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Novel strategy to increase specificity of ALA-Induced PpIX accumulation through inhibition of transporters involved in ALA uptake

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ABSTRACT

Background: Aminolevulinic acid–based photodynamic therapy (ALA-PDT) has emerged as a cancer treatment due to its high specificity and low side effects. In this study, we aimed to identify possible new drugs targeting transporters highly expressed in normal cells but not in cancer cells, to increase the specificity of ALA-PDT.
Method: We used a total of seven cell lines, consisting of two gastric, three prostate, and two lung cell lines, for this purpose. siRNAs and inhibitors of these transporters were added, and PpIX production was evaluated using HPLC to examine the roles of transporters in ALA uptake.
Results: No correlation in the expression of transporters was observed among cell lines of the same origin. Two major findings were obtained: PEPT1 and PAT1 were expressed only in normal lung and prostate cells, respectively, but not in their cancerous counterparts. The inhibition of these transporters saw a significant decrease in PpIX production only in normal cells, but not in cancer cells.
Conclusion: These findings show that the usage of drugs targeted specifically to highly expressed transporters in normal cells is essential for reducing PpIX accumulation in normal cells in order to increase the specificity of ALA-PDT in cancer.

1. Introduction

The concept of photodynamic therapy (PDT) was first established in 1900, when Oscar Raab, a medical student in Germany, accidentally found that paramecia incubated with certain dyes died following exposure to light but survived in the dark [1]. This therapy was quickly adapted in cancer research and several other fields, such as dentistry and dermatology [2–4]. In recent decades, PDT has become an important approach in various cancer clinical trials [5,6].

Aminolevulinic acid (ALA)-PDT is attracting increasing attention as a new alternative in cancer treatment, particularly conventional chemotherapy, due to its high efficiency and extremely low side effects [6–10]. ALA-PDT is known to be highly cancer specific, with its accumulation of protoporphyrin (PpIX) in mitochondria following the administration of exogenous ALA, which triggers phototoxicity in cancer cells but less in normal cells [10,11]. However, recent studies by Xie *et. al.* (2016) found that protoporphyrin (PpIX), a product from ALA in the heme synthesis pathway, accumulate some PpIX in normal cells, suggesting possible undesirable cytotoxicity to surrounding healthy cells [12]. This suggests the need for a customized therapy that can increase specificity. ALA is known as a substrate of four transporters studied in this work, namely, peptide transporter 1 (PEPT1), proton-coupled amino acid 1 transporter (PAT1), taurine transporter (TauT), and GABA transporter 2 (GAT2), suggesting that these transporters may be highly involved in the cellular accumulation of PpIX [13–15]. PAT1 and PEPT1 are commonly found in gastrointestinal tract and are involved in various amino acid uptakes [13,14]. On the other hand, TauT and GAT2 are reported to be highly expressed in neurotransmitters in brains [15]. A schematic illustration of these transporters was depicted in Fig. 1.

In this study, we investigate key transporters involved in the cellular uptake of ALA, especially transporters that are highly expressed in normal cells but not in cancer cells. We explore the potential involvement of all four transporters in both normal and cancer cells *in vitro*. The present study shows that the usage of drugs, targeted specifically to highly expressed transporters in normal cells (but not in cancer cells), is essential for reducing the PpIX accumulation in normal cells in order to increase the specificity of ALA-PDT and ALA-PDD in cancer.

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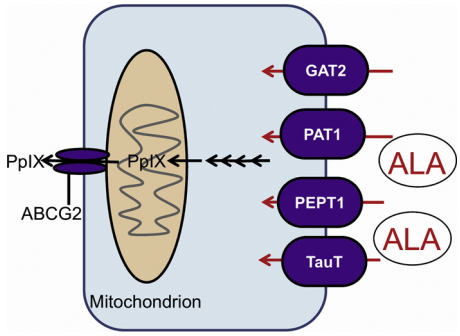

Fig. 1. Schematic illustration on the roles of various uptake transporters, namely PEPT1, PAT1, TauT and GAT2.

2. Materials and methods

2.1. Cells and cell culture

The human gastric-cancer cell line TMK1 was obtained from Assoc. Prof. Dr. Endo Yoshio from Kanazawa University, Japan. The human lung carcinoma cell line, A549, and human lung normal cell line, WI38, were obtained from RIKEN, Japan. The human prostate-cancer cell lines, DU145 and PC3, were obtained from Prof. Inoue Keiji from Kochi University, Japan. PrEC cells were obtained from SBI Pharmaceuticals Ltd., Tokyo, Japan. TMK1, MKN45, and PC3 cells were cultured in RPMI 1640 culture medium. DU145, A549, and WI38 cells were cultured in a DMEM-high glucose culture medium. PrEC cells were cultured in and routinely grown in PrEGM culture medium, supplemented with BPE, hydrocortisone, hEGF, epinephrine, transferrin, insulin, retinoic acid, triidothyronine, and GA-1000. All cell lines were incubated with their respective culture media followed by the addition of 10% FBS and 10% ABAM, at 37 °C in a 5% CO_2 incubator. Experiments were only carried out when cell density reached 50%–80% confluence. Characteristics of these cell lines were tabulated in Table 1.

2.2. Biochemicals

RPMI-1640 culture medium, DMEM-high glucose culture medium, antibiotic–antimycotic mixed stock medium (ABAM), prostate epithelial growth medium (PrEGM), and its supplements were purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Equitech-Bi, Inc. (Kerrville, TX, USA). Aminolevulinic acid (ALA) was purchased from Cosmo Oil Ltd. (Tokyo). Ibuprofen, tryptophan, taurine, and gamma-aminobutyric acid (GABA) were purchased from Sigma Aldrich Corporation (Tokyo, Japan). All inhibitors used were of analytical grade.

2.3. HPLC analysis of PpIX

Cells (0.2×10^6 cells/mL) were incubated with 1 mM ALA in respective media under 5% CO_2 gas at 37 °C in the dark for 24 h. Sample preparation was performed using perchloric acid:methanol (1:1, v/v) solution, as previously described [22]. Extracellular PpIX was determined by recovering culture medium while the remains were collected for determining intracellular PpIX. Samples were quantitatively determined by measuring their fluorescence in a Hitachi Fluorescence Spectrophotometer Type F7000 (Hitachi High-Technologies, Tokyo, Japan). To measure fluorescence intensity, excitation and emission wavelengths of 405 and 605 nm, respectively, were used.

2.4. Western blot analysis

Western blotting analyses were carried out as previously described [30]. We used polyclonal anti-human PAT1 antibody (Novus Biologicals, Littleton, CO, USA; 1:1000), anti-human PEPT1 antibody (Abcam, Cambridge, MA, USA; 1:200), anti-human TauT antibody (Santa Cruz Biotechnology, Dallas, TX, USA; 1:500), anti-human GAT2 antibody (Medical & Biological Laboratories, Nagoya, Japan; 1:1000), and human actin antibody (MP Biomedicals, Santa Ana, CA, USA; 1:200 dilution) as the primary antibody. Secondary antibodies used in this study were horseradish peroxidase (HRP)-conjugated anti-mouse (Cell Signaling Technology, Beverly, MA, USA) and anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA) concentrate, which were diluted 3000 times in TBST solution.

2.5. Establishment of cancer cell lines transiently suppressing transporter expression

To treat DU145 cells and suppress PAT1, PEPT1, and GAT2 expression, 100 nM PAT1 siRNA (sense, 5′-GCGCUUUGGUCAAAGC AAU-3′; anti-sense, 5′-AUUGCUUUGACCAAAGCGC-3′), PEPT1 siRNA (sense, 5′-CAAGAGUGGGAAAGUUUA-3′; anti-sense, 5′-UAAACUUUCC CACUCAUUG-3′) (Sigma Genosys siRNA Service, Tokyo, Japan) and GAT2 siRNA (Factory-prepared siRNA) (Santa Cruz Biotechnology, Dallas, TX, USA) were transfected into DU145 cells using DharmaFECT™ 4 (Dharmacon, Lafayette, CO, USA), in accordance with the manufacturer's instructions. Cells were further incubated for designated periods as described in the figure legends.

3. Results

3.1. PpIX production and expression of transporters in cancer cell lines

The concentration of PpIX was first evaluated on gastrointestinal-cancer cell lines, TMK1 and MKN45, and prostate-cancer cell lines, DU145 and PC3 (Fig. 2a). No significant difference in PpIX levels was observed for these graphs ($p > 0.05$) (Fig. 2a). This study is aimed at identifying which cell lines can take up most ALA following the addition of a fixed ALA concentration, which in turn contributes to the production of PpIX. The efficiency of ALA-PDT is determined by how much PpIX accumulate intracellularly. Extracellular PpIX, believed to be exported out by an efflux transporter and do not directly contribute to PDT response, were also included in this study as to determine the

Table 1
List of characteristics of various cell lines used in this study.

Origin	Cell lines	Description	References
Stomach	TMK1	Human poorly differentiated gastric adenocarcinoma Mutant p53 tumour suppressor gene with rearranged $p15^{MTS2}$	[16]
	MKN45	Human poorly differentiated gastric adenocarcinoma Obtained from 62-year old Japanese female Homozygous deletion of $p16^{CDKN2/MTS1/INK4A}$ and $p15^{MTS2}$	[16]
Prostate	DU145	Human epithelial prostate carcinoma Metastatic cells derived from brain Grow as isolated islands on plastic Petri dishes	[17,18]
	PC3	Human epithelial prostate carcinoma Metastatic cells derived from bone Retain co-regulators to suppress androgen receptor	[18]
	PrEC	Normal human prostate epithelial cells Packed cuboidal morphology Used as control for prostate cancer studies	[19]
Lungs	A549	Human epithelial lung carcinoma Synthesize lecithin with high percentage of desaturated fatty acids utilizing the cytidine diphosphocholine pathway	[20]
	WI38	Human lung fibroblast Extensively studied cell line which is derived from a single donor and can be frozen for indefinite periods of time	[21]

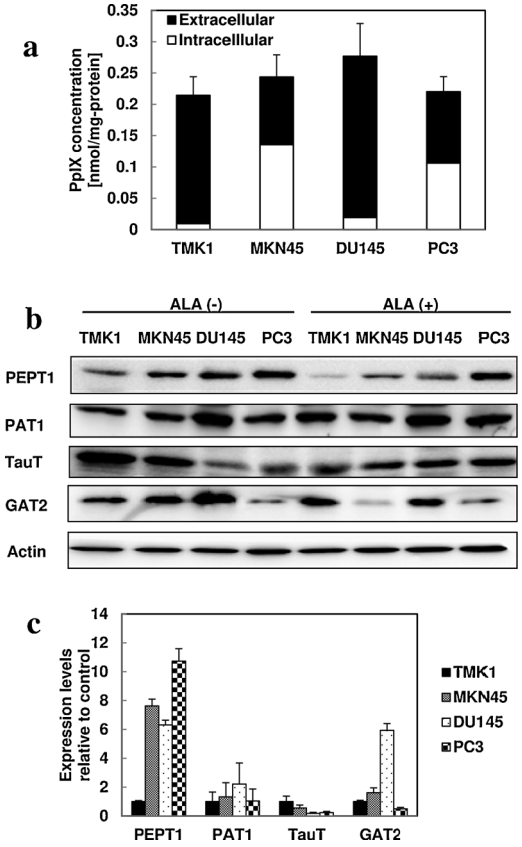

Fig. 2. Relationship of PpIX production and expression levels of transporters in four selected cancer cell lines. The cancer cell lines used in this study were TMK1, MKN45, DU145, and PC3. The transporters used in this study were PEPT1, PAT1, TauT, and GAT2. (a) The production of intracellular and extracellular PpIX following the addition of 1 mM ALA for 24 h. Intracellular and extracellular PpIX were determined as described in materials and methods. (b) Protein expressions of transporters using Western blotting in the absence or presence of incubation of 1 mM ALA for 24 h (c) Band intensity of protein expression of transporters in four selected cell lines using Western blotting without ALA administration. n = 3. Bars represent standard deviation (SD). Blots have been cropped to ease visualization. One representative blot is shown out of three independent experiments. Unprocessed original scans of blots are shown in Supplementary Fig. 1.

total PpIX taken in by the uptake transporters. We considered that a high-expression level of specific transporters would result in higher uptake of ALA, which would subsequently lead to a rise in PpIX production. Protein expression of PAT1 was observed to be higher in DU145 cells than in the other cell lines (Fig. 2b and c). PEPT1 was found to be equally highly expressed in MKN45, DU145, and PC cells. TauT was found to be considerably highly expressed in TMK1 cells, followed by MKN45 cells. Both DU145 and PC3 exhibited relatively low expression levels of TauT. On the other hand, GAT2 was found to be highly expressed only in the DU145 cell line. The results here also indicate that the expression of PEPT1 and TauT transporters was slightly lowered following the addition of ALA, although this effect was reversed in the case of PAT1. Unlike the remaining cell lines that expressed only one transporter, DU145 highly expressed three transporters, namely, PEPT1, PAT1, and GAT2.

3.2. Roles of transporters in ALA uptake in cancer cell lines

PEPT1, PAT1, and GAT2 gene knockdown studies were carried out to identify the dominant transporters responsible for ALA uptake in DU145 cells. DMEM culture medium was used to prevent ABAM substrates from inhibiting the formation of complex in transfection medium. The optimal concentrations for knocking down PEPT1, PAT1, and GAT2 were first determined using Western blotting, as described in the methods. Cytotoxicity tests were carried out beforehand to ensure cell death following siRNAs and Dharmafect addition do not show obvious difference with their respective controls. The concentrations of siPEPT1, siPAT1, and siGAT2 were fixed at 100 nM (Fig. 3a–c). The amount of PpIX produced following PEPT1, PAT1, and GAT2 knockdown showed significant reductions of 70%, 60%, and 90%, respectively, compared to the siNC control ($p < 0.05$) (Fig. 3d–f). This result showed that the inhibition of these transporters significantly decreased PpIX production in DU145 cells, suggesting that these transporters play significant roles in the cellular uptake of ALA in this cell line, which coincided with their high expression levels in DU145 cells.

After showing that all of PEPT1, PAT1, and GAT2 are involved in ALA uptake, inhibitors were added to evaluate the effect on PpIX production of suppressing each transporter's activity. Studies were performed by co-administering ALA and inhibitors in a concentration-dependent manner, using DU145 cells (Fig. 4a–d). Cytotoxicity test were also carried out beforehand to ensure cell death following inhibitors addition do not show obvious difference with their respective controls. There are two types of inhibitors, competitive and non-competitive inhibitors. Competitive inhibitors are substances that share similar structure with substrates to an enzyme (transporter in this study) and compete with them for active site [23]. On the other hand, non-competitive inhibitors bind to allosteric sites of the enzyme, changing the conformation of active site and thus preventing attachment of substrates [23]. Inhibition of all of these transporters showed significant decreases in PpIX levels, suggesting that all four transporters are involved in the cellular uptake of ALA in DU145 cells. The roles of TauT and GAT2 cannot be determined separately, as both taurine and GABA inhibit the action of both TauT and GAT2 [24,25]. Ibuprofen and tryptophan are inhibitors of PEPT1 and PAT1 respectively [26,27].

Inhibition of all four transporters in the remaining cell lines, TMK1, MKN45, and PC3, was also studied, and the obtained data were again plotted in Fig. 4e. In general, all of these results correlated with the expression of all four transporters in all cell lines (except PAT1 inhibition in TMK1, where it was expressed at a low level), whereby the inhibition of transporters that were highly expressed resulted in a significant decrease in PpIX production (Table 2). These findings showed that the cellular uptake of ALA is highly dependent on the expression levels of transporters and is not fixed to a specific transporter. The expression and roles of transporters in normal and cancer cells of the same origin were then studied in order to identify a cancer-specific drug that can increase the specificity of ALA-PDT and ALA-PDD.

3.3. PpIX production and expression of transporters in normal and cancer cell lines

The studies described in this section used several sets of normal and cancer cell lines of the same origin, namely, WI38 (normal) and A549 (cancer) from lungs; and PrEC (normal), PC3, and DU145 (cancer) from the prostate gland. The concentration of PpIX was first evaluated in all of these cell lines. WI38 cells (~0.09 nmol/mg-protein) exhibited PpIX production that was around 35% lower than that of their cancerous counterparts, A549 cells (~0.16 nmol/mg-protein) ($p < 0.05$) (Fig. 5a). No significant difference was observed between the total PpIX produced in the PrEC–PC3 pair and the PrEC–DU145 pair ($p > 0.05$) (Fig. 5b and c). Such accumulation of PpIX in normal cells may result in unwanted cytotoxicity in treatments using ALA. Therefore, the expression of various transporters involved in ALA uptake was then evaluated in order to identify transporters potentially governing ALA uptake in all of these cell lines.

Protein expression of PAT1 and GAT2 was found to be higher in A549 than in WI38, while PEPT1 was found to be highly expressed in WI38 relative to that in A549 (Fig. 5d). The bands representing PAT1 and GAT2 in WI38, together with the PEPT1 band in A549, were barely

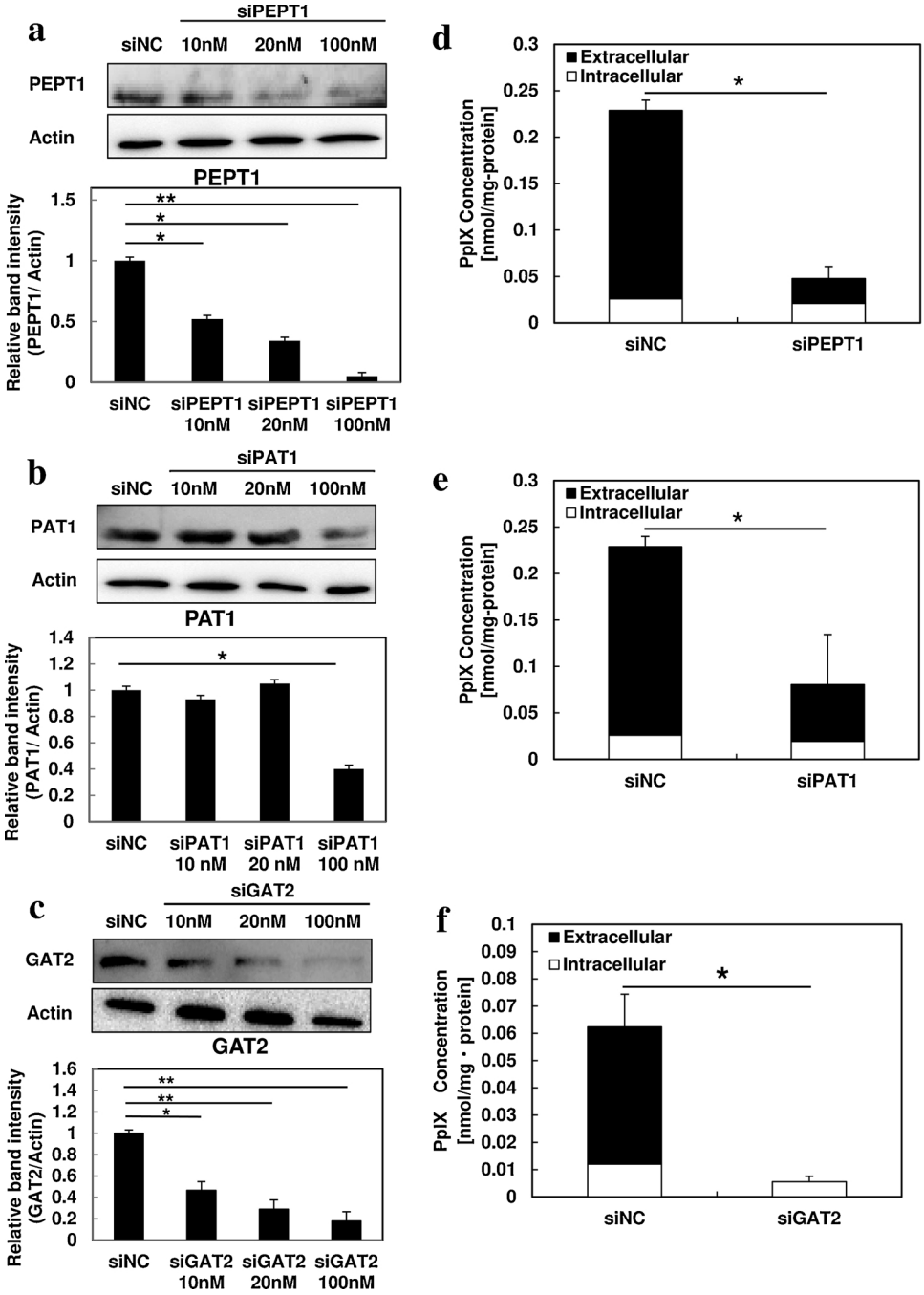

Fig. 3. Effects of PEPT1-, PAT1-, and GAT2-specific siRNAs on the production of PpIX in DU145 cells. Cells were seeded at 2×10^4 cells/ml. The optimal concentrations for (a) siPEPT1, (b) siPAT1, and (c) siGAT2 were fixed at 100 nM. (d–f) Intracellular and extracellular PpIX in DU145 cells treated with 100 nM siRNAs for 26 h, followed by the addition of 1 mM ALA for another 24 h. One-way ANOVA (Tukey's test) was performed for each set of data to show that there were significant differences in mean values between treated and untreated samples, *, $p < 0.05$; **, $p < 0.01$. n = 3. Bars represent standard deviation (SD). Blots have been cropped to ease visualization. One representative blot is shown out of three independent experiments. Unprocessed original scans of blots are shown in Supplementary Fig. 2.

visible, suggesting very low abundance in the respective cell lines. On the other hand, TauT was found to be expressed at a low level in both WI38 and A549 cells. In the case of prostate cells, PEPT1 was the only highly expressed transporter found in PC3 cells (Fig. 5e). PrEC showed high expression levels of PAT1 and PEPT1, while PAT1, PEPT1, and GAT2 were highly expressed in DU145 cells. GAT2 was found to be the only transporter expressed in DU145 cells among the three cell lines. Similar to the results shown in Table 2, PC3 and DU145 did not exhibit similar transporter expression patterns despite both cell lines being cancerous and originating from the same organ; this further highlights the importance of a therapy customized to each patient. Based on the results described directly above and the findings from the previous section, it is believed that transporters that were highly expressed would play a significant role in ALA uptake. Therefore, inhibiting the actions of these highly expressed transporters should result in a significant decrease in PpIX production.

3.4. Difference of transporter roles in ALA uptake in normal and cancer cell lines

In this section, we inhibited the transporters involved in ALA uptake to observe the change in PpIX production in normal and cancer cell lines of similar origins. The difference in ratio of intracellular and extracellular PpIX were believed to due to expression of ABCG2, a PpIX efflux transporter, in some cell lines [28]. The amount of PpIX being exported out is believed to be dependent on the expression level of ABCG2 in the cell. Co-administration of fumitremorgin C, an inhibitor of ABCG2, during ALA-PDT could increase efficiency of the therapy [28]. Therefore, the results were being presented in the form of total PpIX. The addition of ibuprofen, an inhibitor of PEPT1, resulted in a decrease in PpIX production of more than 40% in WI38 cells, but not in A549 cells (Fig. 6a). No significant difference in PpIX production was observed when tryptophan, taurine, and GABA were co-administered

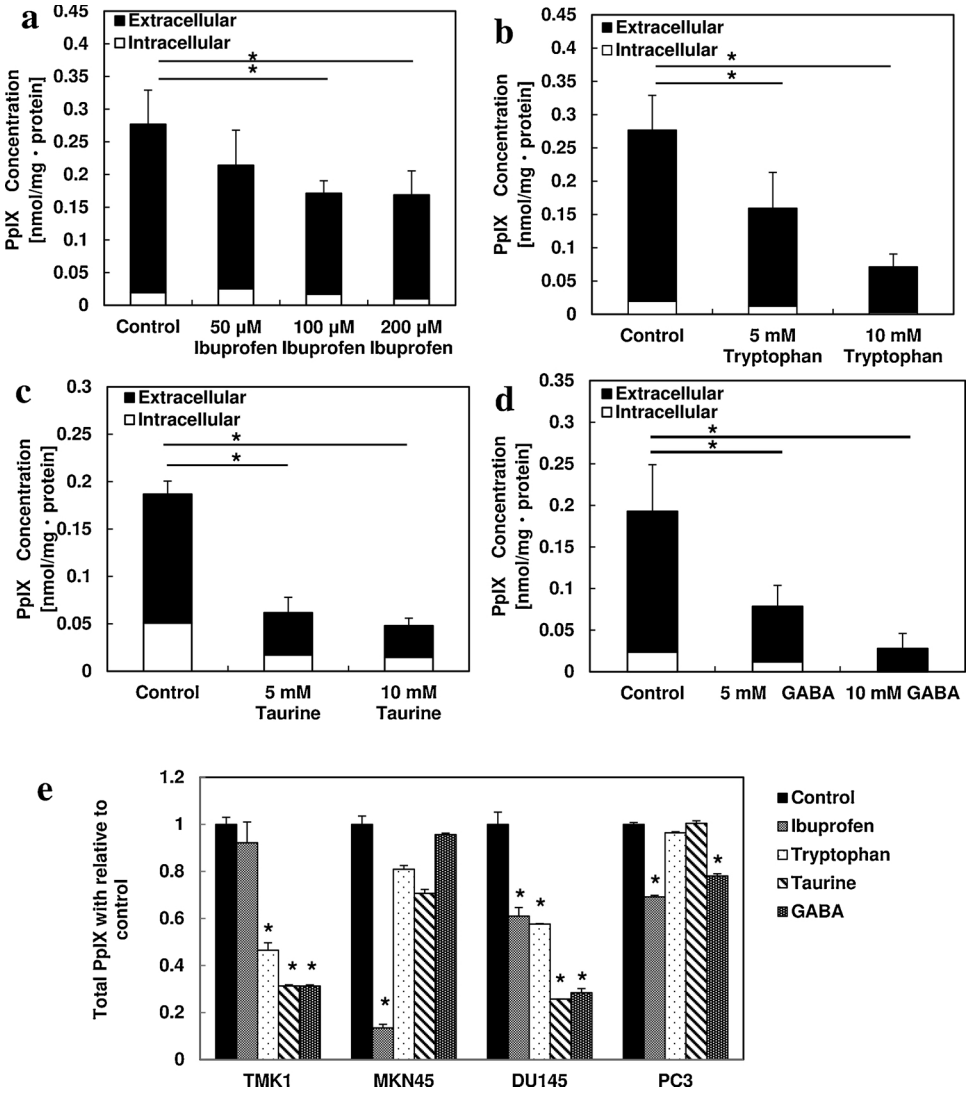

Fig. 4. Changes in the production of intracellular and extracellular PpIX following the co-addition of ALA and transporter inhibitors. Ibuprofen and tryptophan are inhibitors of PEPT1 and PAT1, respectively. Taurine and GABA inhibit both TauT and GAT2. ALA at 1 mM was coadministered with (a) PEPT1 inhibitor, (b) PAT1 inhibitor, and (c–d) TauT and GAT2 inhibitors in the DU145 cell line. (e) Inhibition assays in all four cell lines were carried out using 5 mM transporter inhibitors. One-way ANOVA (Tukey's test) was performed for each set of data to show that there were significant differences in mean values between treated and untreated samples, *, $p < 0.05$. n = 3. Bars represent standard deviation (SD).

Table 2
Relationship between transporters involved in ALA uptake and PpIX production in four selected cancer cell lines. (a) Protein expression of various transporters and (**b**) their effects on PpIX production following co-addition of inhibitors and ALA (Values are relative to transporters which are most highly expressed among four cell lines (1.0)).

Low Medium High

a

Cell lines	TMK1	MKN45	DU145	PC3
PEPT1	0.39	0.88	0.93	1
PAT1	0.45	0.65	1	0.53
TauT	1	0.5	0.17	0.21
GAT2	0.24	0.33	1	0.34

b

Inhibitors	Cell lines			
	TMK1	MKN45	DU145	PC3
Ibuprofen	-	↓	↓	↓
Tryptophan	↓	-	↓	-
Taurine	↓	-	↓	-
GABA	↓	-	↓	-

with ALA, suggesting that PAT1, TauT, and GAT2 are not involved in the ALA uptake in WI38 cells ($p > 0.05$). These results coincide with the Western blotting results shown in Fig. 3d, where transporters other than PEPT1 were expressed at low levels in WI38 cells. The change in the level of PpIX produced following the addition of ibuprofen in both WI38 and A549 cells showed a higher degree of significance ($p < 0.01$) than that in the control ($p < 0.05$). On the other hand, studies using tryptophan, taurine, and GABA in A549 cells showed significant decreases in PpIX production, but this was not the case for ibuprofen ($p < 0.05$), which again coincided with the protein expression results (Fig. 5e). This suggests that the three transporters, with the exception of PEPT1, were involved in the ALA uptake in this cell line. This inhibition assay was then repeated using the same methods in PrEC and PC3 cells originating from the prostate gland (Fig. 6b). The inhibition of PEPT1 in PrEC and PC3 resulted in a significant decrease in PpIX production ($p < 0.05$). The PpIX level decreased significantly following the addition of tryptophan in PrEC, but this trend was not observed in PC3 ($p < 0.05$). No significance difference was observed following the addition of taurine and GABA in both PrEC and PC3. These results coincided with the protein expression levels presented in Fig. 5e.

Studies were carried out again in another pair of prostate cell lines, PrEC and DU145, as shown in Fig. 6c. PpIX production of both cell lines decreased significantly following the inhibition of PEPT1 and PAT1 ($p < 0.05$). There was no significant change in PpIX levels in PrEC cells following the addition of taurine and GABA. An observable decrease in PpIX production was observed in DU145 following the inhibition of TauT and GAT2 ($p < 0.05$). Similar to the findings in two previous studies, the inhibition results correlated with the protein expression levels shown in Fig. 5e.

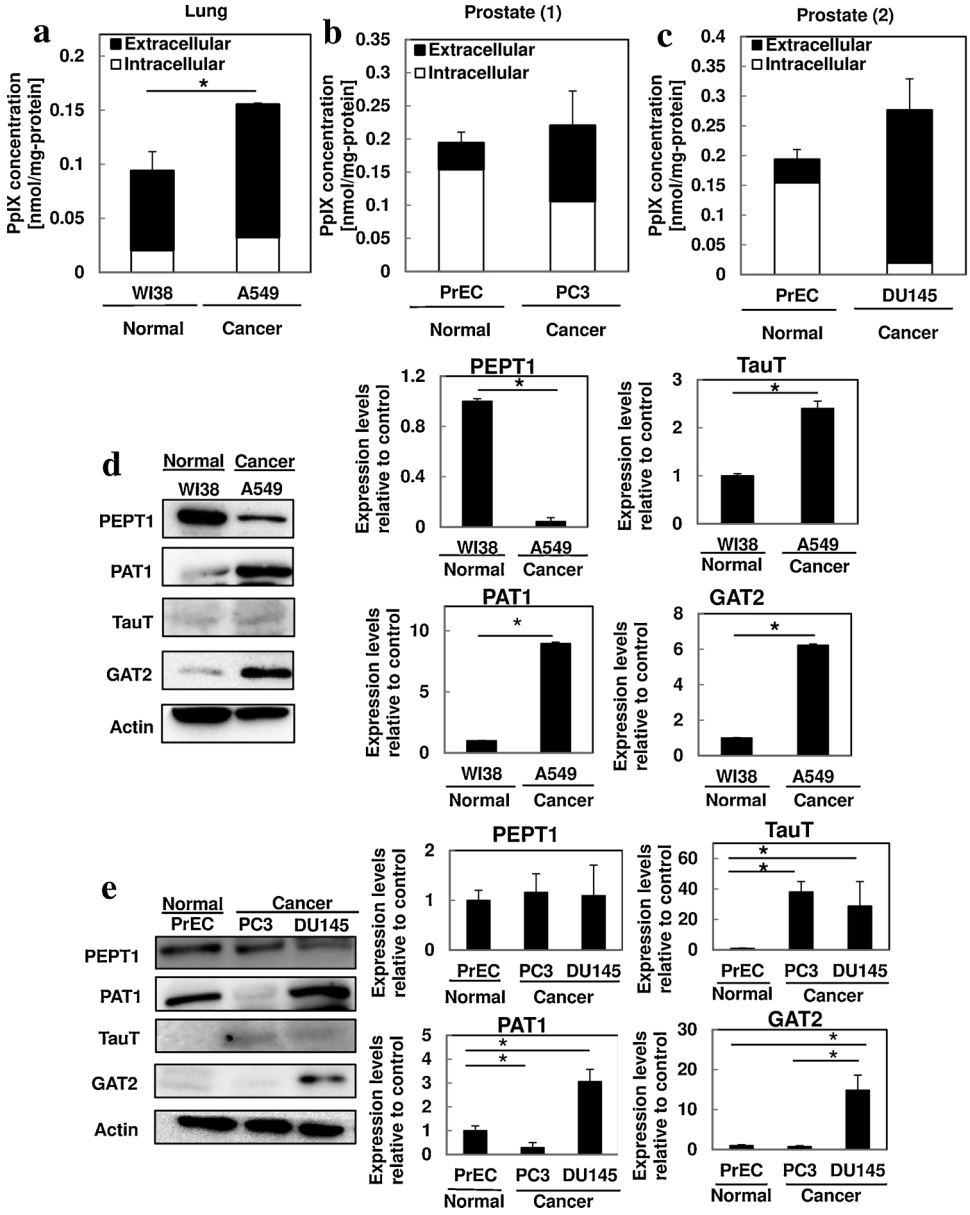

Fig. 5. Relationship of PpIX production and expression levels of transporters in selected cancer cell lines. Cell lines used in this study were lung normal (WI38) and cancer (A549) cells, and prostate normal (PrEC) and cancer (PC3, DU145) cells. Transporters used in this study were PEPT1, PAT1, TauT, and GAT2. (a–c) Production of intracellular and extracellular PpIX following the addition of 1 mM ALA for 24 h in various normal and cancer cell lines. (d) Protein expression of transporters and their band intensities in lung normal and cancer cell lines using Western blotting. (e) Protein expression of transporters and their band intensities in prostate normal and cancer cell lines using Western blotting. n = 3. Bars represent standard deviation (SD). Blots have been cropped to ease visualization. One representative blot is shown out of three independent experiments. Unprocessed original scans of blots are shown in Supplementary Fig. 3 and 4.

Overall, the inhibition of transporters that were highly expressed resulted in a significant decrease in PpIX production, possibly due to a decrease in the cellular uptake of ALA (Table 3). The results described in this subsection show that could potentially be used as drugs for customized therapy to increase cancer specificity in ALA-PDT and ALA-PDD. The results also show the factors affecting cellular uptake are independent of the origin of organ.

4. Discussion

The definition of cellular uptake varies depending on the context. In medicine, it is defined as the absorption and incorporation of a substance by living tissue [29]. Understanding the cellular uptake of ALA, such as identifying the dominant influx transporters and possible naturally occurring inhibitors of it, is important in enhancing the efficacy of ALA-PDT and ALA-PDD.

To understand the role of transporters involved in ALA uptake, their expression levels were being studied. Table 2 shows that the expression of these transporters was independent of the organ of origin, where TMK1 and MKN45 did not exhibit similar transporter expression trends despite both originating from the stomach. Prostate-cancer cells DU145 and PC3 also failed to exhibit similar trends in transporter expression. These findings suggested that the expression levels and activity of these four transporters, namely, PEPT1, PAT1, TauT, and GAT2, might be independent of their origin. The sensitivity of ALA uptake in different cell lines is believed to be dependent on their expression levels, as shown in Tables 2 and 3.

Downregulation of PEPT1, PAT1, and GAT2 *via* siRNA showed a significant decrease in PpIX production, suggesting a decrease in the uptake of ALA by these transporters (Fig. 3). From these results, we believed that these transporters were equally important in ALA uptake, which contrasts with the findings of previous studies [12,28]. The decrease in uptake of ALA is believed to have resulted in a lower ALA pool within the cell, which in turn reduced the production of porphyrin intermediates and finally lowered the production of PpIX. These findings were further verified and validated by inhibition studies of all four transporters using DU145 cells (Fig. 4). Inhibition studies on the remaining three cell lines, TMK1, MKN45, and PC3, generally also showed similar trends, whereby the inhibition of highly expressed transporters caused a decrease in cellular uptake of ALA, leading to lower production of PpIX.

Identifying the dominant transporter is essential in understanding

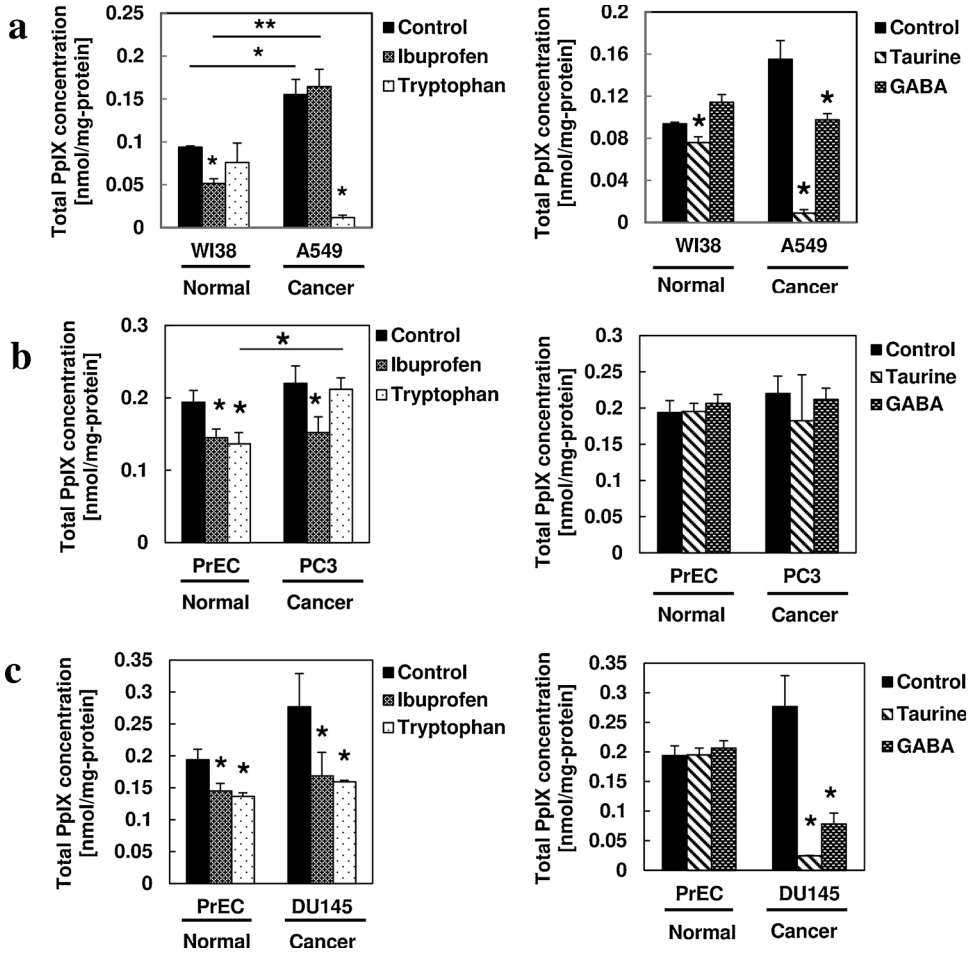

Fig. 6. Changes in the production of intracellular and extracellular PpIX following the co-addition of ALA and transporter inhibitors. Ibuprofen and tryptophan are inhibitors of PEPT1 and PAT1, respectively. Taurine and GABA inhibit both TauT and GAT2. Inhibitor assays were carried out in sets, namely, (a) lung normal (WI38) and cancer (A549) cell lines, (b) prostate normal (PrEC) and cancer (PC3) cell lines, and (c) prostate normal (PrEC) and cancer (DU145) cell lines. One-way ANOVA (Tukey's test) was performed for each set of data to show that there were significant differences in mean values between treated and untreated samples. *, $p < 0.05$; **, $p < 0.01$. n = 3. Bars represent standard deviation (SD).

Table 3
Relationship between transporters involved in ALA uptake and PpIX production in selected normal and cancer cell lines. (a) Protein expression of various transporters and (b) their effect on PpIX production following co-addition of inhibitors and ALA (Values are relative to transporters which are most highly expressed among lung cell lines (1.0); and among prostate and stomach cell lines (1.0)).

Low — Medium — High

a

	Lungs		Prostate gland			Stomach
	Normal	Cancer	Normal	Cancer		Cancer
	WI38	A549	PrEC	PC3	DU145	TMK1
PEPT1	1	0.03	0.98	0.90	1	0.42
PAT1	0.09	1	0.34	0.11	1	0.45
TauT	0.01	0.19	0.01	0.21	0.17	1
GAT2	0.07	1	0.06	0.04	1	0.24

b

Inhibitors	Lungs		Prostate gland		
	Normal	Cancer	Normal	Cancer	
	WI38	A549	PrEC	PC3	DU145
Ibuprofen	↓	-	↓	↓	↓
Tryptophan	-	↓	↓	-	↓
Taurine	-	↓	-	-	↓
GABA	-	↓	-	-	↓

the sensitivity of cells to ALA-induced PpIX accumulation. In contrast to previous *in vitro* and *in vivo* studies in gastric cancer cells by various research groups which focused only on PEPT1, our study showed that expression levels of PEPT1, PAT1, TauT, and GAT2 were correlated with the response to ALA-induced PpIX accumulation in these four cell lines *in vitro*, although further tests are necessary to discover the actual contribution of each transporter [12,28,30].

Addressing the specificity of target transporters is important to ensure maximum exogenous ALA-induced PpIX accumulation only in cancer cells, but not in normal cells. Three sets of cell lines from two different origins were studied in order to obtain more comprehensive results on the cellular uptake of ALA by transporters. Despite no significant difference in the total levels of PpIX among all of these cell sets, differences in intracellular and extracellular PpIX levels between PrEC

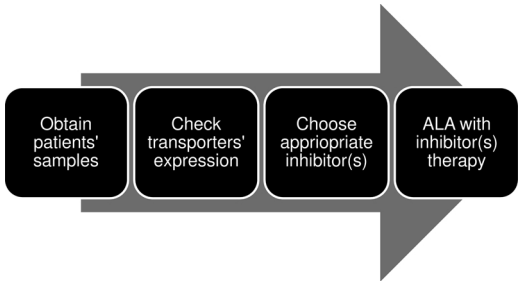

Fig. 7. Flow chart showing chronological order of customized therapy to enhance the specificity of ALA-PDT and ALA-PDD.

and DU145 cells were observed (Fig. 5c). This is believed to have been due to the higher expression level of ATP-binding cassette (ABC) transporter, ABCG2, in DU145 than in PrEC cells. ABCG2, formerly known as breast-cancer-resistant protein 1, contributes to drug resistance in breast cancer [31]. This transporter, expressed in various normal and cancerous tissues, plays a role in PpIX efflux as an efflux transporter [24,32].

Similar to the results described in the previous section, Table 3 a also shows that the expression of these transporters was independent of the organ of origin, where all five cell lines studied in this section did not express similar transporter expression trends despite originating from the same organ. This again suggests that the expression levels and activity of these four transporters may be independent of their origin. The sensitivity of ALA uptake in different cell lines was believed to be dependent on their expression levels, as shown in Table 3, and their expression levels would be vital in a customized ALA-PDT therapy. Two major findings were obtained from these studies: (i) PEPT1 was expressed only in normal lung cells, but not in their cancerous counterparts; and (ii) PAT1 was expressed only in normal prostate cells, but not in their cancerous counterparts (Fig. 5d and e). The inhibition of these transporters in these cell lines resulted in a significant decrease in PpIX production in normal cells, but not in cancer cells (Fig. 6a and b). This suggests that ibuprofen and tryptophan might be useful in customized therapy by increasing the specificity to cancer.

Mathematical calculations showed that the production of PpIX in the controls of A549 and WI38 cells differed, whereby the former produced approximately 1.7 times more PpIX than the latter. However, the difference in PpIX production of these cell lines increased further to about 3.2 times following the inhibition of PEPT1 using ibuprofen, indicating a significant decrease in PpIX production in WI38 cells but not in A549 cells ($p < 0.05$) (Fig. 6a). In the case of prostate cells, despite no significant difference in PpIX production being observed between PrEC and PC3 cells, PpIX production was approximately 1.6 times higher in PC3 than in PrEC cells following the addition of tryptophan, proving that the inhibition of ALA uptake occurred only in PrEC cells ($p < 0.05$) (Fig. 6b). The increase in ratio values of both cases indicated that these two drugs are specifically targeted to normal cells and could be used as a customized therapy together with ALA treatment to increase the specificity of ALA-induced PpIX accumulation in cancer. The flow of a customized therapy for ALA-PDT or PDD is presented in Fig. 7. The administration of these drugs could increase the specificity of the therapy by preventing the cellular uptake of ALA in normal cells.

This study also revealed the abundance of PEPT1 in cancer and normal cells. Based on research by Chung et al. (2013) and Gong *et al.* (2017), PEPT1 are highly expressed in gastric and liver cancer cells compared with the level in normal cells, suggesting that normal cells might exhibit much lower production of PpIX [33,34]. The results in this section suggested otherwise, whereby normal lung cells (WI38) expressed much more PEPT1 than their cancerous counterparts (A549) (Table 3). Normal prostate cells, PrEC, also exhibited levels of PEPT1 equal to those of their cancerous counterparts, PC3 and DU145 (Table 3). Based on these findings, we hypothesized that PEPT1 may be expressed in normal cells, depending on their organ origins.

The importance of specificity in ALA-PDT should not be underestimated, especially with regard to cancers that cannot be treated with ALA-PDT due to the accumulation of PpIX in normal cells, such as esophageal cancer. Several studies by medical doctors provided evidence that PpIX also accumulated in normal cells. A study by Kriegmair et al. (1994) showed that the specificity of ALA uptake in 146 patients with neoplastic urothelium was below 70% [35]. Another study by Zaak et al. (2001) also revealed this phenomenon in 53 bladder-cancer patients and highlighted that further countermeasures to reduce PpIX accumulation in normal cells should be taken [36]. Therefore, it is important that a new drug, administered together with ALA, serves as an inhibitor to block the uptake of ALA in normal cells without affecting the accumulation of PpIX in esophageal-cancer cells. Further studies on the role of transporters, especially in esophageal normal and cancer cells, are required before further clinical studies using ALA can be carried out.

In conclusion, the results obtained here show that the usage of drugs targeted specifically to transporters in normal cells is essential at reducing the PpIX accumulation in normal cells in order to increase the specificity of ALA-PDT and ALA-PDD in cancer. This study also showed that the role of transporters involved in ALA uptake differs among different cell lines (even of the same origin) and is highly dependent on the expression levels of respective transporters. These findings also showed that PEPT1 may be highly expressed in normal cells depending on their organ origin.

5. Author contributions

H.W.L and S.O. wrote the main manuscript. H.W.L., R.S., S.U. and S.O. designed the experiments. H.W.L., R.S. and S.U. collected and processed the data. M.N. and T.T. contributed reagents, materials and analysis tools. H.W.L. and S.O. reviewed and revised paper. All authors approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pdpdt.2019.06.017.

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