaction conditions. There was no significant difference in cell survival following different times of incubation with talaporfin sodium at a fluence of 20 J/cm². Cell survival was significantly higher following 15 min of incubation with talaporfin sodium at a fluence of 10 J/cm². Conversely, cell survival was significantly lower following 180 min incubation with talaporfin sodium at fluences of 5 J/cm².

<Fig. 3>

<Fig. 4>

Morphological changes of myocardial cell nuclei

Morphological changes of myocardial cell nuclei following the photodynamic reaction are shown in Figure 5. Chromatin fragmentation and aggregation in the nucleus were observed 24 h after the photodynamic reaction, after 60-min incubation with talaporfin sodium.

<Fig. 5>

DISCUSSION

Duration of incubation with talaporfin sodium greatly affects cell survival

The rate of cell survival following 15 min of incubation with talaporfin sodium was significantly different to that following 30 min incubation. This change may result from an increase of the intracellular talaporfin sodium concentration from 0.17 to 0.22 µg/mL (see Figure 2). Therefore, concentration of approximately over 0.2 µg/mL intracellular talaporfin sodium induces late cell death. We also observed a significant difference in cell survival when comparing 60 min incubation with talaporfin sodium with 180 min incubation. This may result from an increase in the intracellular talaporfin sodium concentration from 0.41 to 0.47 µg/mL (see Figure 2). We suspect that lesion size extension in the late phase might be induced when the intracellular talaporfin sodium concentration is greater than approximately 0.2 µg/mL.

1 **Uptake characteristics of talaporfin sodium**

2 We converted the values of the intracellular talaporfin sodium concentration to the
3 intracellular talaporfin sodium mass. The diameter of non-adherent myocardial cells
4 was measured as $12.5 \pm 2.2 \mu\text{m}$. The volume of myocardial cells was calculated as $1.02 \times 10^3 \mu\text{m}^3$,
5 assuming that floating cells were spherical. The amount of talaporfin sodium taken up by cells
6 was determined to be $0.5 \times 10^{-3} \text{ pg/cell}$, with an initial talaporfin sodium concentration of 20
7 $\mu\text{g/mL}$ and a incubation duration of 6h. A previous study of talaporfin sodium cellular uptake
8 reported uptake values of 1 pg/cell, with an initial concentration of 10 $\mu\text{g/mL}$ using a
9 KYSE70 esophageal squamous carcinoma cell line, and $2.0 \times 10^{-3} \text{ pg/cell}$, with an initial
10 concentration of 10 $\mu\text{g/mL}$, using a P388 leukemia cell line (12). The uptake amount of
11 various photosensitizers is in the range of $10^{-3} - 10^{-1} \text{ pg/mL}$ (13–15). However, these values
12 vary with the cell species, initial photosensitizer concentration, and incubation time.

13 **Cellular uptake situation and vascular damage *in vivo***

14 The administered talaporfin sodium would migrate from blood to interstitial fluid around the
15 cells, then taken up into the cells *in vivo*, that is, the concentration around the cells changes
16 with time. The investigation of cellular uptake *in vitro* was performed with the incubation with
17 constant talaporfin sodium concentration from the beginning. This point is the limitation of
18 this manuscript since the situation *in vitro* may not exactly reflect the situation *in vivo*. It
19 would be also a limitation that a part of talaporfin sodium may be washed from the cell during
20 the wash procedure *in vitro* but it would not be dominant.

21 Short drug-light interval *in vivo* corresponding to short incubation time with
22 photosensitizer *in vitro* restrict uptake of talaporfin sodium to endothelial cells. So that,
23 photodynamic reaction in the cell does not affect huge. In terms of oxidation effect in the
24 blood adjacent to the endothelial cells, the oxidation capability of generated singlet oxygen as
25 a therapeutic effect may be strongly suppressed by high binding ratio of talaporfin sodium and
26 serum albumin in blood (16). Moreover, since blood flow in myocardial vessel is high, risk of
27 vascular closure by thrombus formation may be reduced. Therefore “Extracellular
28 photodynamic reaction in vessels” may not influence huge damage to endothelial cells and/or

1 risk of vascular closure may be reduced. So, we believe that myocardial vessels would be
2 protected from the extracellular photodynamic reaction. We have confirmed the vascular
3 patency of myocardium tissue in an acute *in vivo* experiment which employed an extracellular
4 photodynamic reaction with talaporfin sodium (7).

5

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10 Scientists (#4672).

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2 **FIGURE CAPTIONS**

3 **Fig. 1** Schematic illustration of the extracellular and intracellular photodynamic reaction

4 definitions in our *in vitro* study setup.

5 **Fig. 2** Change in intracellular talaporfin sodium concentration with duration of incubation

6 with talaporfin sodium. Talaporfin sodium concentration: 20 µg/mL.

7 **Fig. 3** Dependence of cell survival on incubation time with talaporfin sodium, at different

8 fluences. Talaporfin sodium concentration: 20 µg/mL. Error bars represent standard

9 deviations. Cell survival is indicated under the conditions below. ♦: 24 h after the

10 photodynamic reaction with fluence of 10 J/cm². ◇: 2 h after the photodynamic reaction with

11 fluence of 10 J/cm². ●: 24 h after incubation with talaporfin sodium. ○: 2 h after incubation

12 with talaporfin sodium. ▲: 24 h after the intracellular photodynamic reaction with fluence of

13 20 J/cm². **P* < 0.01 IBM SPSS Statistics was used to perform a Student's *t*-test. *n* = 4 with

14 fluence of 7.5 J/cm², *n* = 9 for all other plots.

15 **Fig. 4** Variable photocytotoxicity effect on myocardial cells (24 h post-photodynamic

16 reaction) following differing talaporfin sodium incubation times. Talaporfin sodium

1 concentration: 20 $\mu\text{g/mL}$. Two plots at a fluence of 10J/cm^2 are reproduced plots from Figure
2 3. Standard deviations are shown for 15 and 180 min of incubation with talaporfin sodium.
3 **Fig. 5** Myocardial cell nuclei before and 24 h after the photodynamic reaction, with 60 min of
4 incubation with talaporfin sodium. Talaporfin sodium concentration: $20\text{ }\mu\text{g/mL}$. Fluence: 20
5 J/cm^2 .
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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5