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Quinolone compounds enhance δ-aminolevulinic acid-induced accumulation of protoporphyrin IX and photosensitivity of tumour cells

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Exogenous δ-aminolevulinic acid (ALA)-induced photodynamic therapy (PDT) has been used in the treatment of cancer. To obtain a high efficacy of ALA-PDT, we have screened various chemicals affecting ALA-induced accumulation of protoporphyrin in cancerous cells. When HeLa cells were treated with quinolone chemicals including enoxacin, ciprofloxacin or norfloxacin, the ALA-induced photodamage accompanied by the accumulation of protoporphyrin was stronger than that with ALA alone. Thus, quinolone compounds such as enoxacin, ciprofloxacin and norfloxacin enhanced ALA-induced photodamage. The increased ALA-induced photodamage in enoxacin-treated HeLa cells was decreased by haemin or ferric-nitrilotriacetate (Fe-NTA), suggesting that an increase in iron supply cancels the accumulation of protoporphyrin. On the other hand, the treatment of the cells with ALA plus an inhibitor of haem oxygenase, Sn-protoporphyrin, led to an increase in the photodamage and the accumulation of protoporphyrin compared with those upon treatment with ALA alone, indicating that the cessation of recycling of iron from haem augments the accumulation. The use of quinolones plus Sn-protoporphyrin strongly enhances ALA-induced photodamage. To examine the mechanisms involved in the increased accumulation of protoporphyrin, we incubated ferric chloride with an equivalent amount of quinolones. Iron–quinolone complexes with visible colours with a maximum at 450 nm were formed. The levels of iron-metabolizing proteins in enoxacin- or ciprofloxacin-treated cells changed, indicating that quinolones decrease iron utilization for haem biosynthesis. Hence, we now propose that the use of quinolones in combination with ALA may be an extremely effective approach for the treatment modalities for PDT of various tumour tissues in clinical practice.

Keywords: δ-aminolevulinic acid (ALA)/ciprofloxacin/enoxacin/PDT/photodamage/protoporphyrin/quinolones.

Abbreviations: ALA, δ-aminolevulinic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Fe-NTA, ferric-nitrilotriacetate; HO, haem oxygenase; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; PDT, photodynamic therapy; PVDF, poly(vinylidene difluoride); SDS–PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis.

Photodynamic therapy (PDT) for cancer patients is widely used to treat non-melanoma skin tumours and preneoplastic skin lesions. PDT involves the activation of photosensitizer, which causes the release of singlet oxygen and other reactive oxygen species upon exposure to light, resulting in photodamage and subsequent tissue destruction (*1*). PDT is performed with ALA, which is converted to the active photosensitizer protoporphyrin IX within cells (*2*). In tumour cells, via the haem biosynthesis pathway, protoporphyrin is synthesized from a large amount of exogenous ALA and accumulates in a specific manner. The application of ALA following PDT treatment has been used in the treatment of skin diseases and has advantages over systemic administration in that the entire body does not face sensitization. ALA-induced PDT has been successfully applied in various medical fields including urology, gastroenterology and dermatology (*3*, *4*). Although there are reports that ALA-induced PDT can also be used as a fluorescence detection marker for the photodiagnosis of tumours (*5*, *6*), the mechanisms involved in the specific accumulation of protoporphyrin in cancerous tissues have not been clearly demonstrated. We previously reported that protoporphyrin accumulates owing to the limited capacity for ferrochelatase reaction (*7*, *8*) where the enzyme catalyses the insertion of ferrous ions into protoporphyrin IX to form protohaem. Additionally, we also reported the increase in the uptake of ALA by cancerous cells (*7*).

The death rate from non-melanoma skin cancer is reduced compared with that from other kinds of malignancies, but both mortality and incidence are rising in tropical regions and owing to the thinner ozone layer (*9*). Nonmelanoma skin cancer is classified into two general groups. One is basal cell carcinoma and the

other is squamous cell carcinoma (*10*). Although many studies on PDT carried out over the past decade show efficiency in treating non-melanoma skin cancer and preneoplastic skin lesion, the results of ALA-PDT in the treatment of them appear to be inadequate (*11*). Since ALA-PDT alone seems to be relatively insufficient for the treatment of non-melanoma skin cancer and preneoplastic skin lesion, advanced treatment options are required to significantly improve the therapeutic effectiveness of ALA-PDT.

Quinolone chemicals are widely used as synthetic anti-bacterial agents to treat respiratory tract infection (*12*). They have a broad anti-bacterial spectrum of activity against Gram-positive and -negative bacteria. Anti-bacterial activities of quinolones are involved in their inhibitory activities against DNA gyrase and topoisomerase IV (*13*, *14*). Both enzymes are members of the type II topoisomerase family that regulates bacterial DNA topology by passing a DNA double helix through another. In addition, the quinolones are used not only as anti-bacterial agents but also as an option for chemotherapy (*15*). During a study on enhancement of the phototoxic effect of ALA-induced photodamage, we tried to improve the sensitivity of ALA-PDT in cancerous cells by screening the increase in the accumulation of protoporphyrin, using many kinds of additional agents. We here report that quinolone agents including enoxacin and ciprofloxacin can enhance the accumulation of protoporphyrin as well as the photodamage in ALA-treated cancerous cells, and the importance of the recycling of iron from haem in cancerous cells upon ALA-induced photodamage is also shown.

Materials and Methods

Materials

Protoporphyrin IX and Sn-protoporphyrin were purchased from Frontier Scientific Co. (Logan, UT, USA). Enoxacin and lomefloxacin were products of Tokyo Kasei Chemicals (Tokyo, Japan) and Merck Biosciences (Darmstadt, Germany), respectively. Norfloxacin, and ciprofloxacin were from WAKO Chemicals (Tokyo). The antibodies for ferrochelatase and actin used were as previously described (*7*, *16*). Anti-transferrin receptor-1 and ferritin were products of Santa Cruz Co. (Santa Cruz, CA, USA) and DAKO Ltd. (Glostrup, Denmark), respectively. All other chemicals used were of analytical grade.

Cell cultures

Human epithelial cervical cancer HeLa and epidermoid carcinoma A431 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% foetal calf serum (FCS) and antibiotics. The cells (1×10^5) in a 1.5-cm-diameter dish were then incubated in the absence or presence of ALA (0.5–1 mM) for 16 h before being exposed to light. Treatment of cells with quinolones was performed for 16–24 h, followed by the addition of ALA to the cell culture (*7*, *8*).

Exposure of the cells to light

The cells were incubated with a specific concentration of ALA for 8–16 h, and 1.0 ml of fresh drug-free medium was then added. Irradiation with visible light was carried out under sterile conditions, using a fluorescence lamp, in a CO_2 incubator. The light was filtered through a glass plate to omit UV light and applied from the bottom of the plate to achieve uniform delivery to the entire plate. The increase of the temperature was confirmed to be $<2^{\circ}C$ by using a thermo-couple device during exposure to light. The power was calibrated with a power metre, and the period of irradiation was adjusted to obtain fluences of 0.54 J/cm^2. Cell viability was measured by trypan-blue exclusion after trypsinization. The cell activity was also examined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Each experiment was carried out in triplicate or quadruplicate. Controls were as follows: (i) cells exposed to ALA but not exposed to light (dark cytotoxicity), (ii) cells untreated with ALA but exposed to light and (iii) cells exposed to neither ALA nor light. Cell viability (cell survival) was expressed as a percentage of control cells. Porphyrins were extracted from the cells with 96% ethanol containing 0.5 M HCl (*7*). The amount of protoporphyrin was determined by fluorescence spectrophotometry, as previously described (*7*, *17*).

Immunoblotting

The lysates from HeLa cells were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). Immunoblotting was carried out with antibodies for ferrochelatase, ferritin and transferrin receptor-1 as the primary antibodies (*8*).

Absorption spectra

Ferric chloride (100 μM) was incubated with quinolones (100 μM). Absorption spectra of iron–quinolone complexes were measured using a JASCO V-530 spectrophotometer (Tokyo).

Statistics

Two-sample *t*-tests were used to compare the amount of protoporphyrin and photosensitivity between treated and untreated controls. Comparison of data from different treatment groups was conducted using 1-way analysis of variance (ANOVA). Analysis was performed using Microsoft Excel 2003 software.

Results

Quinolones enhances ALA-induced photodamage

We (*7*, *8*) have previously shown that the ALA-induced accumulation of protoporphyrin is inversely related to the level of ferrochelatase. To obtain a high efficacy of ALA-PDT, the various chemicals affecting the ALA-induced accumulation of protoporphyrin were screened using HeLa cells. The fluorescence pattern with ethanol extracts of the cells treated with 1 mM ALA for 16 h showed a maximum peak at 637 nm with excitation at 400 nm, which was consistent with that of standard protoporphyrin. First, effects of quinolone chemicals, including enoxacin and ciprofloxacin, on the ALA-induced accumulation of protoporphyrin were examined. As shown in Fig. 1A, enoxacin, ciprofloxacin and norfloxacin increased the accumulation of protoporphyrin by incubation of HeLa cells with ALA, in a dose-dependent manner. In the absence of ALA, no accumulation of protoporphyrin in enoxacin- or ciprofloxacin-treated cells was observed. When pre-incubation of cells with enoxacin was carried out, the accumulation increased by 1.4- and 1.9-fold with 16 and 24 h incubation, respectively (Fig. 1B). The pre-treatment with ciprofloxacin, norfloxacin or lomefloxacin was also effective for the accumulation. The growth of cells did not change by the treatment with 100 μM quinolone compounds for 48 h (data not shown). When a concentration of >200 μM these quinolones was used, the accumulation of protoporphyrin gradually declined due to cytotoxicity. To evaluate the effect of enoxacin on ALA-induced photosensitivity of HeLa cells, they were treated with 1 mM ALA for 16 h and then exposed to visible light. The viability of the cells pre-treated with ALA plus 50 and 100 μM

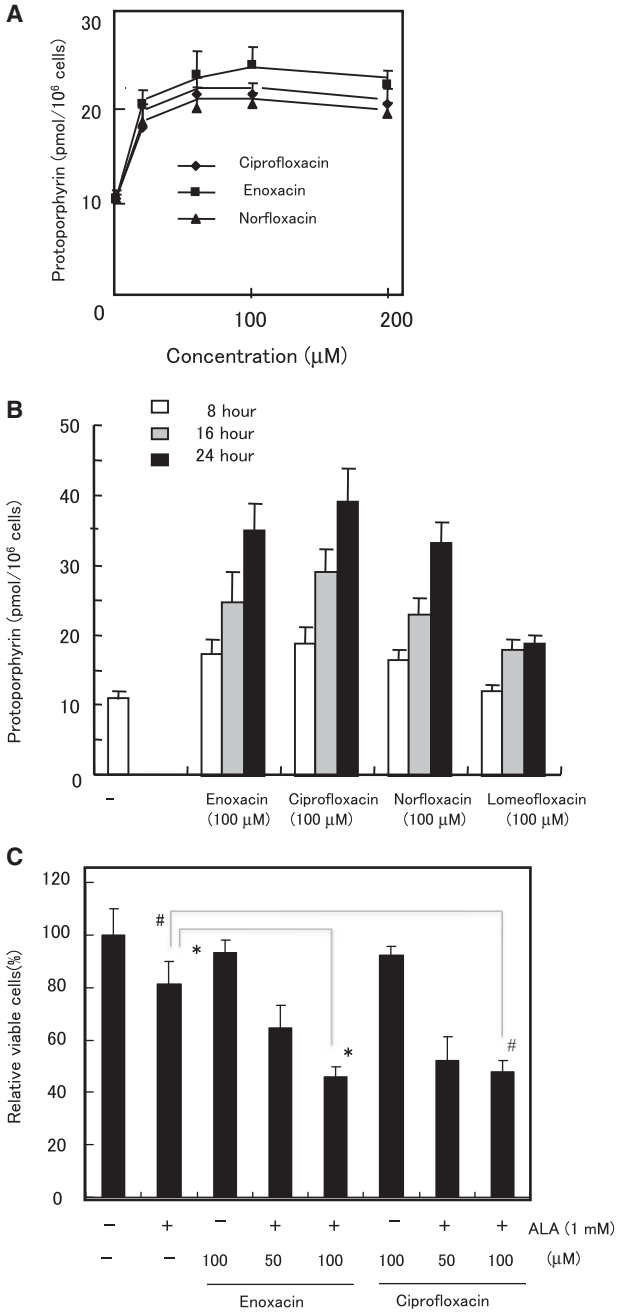

Fig. 1 The increase by quinolone compounds of the accumulation of protoporphyrin and photosensitivity in ALA-treated HeLa cells. (A) Effect of quinolones on the ALA-induced accumulation of protoporphyrin. HeLa cells were incubated with 1 mM ALA plus the indicated concentration of enoxacin, ciprofloxacin or norfloxacin for 16 h. Porphyrin was extracted from the cells and measured using a fluorospectrophotometer. (B) Effect of pre-treatment. HeLa cells were pre-treated with quinolone compounds (100 μM) for the indicated period, and changed to a fresh medium with 1 mM ALA, followed by incubation for 16 h. The concentration of protoporphyrin was determined. (C) Effect of quinolones on ALA-induced photodamage. The cells pre-treated with the indicated concentration of enoxacin or ciprofloxacin for 24 h were incubated without or with 1 mM ALA for 16 h, and then exposed to visible light. Light dose = 0.54 J/cm^2. After trypsinization, the living cells were counted. More than 500 living cells without any treatment were counted. The data are expressed as the average ± SEM for three to four experiments. Two-sample *t*-test was carried out: $^*P < 0.005$ and $^\#P < 0.005$ versus ALA alone.

enoxacin decreased to 62 and 43%, respectively, which were significantly different compared with that with ALA alone (78%) (Fig. 1C). Ciprofloxacin was also effective for the photodamage. When the cells treated with enoxacin or ciprofloxacin alone were exposed to light, virtually no cell death was observed. No cell death of ALA-treated cells was also observed without exposure to light. In separate experiments, when A431 cells were pre-treated with 100 μM enoxacin, ciprofloxacin or norfloxacin for 24 h, the ALA-induced photodamage was stronger than that with ALA alone (Fig. 2A). In parallel, the ALA-induced accumulation of protoporphyrin in quinolone-treated cells was higher than that in control cells (Fig. 2B). Thus, quinolone compounds such as enoxacin, ciprofloxacin and norfloxacin facilitate ALA-induced photodamage.

The inhibitory effect of haemin and Fe-NTA on ALA-PDT

Figure 3A shows the decrease of the ALA-induced accumulation of protoporphyrin in haemin-treated HeLa cells, suggesting that iron from haem can be reused as the substrate of the ferrochelatase reaction. The addition of Fe-NTA also led to a decrease of the

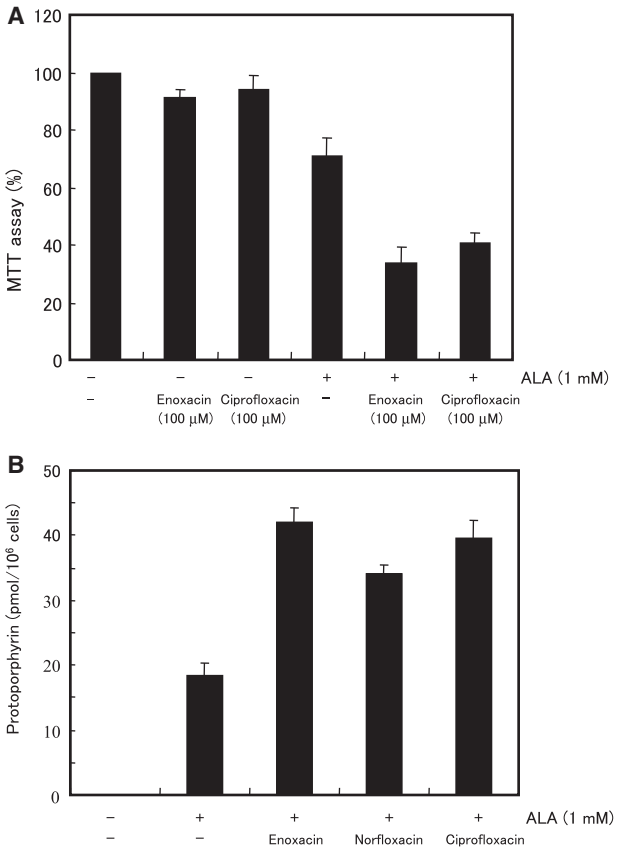

Fig. 2 The increase by enoxacin, norfloxacin or ciprofloxacin of the accumulation of protoporphyrin and photodamage in ALA-treated A431 cells. (A) Treatment of A431 cells with indicated quinolone chemicals (100 μM), in combination with 1 mM ALA. The cells as above were irradiated, and survival of the cells was analysed by the MTT assay. The data are expressed as the average ± SEM for at least three independent experiments. (B) The concentration of protoporphyrin in the cells was determined, similar to those shown in the legend of Fig. 1.

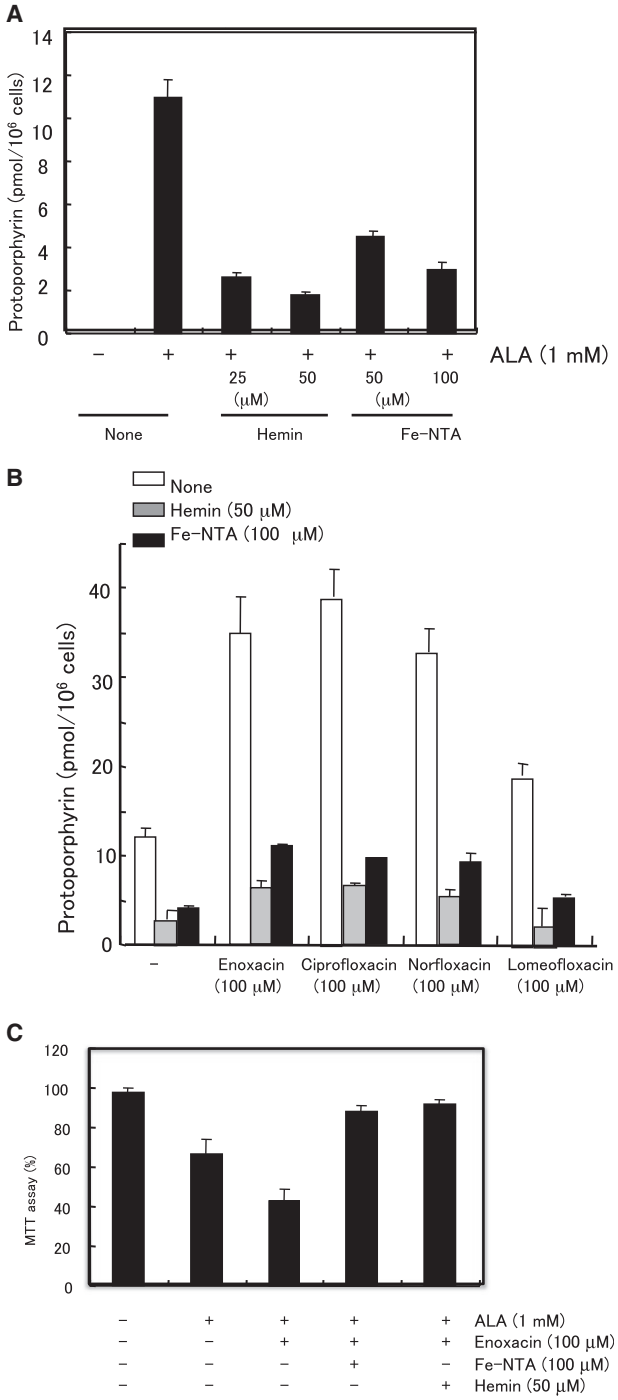

Fig. 3 Effect of haemin and Fe-NTA on ALA-induced accumulation of protoporphyrin and photodamage. (A) The inhibitory effect of haemin and Fe-NTA on the ALA-induced accumulation of protoporphyrin. HeLa cells were incubated with 1 mM ALA plus the indicated concentration of haemin and Fe-NTA for 16 h. Porphyrin was extracted from the cells and measured using a fluorospectrophotometer. (B) Effect of haemin and Fe-NTA on the quinolone-dependent increased accumulation of protoporphyrin. HeLa cells were pre-treated with the indicated concentration of quinolones for 24 h, and then incubated with 1 mM ALA, in combination with haemin or Fe-NTA for 16 h. The accumulated protoporphyrin was measured. (C) Effect of haemin, Fe-NTA and enoxacin on ALA-induced photodamage. The cells pre-treated with enoxacin plus haemin or Fe-NTA as above were irradiated, and survival of the cells was analysed by the MTT assay.

accumulation of protoporphyrin. These results suggest that iron supply for the ferrochelatase reaction is limited in cancerous cells. Then, we examined the effect of haemin or Fe-NTA on the increased accumulation of protoporphyrin by quinolones. As shown in Fig. 3B, enoxacin-, ciprofloxacin-, norfloxacin and lomefloxacin-dependent increases in the accumulation of protoporphyrin were cancelled by the treatment with haemin or Fe-NTA. To evaluate the inhibitory effect of haemin on photosensitivity, the cells treated with enoxacin and haemin were exposed to light. The ALA-induced photodamage in enoxacin-pre-treated HeLa cells was decreased by 50 μM haemin (Fig. 3C). Fe-NTA (100 μM) also reduced the rate of cell death.

Synergistic effect of Sn-protoporphyrin and quinolones on ALA-induced photodamage

We next examined the effect of Sn-protoporphyrin, an inhibitor of haem oxygenase (HO), on ALA-induced accumulation of protoporphyrin. The accumulation was markedly increased in 20 μM Sn-protoporphyrin-treated A431 cells, while the increase was cancelled by co-incubation with 100 μM Fe-NTA and 20 μM Sn-protoporphyrin (Fig. 4A). The addition of 50 μM haemin or 50 μg/ml myoglobin diminished the accumulation in Sn-protoporphyrin-treated cells (Fig. 4A). These results indicated that degradation of haem by HO and subsequent utilization of the released iron for the ferrochelatase reaction contributes to the reduction of the accumulation of protoporphyrin. The photodamage was then evaluated using Sn-protoporphyrin-treated cells. As shown in Fig. 4B, the cell death of Sn-protoporphyrin- and ALA-treated cells was severer than that of ALA-treated cells. Treatment with haemin or Fe-NTA in combination with Sn-protoporphyrin resulted in less photosensitivity. Conversely, pre-treatment of A431 with enoxacin or ciprofloxacin, followed by the treatment with Sn-protoporphyrin and ALA for 16 h led to a marked increase in the photodamage and accumulation of protoporphyrin, as compared with those upon pre-treatment with quinolones alone or treatment with Sn-protoporphyrin alone (Fig. 4C and D). Thus, the use of quinolones plus Sn-protoporphyrin strongly enhances ALA-induced photodamage.

Iron-chelating activity of quinolones

We finally examined the mechanisms involved in the increase in the ALA-induced accumulation of protoporphyrin by quinolones. Figure 5A shows absorption spectra of the iron–quinolone complexes formed by incubation of ferric chloride with an equivalent amount of enoxacin, ciprofloxacin and norfloxacin. Iron–quinolone complexes with visible colours with a maximum at 450 nm were formed, indicating that quinolones function as iron-chelators (*18*). Therefore, to examine changes of cellular metabolism upon quinolone treatments, the levels of iron-metabolizing proteins in enoxacin- or ciprofloxacin-treated cells were analysed by immunoblotting. The level of transferrin receptor-1 in enoxacin- or ciprofloxacin-treated HeLa cells increased compared with that of untreated cells, while ferritin in quinolone-treated cells slightly

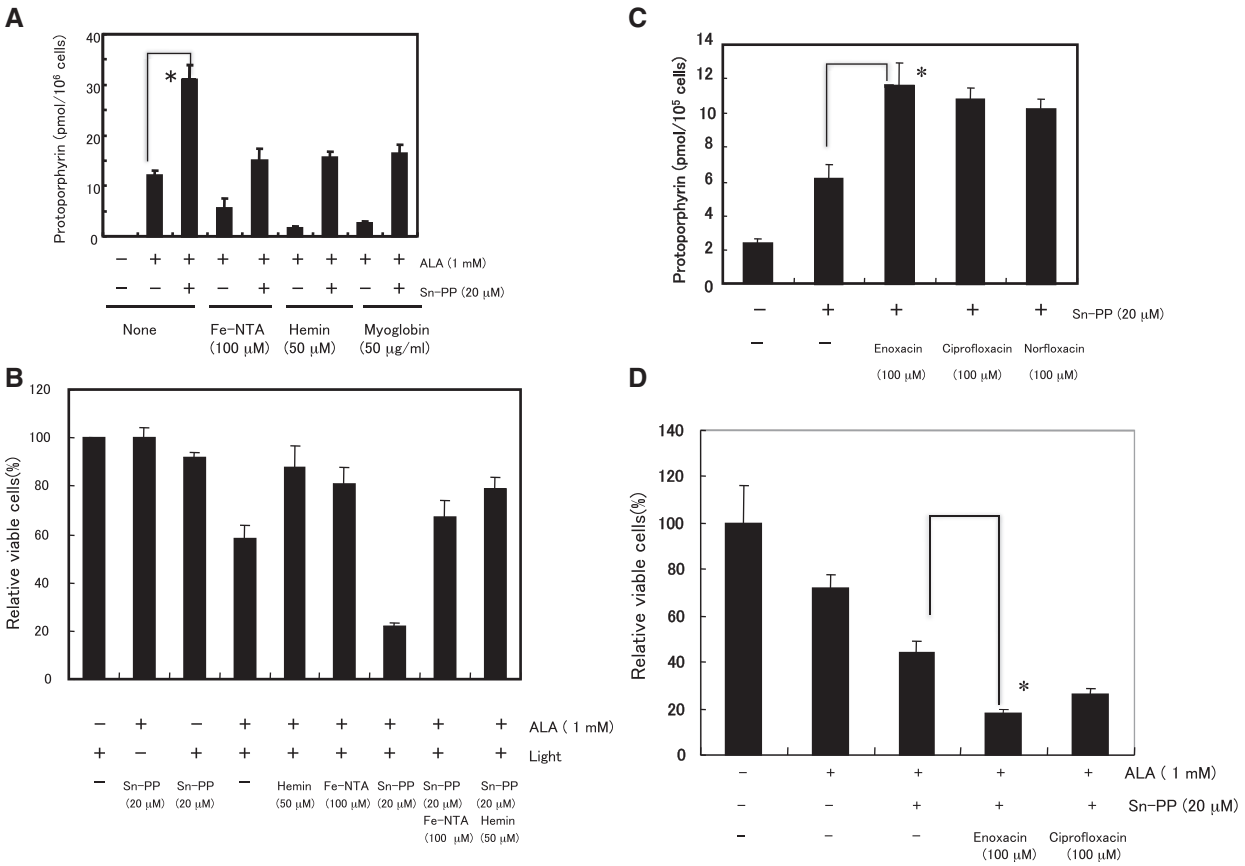

Fig. 4 Effect of Sn-protoporphyrin and quinolone chemicals on ALA-induced accumulation of protoporphyrin and photodamage. (A) Effect of Sn-protoporphyrin on the accumulation of protoporphyrin. A431 cells were incubated with 1 mM ALA in the absence or presence of 20 μM Sn-protoporphyrin (Sn-PP) for 16 h. The indicated cells were simultaneously incubated without or with Fe-NTA, haemin or myoglobin. The concentration of protoporphyrin in the cells was determined. The data are expressed as the average ± SEM for at least three independent experiments. $*P<0.01$. (B) The photosensitivity of the cells. The cells treated as above were exposed to light and the living cells were determined by counting living cells, using trypane blue. More than 500 living cells were counted in control. (C) Effect of quinolones and Sn-protoporphyrin on the accumulation of protoporphyrin. A431 cells were pre-treated with 100 μM enoxacin, 100 μM ciprofloxacin or 100 μM norfloxacin for 24 h, and were incubated with 1 mM ALA in the absence or presence of 20 μM Sn-protoporphyrin (Sn-PP) for 16 h. Protoporphyrin in the cells was examined. The data are expressed as the average ±SEM for three to four independent experiments. $*P<0.005$. (D) The photosensitivity of the cells. The living cells upon exposure to light were measured by the trypane-blue exclusion method. $*P<0.01$.

decreased (Fig. 5B). Since the expression of these proteins changes in a manner dependent on the intracellular level of iron (*19*), quinolones decrease iron utilization in cells. In contrast, virtually no change in the level of ferrochelatase in enoxacin-treated cells was observed although the expression of ferrochelatase was low by the treatment with iron-chelators (*20*), indicating that iron chelation with enoxacin or ciprofloxacin was weak.

Discussion

Since cancer development and progression are highly complex, it is evident that often no single therapeutic modality can be curative. Understanding the cellular and molecular events contributing to PDT-induced apoptosis and recovery from cell death leads to the development of more sophisticated approaches to drug design and therapy. A new molecular-based therapeutics including multiple regimens is more likely to eradicate malignant tissues. The present study first demonstrated that anti-bacterial drugs, quinolones, could enhance the ALA-induced photosensitivity of two kinds of human cancer cells. The effect of quinolones was also seen with colon cancer Colo201 and breast cancer MCF-7 cells. Results based on chemical extraction of porphyrin from cells confirmed the increased production of protoporphyrin upon treatment with quinolones including enoxacin, norfloxacin, ciprofloxacin and lomefloxacin, followed by incubation with ALA. In the absence of exogenously added ALA, neither photodamage nor the accumulation of protoporphyrin was observed even with quinolone-treated cells. The concentration of quinolones up to 100 μM used in this study neither affected cell growth nor showed cytotoxicity. Thus, the treatment of tumour cells with quinolones can enhance efficacy of ALA-induced photodamage.

We (*7*) previously reported that desferrioxamine, an iron-chelator, markedly enhanced ALA-induced photosensitivity *in vitro*. Quinolones act as chelating agents with metal ions such as calcium and magnesium (*21*, *22*). We showed the formation of enoxacin-, norfloxacin- and ciprofloxacin-iron complexes by determining absorption spectra (Fig. 5A), indicating that chelation of iron with quinolones occurred. This

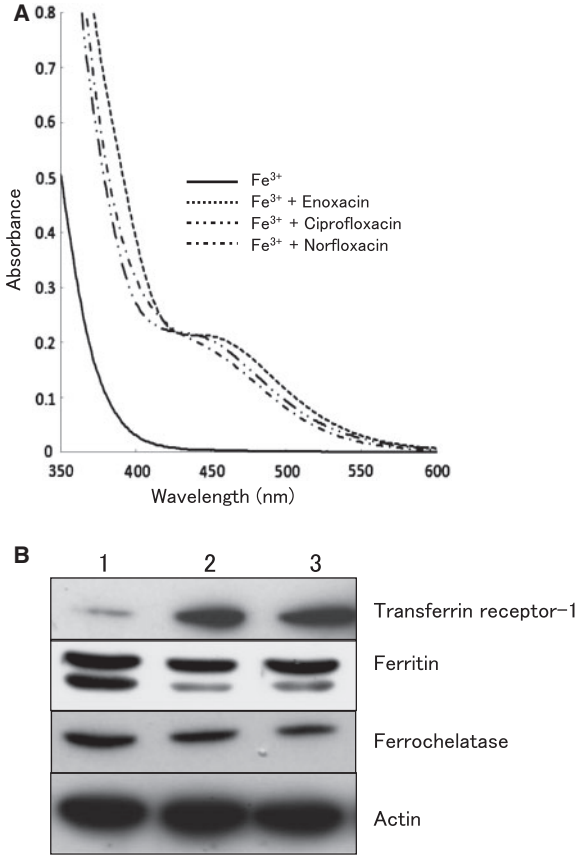

Fig. 5 Quinolone chemicals chelate with iron and affect the expression of iron-metabolizing proteins. (A) Spectroscopic scan of quinolone chemicals complexed with ferric ion. Ferric chloride (100 μM) was incubated without or with 100 μM enoxacin, 100 μM ciprofloxacin or 100 μM norfloxacin in phosphate-buffered saline at room temperature for 5 min. Absorption spectra of the formed iron–quinolone complexes were measured. Lower scan is a comparison of ferric chloride without quinolones. (B) Immunoblot analysis of transferrin receptor-1, ferritin and ferrochelatase in enoxacin- or ciprofloxacin-treated HeLa cells. The cells were treated without (lane 1) or with 100 μM enoxacin (lane 2) or 100 μM ciprofloxacin (lane 3) for 24 h, washed twice with phosphate-buffered saline, and then lysed. The cellular proteins were analysed by SDS-PAGE, and immunoblotting was performed using antibodies for transferrin receptor-1, ferritin, ferrochelatase and actin, as the primary antibodies.

observation was similar to that seen in case of desferrioxamine-iron complex (*23*). Immunoblot analysis revealed that the expression of transferrin receptor-1 was high and that of ferritin was low in enoxacin- and ciprofloxacin-treated cells compared with those of untreated cells. On the Basis of the fact that the alteration of expressions of both proteins occurs in a manner dependent on the level of intracellular iron (*19*), the treatment of cells with quinolones leads to iron deficiency. We (*20*) previously found that ferrochelatase is an iron-sulphur-containing enzyme and its expression was markedly decreased in iron-deficient cells. The decrease of the level of ferrochelatase was essential for ALA-PDT (*7*, *8*). The present data showed that the expression of ferrochelatase in enoxacin- and ciprofloxacin-treated cells was basically unchanged. Moreover, neither enoxacin nor ciprofloxacin inhibited iron-chelating ferrochelatase activity (Y. Ohgari & S. Taketani, unpublished results), suggesting that the iron deficiency in cells by the treatment with quinolones can be weak. Therefore, since the utilization of iron for ferrochelatase reaction *in vivo* is liable to drop by the treatment with quinolones, it is possible that the accumulation of protoporphyrin is, in contrast, increased.

We (*7*) previously reported that the low level of ferrochelatase in tumour cells could contribute to the ALA-induced accumulation of protoporphyrin and subsequent photodamage *in vitro*. The production of NO increased the ALA-induced photodamage by decreasing the levels of mitochondrial iron-containing enzymes including ferrochelatase and NADH dehydrogenase (*8*). Furthermore, the irradiation of epidermal cells led to a decrease of the level of ferrochelatase, which is mediated by the generation of reactive oxygen species (*24*). Thus, the ferrochelatase reaction is deeply related to mitochondrial functions. The present study showed that the level of ferrochelatase in enoxacin- or ciprofloxacin-treated cells was not decreased since these chemicals showed low cytotoxicity and caused partial iron deficiency. However, it is possible that quinolones target some unidentified proteins in mitochondria, which are related to iron metabolism, and then the utilization of iron to haem biosynthesis can be down-regulated.

In addition of the enhancement of *in vitro* ALA-induced photodamage by quinolones, we showed that Sn-protoporphyrin, an inhibitor of HO (*25*), increased the ALA-induced accumulation of protoporphyrin and photosensitivity. In humans, two isoforms of HO have been characterized: a constitutive expressed form, HO-2, and an inducible form, HO-1. The reduced expression of HO-1 mRNA by siRNA increased cell death upon ALA-PDT (*26*). Therefore, the decrease of HO function can contribute to the ALA-induced accumulation of protoporphyrin. We have showed that HO-1 is markedly induced not only by agents and chemicals that produce oxidative stress involving the generation of reactive oxygen species but also by the substrate haem (*27*, *28*). Then, through ALA-induced photodamage, HO-1 in ALA-treated cells was induced in time- and dose-dependent manners, and the induction of HO-1 was seen in the protoporphyrin-accumulated cells (*7*). It is considered that uncommitted haem in the cells is very dangerous for the maintenance of living systems, and reutilization of iron, including degradation of the haem, catalysed by HO, is essential for the homeostasis of iron in cells (*19*). Then, excess haem produced from ALA may induce HO-1. It was also considered possible that the accumulated protoporphyrin may generate reactive oxygen species via autoxidation (*29*), which leads to the induction of HO-1. This possibility was ruled out by the observations that the increased accumulation of protoporphyrin by Sn-protoporphyrin was cancelled by the exogenously added haemin or Fe-NTA. On the other hand, on the basis of the fact that HO degrades haem, producing iron, CO and biliverdin (*20*), the supply of iron for its reutilization is stopped by the inhibition of the HO reaction with Sn-protoporphyrin, leading to the increase in the

production of protoporphyrin. The present data revealed that enoxacin and Sn-protoporphyrin in combination exhibited a synergistic effect on ALA-induced accumulation of protoporphyrin as well as photodamage. Not only the decrease of reutilization of iron by HO but also the decrease of the utilization of mitochondrial iron for haem biosynthesis can further promote ALA-PDT. Nevertheless, it is still possible that there are other effects of quinolones on cellular metabolism.

Quinolones target several kinds of bacterial topoisomerases, resulting in inhibition of DNA synthesis (*13*, *14*), but several types of quinolones exhibit negative side effects, including significant phototoxicity (*30*). Quinolone-dependent phototoxicity is probably not due to the accumulation of protoporphyrin but instead to the generation of phototoxic metabolites of the drugs in cells, leading to DNA fragmentation and modification (*31*). The present data show that the augmentation of ALA-induced photodamage by quinolones is not responsible for the phototoxicity of quinolones, since the sole treatment with 100 μM enoxacin, ciprofloxacin or norfloxacin did not show photosensitivity because of the low concentration of drugs as well as a low dose of light (Fig. 1C). In addition, it is possible the phototoxicity of quinolones may be helpful to further augment the efficacy of ALA-PDT.

Quinolones were chosen as an attractive candidate agent for PDT because quinolones are widely used in clinical contexts, show relatively low toxicity (*13*), and can be easily administered orally with large volume of distribution and good tissue penetration (*14*). The concentration of quinolones used in this study was equivalent to that in blood of patients (10–250 μM) by clinically conventional therapy (*32*). These properties of quinolones are safer than those of iron-chelators including desferrioxamine. Other studies have recently shown their anti-tumour activity in a variety of human tumour cells by inhibiting the proliferation of tumour cells and promoting apoptosis (*33*, *34*). Furthermore, very recently, quinolone compounds were shown to enhance RNAi by interacting with trans-activating responsive region RNA-binding protein and may be useful as RNAi enhancers in the development of research tools and therapeutics (*35*). Taken together, the application of anti-bacterial agents, quinolones, may be extremely effective for ALA-PDT and their use in multiple combinations with other therapeutic reagents can optimize the treatment modalities for PDT of various tumour tissues.

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Conflict of interest
None declared.

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