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Evaluation of aminolevulinic acid-mediated protoporphyrin IX fluorescence and enhancement by ABCG2 inhibitors in renal cell carcinoma cells

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Abstract

Aminolevulinic acid (ALA) has been approved as an intraoperative molecular imaging probe for protoporphyrin IX (PpIX) fluorescence-guided resection of glioma. Here we explored its potential application for renal cell carcinoma (RCC) that is showing increased incidence in recent years. ALA-mediated PpIX in cell lysates (intracellular) and culture medium was measured in five human RCC cell lines (786-O, 769-P, A-704, Caki-1, Caki-2) and a non-tumor human kidney epithelial cell line HK-2 by spectrofluorometry and flow cytometry. The activity of PpIX bioconversion enzyme ferrochelatase (FECH) and PpIX efflux transporter ABCG2 was determined to correlate with the PpIX level. We found that ALA-PpIX fluorescence was highly variable among RCC cell lines and A-704 was the only RCC cell line exhibiting significantly higher intracellular PpIX than HK-2 cells. Neither the intracellular PpIX level nor the total amount of PpIX (including PpIX in cell lysates and the medium) had significant correlation with the activity of FECH or ABCG2. To enhance the intracellular PpIX, cells were treated with Ko143, a pharmacological inhibitor of ABCG2. Ko143 significantly increased the intracellular PpIX in cell lines with ABCG2 activity, but not in cell lines with little ABCG2 activity. In fact, there was a positive correlation between the ABCG2 activity and Ko143-induced PpIX enhancement across kidney cell lines. To identify clinically relevant ABCG2 inhibitors, small molecule inhibitors targeting various cell signaling pathways, some of which are known to inhibit ABCG2, were evaluated for the enhancement of ALA-PpIX in Caki-2 cells that had the highest ABCG2 activity in the RCC cell panel. Our screening led to the identification of several clinically available inhibitors that significantly increased the intracellular PpIX. Particularly, kinase inhibitor lapatinib exhibited the strongest enhancement effect. These clinical inhibitors can be used for the enhancement of ALA-PpIX fluorescence in tumors with elevated ABCG2 activity.

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Keywords

aminolevulinic acid (ALA); protoporphyrin IX (PpIX); photodynamic therapy (PDT); fluorescence-guided tumor resection; ABCG2; renal cell carcinoma (RCC); lapatinib

INTRODUCTION

Aminolevulinic acid (ALA, Gleolan) was recently approved by the FDA as an intraoperative probe for fluorescence-guided resection of high-grade glioma (1). Although ALA has no fluorescence on its own, it is metabolized in the heme biosynthesis pathway to produce protoporphyrin IX (PpIX) with strong red fluorescence. The accumulation of PpIX in tumor tissue enables the use of its fluorescence to guide tumor resection. Enhanced visual difference between tumor and normal tissues revealed by real-time PpIX fluorescence imaging allows surgeons to gain increased tumor detection certainty and dissect tumors that are otherwise invisible, resulting in improved surgical outcomes (2, 3). ALA-PpIX fluorescence-guided tumor surgery has been shown to increase progression-free survival in patients with high-grade glioma by dissecting more tumor tissue than conventional white light surgery (4). Not only being a fluorescent agent for tumor imaging, PpIX is also an effective photosensitizer. Tumor accumulation of PpIX after ALA has led to the use of ALA-photodynamic therapy (PDT) for cancer treatment, which is now commonly used for precancerous and cancerous skin lesions (5).

Effective use of ALA for fluorescence-guided tumor resection and PDT depends on preferential tumor accumulation of PpIX, which is determined by several dynamic biological processes including its biosynthesis from ALA, bioconversion to non-fluorescence heme, and efflux by membrane transporters (6). PpIX is produced in mitochondria from ALA in the heme biosynthesis pathway composed of multiple enzyme-catalyzed steps in the cytosol and mitochondria. The final step of the pathway is the formation of heme from PpIX and ferrous iron, an enzymatic reaction catalyzed by the ferrochelatase (FECH) in mitochondria. PpIX can be transported outside of cells by efflux transporters such as ATP-binding cassette sub-family G member 2 (ABCG2), also known as breast cancer resistance protein (BCRP). Though increased activity of some enzymes involved in PpIX biosynthesis can increase PpIX production (7, 8), elevation of FECH enzyme (9) or ABCG2 activity (10) results in reduced PpIX level.

The approval of ALA for guiding glioma resection has encouraged the exploration of this molecular imaging probe for other oncological surgeries (11). One potential application is for the resection of renal cell carcinoma (RCC). As the third most common genitourinary tumor and the seventh most common cancer in the US, the incidence of RCC is increasing over the past years (12), and the majority (up to nearly 70%) of newly diagnosed RCC cases are early localized diseases (13). Surgery is the standard of care for the localized RCC and kidney-preserving surgery is preferred due to the preservation of kidney function (12). A prospective, non-randomized single-center clinical trial shows that laparoscopic kidney-preserving resection of RCC under ALA-PpIX fluorescence guidance has excellent surgical outcomes with sensitivity and specificity of 95% and 94%, respectively (14). PpIX

fluorescence-guided RCC resection significantly reduces the operating time and kidney ischemia time during surgery, which is conducive to the preservation of kidney function. The possibility of using PpIX fluorescence to assess tumor margin further helps to reduce surgery time by avoiding the time-consuming frozen tumor section-based margin evaluation. This promising clinical study together with a preclinical evaluation from the same group (15) indicate the benefit of using ALA-PpIX fluorescence for guiding RCC tumor resection.

However, to the best of our knowledge, ALA-PpIX production has not been well studied in human RCC cell lines. There is only one report showing enhanced ALA-PpIX in one RCC cell line compared with non-tumor kidney cells (16). It is not yet known whether this is the case for other RCC cell lines. In this study, we evaluated ALA-PpIX level in a panel of human RCC cell lines and non-tumor kidney cells and found that RCC cell lines exhibited significant heterogeneity in PpIX level. Inhibition of ABCG2 greatly increased the intracellular PpIX in RCC cell lines with elevated ABCG2 activity, but not in cells with no activity. Our results indicate a strong positive correlation between ABCG2 activity and the response to ABCG2 inhibition for PpIX enhancement across RCC cell lines. Importantly, several clinically used small molecule inhibitor with activity of enhancing intracellular PpIX fluorescence were identified.

MATERIALS & METHODS

Chemicals

Delta-aminolevulinic acid hydrochloride (ALA) from Frontier Scientific Inc. (Logan, UT) was dissolved in phosphate-buffered saline (PBS) solution. PpIX and Pheophorbide A (Pha) were obtained from Frontier Scientific Inc. and dissolved in DMSO. Ko143 and palmitic acid were purchased from Sigma (St. Louis, MO) and dissolved in DMSO. Ethylenediaminetetraacetic acid (EDTA), zinc acetate, sodium dodecyl sulfate (SDS) and Triton X-100 were purchased from Sigma and dissolved in deionized water. Protease inhibitor cocktail (1× working solution) includes aprotinin (2 µg/mL), leupeptin (2 µg/mL), pepstatin A (1 µg/mL) and phenylmethanesulfonyl fluoride (PMSF, 1 mM), all from Sigma. All chemicals were sterilized through filtration using 0.22-µm pore size filters and stored in a -20°C freezer.

Cell culture

Human RCC cell lines 786-O, 769-P, and non-tumor kidney proximal tubular epithelial cell line HK-2 were maintained in RPMI 1640 medium supplemented with 9% fetal bovine serum (FBS, Atlanta Biologicals) and 1% antibiotics and antimycotics. Human RCC cell lines Caki-1 and Caki-2 were maintained in McCoy's 5A medium supplemented with 9% FBS and 1% antibiotics and antimycotics. Human RCC cell line A-704 was maintained in EMEM supplemented with 9% FBS and 1% antibiotics and antimycotics. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cell culture medium were purchased from Mediatech (Manassas, VA). Cells were cultured at 37°C in a humidified incubator with 5% CO₂ and inspected almost daily. Cells less than 30 passages were used for experiments.

Spectrofluorometric analysis of PpIX

Cells were implanted in 6-well culture plates in complete medium and cultured for overnight. Cells were treated with ALA (1 mM) alone, Ko143 (1 μ M) alone, or ALA (1 mM) combined with Ko143 (1 μ M) in complete RPMI 1640 medium for 4 h. Controls received no treatment. Cell culture medium was collected after 4-h incubation. Cells were washed twice with PBS and lysed in 0.1% Triton X-100 in PBS containing 100 μ M EDTA and 1 $\times$ protease inhibitor cocktail to obtain cell lysates. Cell lysates and medium were centrifuged and the supernatants were collected. The protein concentration of cell lysates was measured with the Bradford method using the Bio-Rad protein assay reagent. Fluorescence emission spectra of supernatants were measured with Fluoromax-3 fluorescence spectrometer (Horiba JY, Edison, NJ) with excitation wavelength at 400 ± 2.5 nm. The fluorescence of untreated control was subtracted from that of treated samples to obtain the PpIX fluorescence in cell lysates and medium, which was used to calculate the amount of PpIX based on corresponding standard curves generated with the same setting. The amount of PpIX in cell lysates and medium was normalized to the amount of protein and reported as nmol PpIX per gram cell protein.

FECH activity assay

FECH activity was determined as described previously (17). Cells were implanted in 100-mm dishes and allowed to grow to 70% confluency. Cells were lysed with 0.1% Triton X-100 in PBS containing 200 μ M palmitic acid and 1 $\times$ protease inhibitor cocktail. Lysates were left at room temperature for 5 min and then centrifuged for 5 min at 3,000 RCF at room temperature to obtain the supernatants. The protein concentration was measured with the Bradford method using the Bio-Rad protein assay reagent. Supernatants of 490 μ L were transferred into 1.7mL microcentrifuge tubes, mixed with 5 μ L of zinc acetate (500 μ M) and 5 μ L of PpIX (100 μ M), and incubated for 10 min at 37°C immediately afterwards. Resultant Zn-PpIX was detected using Fluoromax-3 fluorescence spectrometer with excitation wavelength at 400 ± 2.5 nm and emission wavelength at 590 nm. The amount of Zn-PpIX was obtained using a standard curve generated with the same setting and normalized to the amount of protein to indicate FECH activity as nmol Zn-PpIX per gram cell protein per minute.

ABCG2 activity assay

ABCG2 activity was assessed by the ability of cells to efflux transport Pha, an ABCG2 substrate (18). Cells were implanted in 12-well culture plates in complete medium and cultured for overnight. Cells were treated with Pha (500 nM) alone or Pha (500 nM) in combination with Ko143 (1 μ M) for 2 h. After 2-h incubation, the medium was removed and cells were washed twice with PBS. Cells treated with Pha alone were incubated with fresh culture medium, and cells treated with Pha in combination with Ko143 were incubated with fresh medium containing Ko143 (1 μ M). After incubation for 20 min at 37°C, the medium was removed and cells were lysed with 0.1% sodium dodecyl sulfate (SDS) in PBS. Cell lysates were centrifuged at 3,000 RCF for 10 min at room temperature. Supernatants were collected and Pha fluorescence was detected using Fluoromax-3 fluorescence spectrometer with excitation wavelength at 400 ± 2.5 nm and emission wavelength at 677 nm. Pha

fluorescence of cells treated with Pha in combination with Ko143 was normalized to that treated with Pha alone to indicate ABCG2 activity, a high percentage increase indicating a high ABCG2 activity.

Flow cytometry for PpIX measurement

PpIX was determined by flow cytometry as described (19). Briefly, cells were implanted in 60-mm dishes and allowed to grow to 70% confluency. Cells were incubated with complete RPMI 1640 medium containing no drug (control), ALA (1 mM) alone, Ko143 (1 μ M) alone, or ALA (1 mM) combined with Ko143 (1 μ M) for 4 h. After the incubation, cells were washed twice with PBS, trypsinized, and suspended in PBS. Cell suspensions were centrifuged at 200 RCF for 5 min, and cell pellets were resuspended in cold PBS. Cell fluorescence was measured with a FACScalibur flow cytometer (BD Biosciences) in the FL3 channel (488 nm excitation, 650 nm long-pass emission). About 20,000 cells were measured and recorded for each experiment.

Evaluation of small molecule inhibitors for ALA-PpIX enhancement

Effects of small molecule inhibitors on PpIX in cell lysates and medium were examined in Caki-2 cells. The inhibitors evaluated include lapatinib, gefitinib, sunitinib, vismodegib, vemurafenib, sorafenib, everolimus, alpelisib, palbociclib, BEZ-235, BKM-120, and PD-325901, which are all kinase inhibitors except vismodegib. Alpelisib, BKM-120 and BEZ-235 are gifts from Novartis and all other inhibitors were purchased from LC Laboratories (Woburn, MA). All inhibitors were dissolved in DMSO and sterilized using 0.22- μ m pore size filters. Cells were incubated with ALA (1 mM) alone, inhibitors (all at 1 μ M) alone, or ALA (1 mM) in combined with inhibitors (1 μ M) in complete RPMI 1640 medium for 4 h. Ko143 (1 μ M) was used as a positive control in the screening. The level of PpIX in cell lysates and medium after treatment was measured with spectrofluorometry as described above.

Statistical analysis

One-way or Two-way ANOVA test followed by multiple comparisons was used to determine the statistical difference. Person correlation analysis was used for correlation assessment. Statistical significance was accepted at $P < 0.05$.

RESULTS

The amount of PpIX in cell lysates and cell culture medium after 4-h incubation with ALA was measured in a panel of human RCC cell lines together with HK-2 cells, a non-tumor human kidney proximal tubular epithelial cell line. Both the cell lysate PpIX and total amount of PpIX, which includes PpIX retained in the cell lysate and PpIX released into the culture medium, were highly variable among RCC cells (Fig 1A). One-way ANOVA test indicates that the total amount of PpIX in three RCC cell lines (786-O, A-704, Caki-2) was significantly higher than that in HK-2 cells ($P < 0.001$). Two other RCC cell lines (769-P, Caki-1) had a similar total PpIX level as HK-2 cells ($P > 0.05$). When the amount of cell lysate PpIX was compared between RCC and HK-2 cells, only A-704 cells showed significantly higher PpIX level than HK-2 cells ($P < 0.05$).

FECH enzyme activity and ABCG2 transport activity were determined to assess possible correlation with ALA-PpIX level. FECH activity was highly heterogeneous in five RCC cell lines, three of which (769-P, A-704, Caki-2) exhibited significantly higher activity than HK-2 cells ($P < 0.001$, Fig 1B). The other two (786-O, Caki-1) showed the trend of increased FECH activity, albeit without statistical significance ($P > 0.05$). Three RCC cell lines (786-O, Caki-1, Caki-2) exhibited higher ABCG2 activity than HK-2 cells. However, only Caki-2 reached the statistical significance ($P < 0.05$, Fig 1C). Neither the cell lysate PpIX nor the total PpIX level was correlated with the FECH activity ($P > 0.05$) or ABCG2 activity ($P > 0.05$), as indicated by the Pearson correlation analysis.

To assess the effects of ABCG2 inhibition on ALA-PpIX level, cells were treated with ABCG2 inhibitor Ko143 and the amount of PpIX in cell lysates and medium was measured. Compared with ALA alone, Ko143 significantly increased cell lysate PpIX level in 786-O, Caki-1, Caki-2 and HK-2 cells ($P < 0.001$), but had no effects in 769-P and A-704 cells ($P > 0.05$, Fig 2A). Medium PpIX level was significantly reduced in 786-O, Caki-1 and Caki-2 cells ($P < 0.01$) after Ko143 treatment (Fig 2B). Medium PpIX was barely detectable in 769-P and HK-2 cells treated with ALA alone and not detectable after treatment with ALA in combination with Ko143. To indicate the cell response to Ko143 treatment, cell lysate PpIX level after ALA in combination with Ko143 was normalized to the level after ALA treatment alone (Fig 2C). Pearson correlation analysis suggests that the cell response to Ko143 was positively correlated with the ABCG2 activity ($P < 0.05$, Fig 2D). RCC cells with enhanced ABCG2 activity such as Caki-2 cells showed good response to Ko143, while cells with no or minimal activity such as 769-P and A-704 cells did not respond to the treatment. The ratio of cell lysate PpIX between tumor cells and non-tumor HK-2 cells was shown in Fig 2E. Ko143 significantly increased tumor to non-tumor fluorescence ratio in Caki-2 cells and had no effect in 786-O and Caki-1 cells. However, it significantly reduced the ratio in 769-P and A-704 cells.

To confirm this finding based on the spectrofluorometric measurements of PpIX, flow cytometry was used to determine cell fluorescence after treatment with ALA alone or in combination with Ko143. Similar to the spectrofluorometric results (Fig 2A), flow cytometer measurements also demonstrate that ALA in combination with Ko143 resulted in significantly higher cell fluorescence than ALA treatment alone in 786-O, Caki-1, Caki-2 and HK-2 cells ($P < 0.001$), but not in 769-P and A-704 cells (Fig 3A). Cell fluorescence after ALA in combination with Ko143 was normalized to the fluorescence after ALA treatment alone to indicate the cell response to Ko143 treatment (Fig 3B). Pearson correlation analysis indicates a strong positive correlation between the cell response to Ko143 and ABCG2 activity ($P < 0.05$, Fig 3C), confirming the spectrofluorometry results. Fig 3D shows the ratio of fluorescence between tumor cells and non-tumor HK-2 cells. Similar to Fig 2E, the ratio was significantly increased in Caki-2 cells but reduced in 769-P and A-704 cells after Ko143 treatment.

Since there is currently no ABCG2 inhibitor available for clinical application, we evaluated small molecule inhibitors for the enhancement of ALA-PpIX with the goal of possible drug repurposing. Caki-2 cell line was chosen for the evaluation because it had the highest ABCG2 activity among all RCC cell lines examined in this study. Effects of a dozen

inhibitors (mostly kinase inhibitors) on the level of PpIX in cell lysates and medium were evaluated and compared with ALA treatment alone (Ctrl, Fig 4). Six inhibitors were found to significantly increase cell lysate PpIX level (Fig 4A, 4B). Treatment with lapatinib, gefitinib and sunitinib resulted in cell lysate PpIX increase by 170%, 108% and 91%, respectively (Fig 4C). Notably, lapatinib induced the highest increase of cell lysate PpIX among all cell signaling inhibitors evaluated ($P < 0.001$), although it was still inferior to the positive control Ko143. Lapatinib in combination with ALA led to significantly lower PpIX level in the medium than ALA treatment alone ($P < 0.01$, Fig 4D, 4E). All inhibitors did not significantly change the total amount of PpIX, which includes PpIX in cell lysates and medium ($P > 0.05$).

DISCUSSION

ALA-mediated PpIX production was evaluated in human RCC cell lines and non-tumor HK-2 cells in this study. We measured PpIX in both cell lysates and cell culture medium to assess the intracellular PpIX level (in cell lysates) and total PpIX production (in cell lysates and medium). Intracellular PpIX is known to be critical for the cytotoxicity induced by ALA-PDT (20) and likely tumor fluorescence-guided resection as well. Both intracellular and total PpIX showed significant variation in RCC cell lines and, surprisingly, A-704 was the only one with significantly higher intracellular PpIX than non-tumor HK-2 cells among five RCC cell lines evaluated.

Because FECH-catalyzed PpIX bioconversion to heme and ABCG2-mediated PpIX efflux are two predominant biological processes for reducing intracellular PpIX, we determined the activity of FECH and ABCG2 and tried to correlate them with PpIX level. Identifying biological processes or biomarkers that have correlation with PpIX level will be important for patient selection for optimal treatment response. Similar to previous studies in different tumor cell lines (19, 21), we noticed that RCC cells with high FECH activity (769-P) or high ABCG2 activity (Caki-2) tended to have low intracellular PpIX. However, there was no significant correlation between the intracellular PpIX and FECH or ABCG2 activity across the RCC cell lines. No correlation was found for the total level of PpIX either. Our results indicate that neither FECH nor ABCG2 activity alone seemed to be able to predict PpIX level in RCC cells, suggesting that PpIX level is determined by a complex interaction between various biological parameters.

The finding that most of the PpIX produced by 786-O and Caki-2 cells was released into the medium prompted us to enhance intracellular PpIX with an ABCG2 inhibitor Ko143. As expected, Ko143 treatment greatly increased the intracellular PpIX level and reduced PpIX release into the medium, supporting the role of ABCG2 transporters in PpIX efflux. Although other mechanisms may be involved as well (22), our present results together with previous reports (10, 18, 23) demonstrate that ABCG2 plays an important role in PpIX efflux. Furthermore, both spectrofluorometric and flow cytometric measurements of PpIX demonstrate strong positive correlation between ABCG2 activity and the cell response to Ko143 across RCC cell lines, which suggests the use of ABCG2 activity as a biomarker for predicting therapeutic response to ABCG2 inhibitors for PpIX enhancement. We did not determine ABCG2 protein level and gene expression in the study because it is the

transporter function that really matters in PpIX efflux, and ABCG2 activity is known to not always correlate with its protein level or expression due to alterations in protein folding, membrane trafficking and degradation (24). Use of ABCG2 protein or expression data, which can be obtained from omics analysis of tissue samples, for therapeutic response prediction should be carried out with the precaution that they may not reliably indicate ABCG2 transport function.

The lack of clinical ABCG2 inhibitors for the enhancement of intracellular PpIX has simulated the repurposing of existing drugs particularly kinase inhibitors as potential ABCG2 inhibitors. Three kinase inhibitors have been examined so far. Imatinib was the first kinase inhibitor shown to increase ALA-PpIX in tumor cells (25). Another kinase inhibitor gefitinib was found to increase intracellular PpIX and cytotoxicity induced by ALA-PDT in human glioma cell lines (26). Genistein, a kinase inhibitor and isoflavone phytoestrogen, has recently been shown to increase ALA-PpIX in a lung cancer tumor model (27) and human glioma cells (28).

We evaluated more than a dozen small molecule inhibitors for its activity of enhancing intracellular PpIX in Caki-2 cells, which had the most robust ABCG2 among RCC cell lines tested. The inhibitors we chose target a variety of cell signaling pathways including human epidermal growth factor (HER) family receptor signaling (gefitinib, lapatinib), vascular endothelial growth factor receptor (VEGFR) signaling (sunitinib, sorafenib), phosphoinositide 3-kinases (PI3K) pathway (alpelisib, BKM-120, BEZ-235), mammalian target of rapamycin (mTOR) signaling (everolimus, BEZ-235), mitogen-activated protein kinase (MAPK) pathway (vemurafenib, PD-325901), cell cycle signaling (palbociclib) and hedgehog signaling pathway (vismodegib). Except BKM-120, BEZ-235 and PD-325901, all are clinically approved drugs including sunitinib, sorafenib and everolimus that are commonly used for RCC treatment. Our evaluation revealed six clinically available inhibitors that were able to significantly enhance the intracellular PpIX in Caki-2 cells, which includes lapatinib, gefitinib, sunitinib, vismodegib, vemurafenib, and sorafenib. We found that lapatinib was most efficacious for enhancing intracellular PpIX and reducing PpIX efflux. This finding is in good agreement with a recent study in ABCG2-overexpressing cells showing that lapatinib has the most potent inhibitory effect on ABCG2 activity among all ABCG2-interacting kinase inhibitors evaluated (29).

The mechanism involved in ABCG2 inhibition by ABCG2-interacting kinase inhibitors such as imatinib, gefitinib and lapatinib has been extensively investigated. It is now known that these kinase inhibitors, being ABCG2 substrates on their own, share common binding sites such as phenylalanine 439 in ABCG2 molecules with other ABCG2 substrates, thus functioning as competitive inhibitors of substrates (29, 30). These ABCG2-interacting kinase inhibitors have been shown to increase the intracellular accumulation of other ABCG2 substrates including chemotherapy drugs (30, 31). In addition, some inhibitors including imatinib (32) and vismodegib (33, 34) are able to inhibit ABCG2 activity by inducing the downregulation of ABCG2 expression. In contrast to ABCG2-interacting kinase inhibitors that suppress ABCG2 activity, genistein is known to activate ABCG2 activity by upregulating ABCG2 expression (35). The mechanism for its enhancement of

ALA-PpIX was shown due to the increased expression of enzymes involved in PpIX biosynthesis (27).

Our study with RCC cell lines implies that the application of ALA and its enhancement strategies for PpIX fluorescence-guided tumor resection needs to be personalized. Most RCC cell lines we evaluated did not display enhanced ALA-PpIX fluorescence compared with non-tumor HK-2 cells and did not show the same response to ABCG2 inhibitor-induced PpIX enhancement. Given the known genetic complexity and diversity of RCC (36), the same heterogeneity might be expected in RCC patients as well. Thus, biomarkers that can predict enhanced tumor PpIX fluorescence and tumor response to PpIX enhancement treatments are needed for optimal treatment outcomes. Although ABCG2 and FECH activity alone could not predict tumor cell PpIX level, tumor cells with high ABCG2 activity were more likely to show good response to ABCG2 inhibitors for PpIX enhancement. Therefore, tumor ABCG2 activity may serve as a biomarker for the enhancement of tumor PpIX fluorescence with ABCG2 inhibitors. As showed in the present study, only tumor cells (Caki-2) with high ABCG2 activity benefited from this enhancement strategy, resulting in increased PpIX ratio between tumor and non-tumor cells. For tumor cells with low or little ABCG2 activity (A-704), inhibition of ABCG2 led to no enhancement of PpIX fluorescence and even reduced the tumor to non-tumor PpIX ratio.

In summary, our study indicates that ALA-PpIX fluorescence was highly variable among RCC cell lines. Although FECH or ABCG2 activity alone did not correlate with PpIX level, ABCG2 activity was able to predict tumor cell response to ABCG2 inhibition for PpIX enhancement. The identification of several clinical drugs as effective ABCG2 inhibitors for the enhancement of ALA-PpIX, lapatinib in particular, may lead to the use of these inhibitors in combination with ALA for guiding the surgery of RCC and other tumors with elevated ABCG2 activity. Future efforts will focus on evaluating the efficacy of this combination treatment in tumor models.

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Figure 1.

Determination of ALA-PpIX production (A), FECH enzyme activity (B) and ABCG2 transport activity (C) in human RCC cell lines and non-tumor human kidney HK-2 cells. Cells were treated with 1 mM ALA for 4 h and the amount of PpIX in cell lysates and medium was measured. FECH enzyme and ABCG2 transport activities were determined as described in the *Materials & Methods* section. Data are presented as mean ± SD from at least 3 independent experiments. * $P < 0.05$, *** $P < 0.001$, compared with the corresponding HK-2 cells by One-way ANOVA test.

Figure 2.

Effects of ABCG2 inhibitor Ko143 on ALA-PpIX determined by spectrofluorometry. Human RCC cell lines and HK-2 cells were treated with ALA (1 mM) alone or in combination with Ko143 (1 μ M) for 4 h, and the amount of PpIX in cell lysates (A) and medium (B) was measured by spectrofluorometry. PpIX was not detectable in the medium of 769-P and HK-2 cells after combination treatments. Cell lysate PpIX level after ALA in combination with Ko143 was normalized to the level after ALA treatment alone to indicate the cell response to Ko143 (C). Pearson correlation analysis showing strong positive correlation between ABCG2 activity and the cell response to Ko143 (D). The ratio of cell lysate PpIX between tumor cells and non-tumor HK-2 cells (E). Data are presented as mean $\pm$ SD from at least 3 independent experiments. ** $P < 0.01$, *** $P < 0.001$, compared with the corresponding ALA alone by Two-way ANOVA test.

Figure 3.

Effects of ABCG2 inhibitor Ko143 on ALA-PpIX determined by flow cytometry. Human RCC cell lines and HK-2 cells were treated with ALA (1 mM) alone or in combination with Ko143 (1 μ M) for 4 h, and the average cell fluorescence was measured by flow cytometry (A). Cell fluorescence after ALA in combination with Ko143 was normalized to that after ALA treatment alone to indicate the cell response to Ko143 (B). Pearson correlation analysis showing strong positive correlation between ABCG2 activity and the cell response to Ko143 (C). The ratio of fluorescence between tumor cells and non-tumor HK-2 cells (D). Data are presented as mean $\pm$ SD from at least 3 independent experiments. *** $P < 0.001$, compared with the corresponding ALA alone by Two-way ANOVA test.

Figure 4.

Effects of small molecule cell signaling inhibitors on ALA-PpIX in Caki-2 cells. Cells were treated with ALA (1 mM) alone (Ctrl) or in combination with inhibitors (all at 1 μ M including the positive control Ko143) for 4 h, and the amount of PpIX in cell lysates and medium was measured by spectrofluorometry. **(A)** Fluorescence spectra of cell lysates after ALA alone or in combination with selected inhibitors. **(B)** The amount of PpIX in cell lysates after ALA alone or in combination with inhibitors. **(C)** Cell lysate PpIX level after treatment with ALA in combination with inhibitors was normalized to that after ALA alone to indicate the effects of inhibitors on ALA-PpIX enhancement. **(D)** Fluorescence spectra of cell culture medium after ALA alone or in combination with selected inhibitors. **(E)** The amount of PpIX in medium after ALA alone or in combination with inhibitors. **(F)** The total amount of PpIX (PpIX in cell lysates and medium) after ALA alone or in combination with inhibitors. Data are presented as mean $\pm$ SD from at least 3 independent experiments. ** $P < 0.01$, *** $P < 0.001$, compared with ALA alone (Ctrl) by One-way ANOVA test.