学位論文

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Invention of a novel photodynamic therapy for tumors using a photosensitizing PI3K inhibitor

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XL147 (SAR245408, pilaralisib), an ATP-competitive pan-class I phosphoinositide 3-kinase (PI3K) inhibitor, is a promising new anticancer drug. We examined the effect of the PI3K inhibitor on PC3 prostate cancer cells under a fluorescence microscope and found that XL147-treated cancer cells are rapidly injured by blue wavelength (430 nm) light irradiation. During the irradiation, the cancer cells treated with 0.2–2 μM XL147 showed cell surface blebbing and cytoplasmic vacuolation and died within 15 min. The extent of cell injury/death was dependent on the dose of XL147 and the light power of the irradiation. These findings suggest that XL147 might act as a photosensitizing reagent in photodynamic therapy (PDT) for cancer. Moreover, the cytotoxic effect of photosensitized XL147 was reduced by pretreatment with other ATP-competitive PI3K inhibitors such as LY294002, suggesting that the cytotoxic effect of photosensitized XL147 is facilitated by binding to PI3K in cells. In a single-cell illumination analysis using a fluorescent probe to identify reactive oxygen species (ROS), significantly increased ROS production was observed in the XL147-treated cells when the cell was illuminated with blue light. Taken together, it is conceivable that XL147, which is preferentially accumulated in cancer cells, could be photosensitized by blue light to produce ROS to kill cancer cells. This study will open up new possibilities for PDT using anticancer drugs.

Photodynamic therapy (PDT) is an alternative cancer treatment modality in which a patient is administered a photosensitizing drug that is sensitive to a specific wavelength of light. The interaction of the drug with this particular wavelength of light results in the production of reactive oxygen species (ROS) that kill targeted tumor cells. In PDT, the photosensitizer does less damage to normal cells than to tumor cells, where it preferentially accumulates.[1] Thus, PDT has every expectation of being an ideal cancer treatment modality because it is minimally invasive and has few side effects, and because it treats lesions selectively with low-energy laser irradiation without seriously affecting normal tissue.

Photosensitizers can be divided into three groups based on their chemical structures and origins: porphyrins, chlorophylls, and dyes. Tumor-affinity photosensitizers, such as porfimer sodium and talaporfin sodium, 5-aminolevulinic acid, and methyl aminolevulinate, are now used for the treatment of several cancers;[2–11] however, their range of applications is still limited. Many chemical compounds have photosensitivity,[12–14] but only few can be administered to patients. Of those that can safely be administered to patients, the tumor selectivity, in many cases, is not sufficiently high. Therefore, it would be useful to explore novel photosensitizers in existing oncotropic drugs.

Among the three classes of phosphoinositide 3-kinase (PI3K), class I PI3K is important in regulating tumor proliferation, survival, angiogenesis, invasion, and dissemination. Dysregulation of the PI3K pathway component is observed in many cancers and is thought to promote tumor growth and survival.[15,16] *PIK3CA*, the gene encoding the p110α subunit of class I PI3K, is often mutated or amplified in human cancers.[17–19] It is also thought to be attributed to resistance to anticancer therapies. Therefore, significant effort has been made to generate PI3K inhibitors for cancer chemotherapy.

XL147 has recently been developed as an ATP-competitive inhibitor of class I PI3K isoforms. XL147 reversibly binds to p110α, p110β, p110γ, and p110δ with half-maximal inhibitory concentration (IC_{50}) values of 48, 617, 10, and 260 nM/L, respectively,[20] and has been reported to

Key words: photodynamic therapy, photosensitizer, PI3K inhibitors, PC3 prostatic cancer cells, live-cell imaging, XL147

Abbreviations: PDT: photodynamic therapy; PDK1: phosphoinositide-dependent kinase-1; PI3K: phosphoinositide 3-kinase; PIP_2: phosphatidylinositol 4,5-bisphosphate; PIP_3: phosphatidylinositol 3,4,5-trisphosphate; ROS: reactive oxygen species

Additional Supporting Information may be found in the online version of this article.

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What's new?

Photodynamic therapy is an alternative cancer treatment modality in which patients are administered a photosensitizing drug that preferentially accumulates in tumor cells. Here, using live-cell imaging, the authors demonstrate that XL147-treated PC3 prostate cancer cells are rapidly injured by 430 nm light illumination. New anticancer drug XL147 has previously been developed as a pan-class I PI3K inhibitor. Marked reactive oxygen species (ROS) production was found in the cytoplasm of XL147-treated PC3 prostate cancer cells when illuminated, suggesting such XL147 cytotoxicity may be attributed to ROS. This study will potentially open up new possibilities for photodynamic therapy using existing anticancer drugs.

show an inhibitory effect on the growth of various cancer cell lines in a dose-dependent manner.[21] In addition, XL147 strongly inhibits the PI3K/AKT/mTOR pathway in tumor xenograft models and displays robust antitumor activity in tumor-bearing mice.[20] Furthermore, the first-in-human phase I study of XL147 has shown a favorable safety profile, demonstrable pharmacodynamic effects, and preliminary antitumor activity in patients with advanced solid tumors.[22] XL147 is currently being investigated in phase II studies in patients with advanced or recurrent endometrial cancer[23] and metastatic HER2-positive breast cancer.[24]

While examining the effects of various PI3K inhibitors on PC3 prostate cancer cells under a confocal laser microscope, we noticed that the cancer cells treated with XL147 were injured by blue laser irradiation within a few minutes. On the basis of this initial observation, we set out to determine whether XL147 could be used as a novel photosensitizer for PDT. Because XL147 inhibits cancer growth predominantly by perturbing PI3K pathways, synergic antitumor effects might also be expected if XL147 has photosensitizing properties for PDT. In this study, we used image-based cellular analysis with an automated photoactivation fluorescence microscope to show that blue light illumination of PC3 prostate cancer cells in the presence of XL147 causes severe cell damage. This study introduces the potential of the anticancer drug XL147 for a photosensitizer in PDT.

Material and Methods

Cell culture and drug treatments

Two human prostate cancer cell lines PC3 and DU145 were obtained from ATCC (Manassas, VA). Human epidermoid carcinoma A431 cells and mouse macrophage-like RAW264 cells were obtained from Riken Cell Bank (Tsukuba, Japan). In accordance with the supplier protocols, PC3 and DU145 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (10 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2. For A431 and RAW264 cell culture, Dulbecco-modified essential medium was used instead of RPMI 1640. For live-cell imaging, the cells were seeded on 25-mm coverslips in 35-mm dishes at a density of 2.0×10^4 cells per dish and were incubated for 48–72 hr before experiments.

XL147 (SAR245408, MW. 448.5, Formula $C_{21}H_{16}N_6O_2S_2$, CAS No. 956958–53-5) was purchased from SYNkinase (San Diego, CA) or AdooQ BioScience (Irvine, CA), dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM, and stored in small aliquot tubes at −20°C until experimental use. Other PI3K inhibitors LY294002 and wortmaninn were purchased from Sigma Chemicals (St. Louis, MO). The cells were treated with the indicated concentration of XL147 or one of the other inhibitors at least 10 min prior to observation. As a control, 0.1% DMSO (vehicle) was added to the cells.

Photoactivation and live-cell imaging

At 48–72 hr after seeding, the culture medium was replaced with Ringer's buffer (RB) consisting of 155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM Na_2HPO_4, 10 mM glucose, 10 mM HEPES pH 7.2, and 0.5 mg/mL bovine serum albumin. The 25-mm coverslips were placed in an RB-filled chamber on a 37°C thermo-controlled stage (Tokai Hit INU-ONI, Shizuoka, Japan). Photoactivation and live-cell imaging were performed using a Leica DMI 6000B inverted microscope operated with a MetaMorph imaging system (Molecular Devices, Sunnyvale, CA). We automated the microscope for photoactivation using the macroprogramming capability of the MetaMorph software.[25] An external light source (Leica EL6000) with a high-pressure short-arc mercury lamp (Osram HXP R 120 W/45C VIS) was used for fluorescence excitation. We mainly used a 430-nm cyan fluorescent protein (CFP) excitation filter (ET-ECFP; Chroma Technology, Bellows Falls, VT) to photosensitize XL147. The entire field of view of the microscope or a selected single-cell area was repeatedly illuminated using a 430-nm wavelength light at 23–138 mW/cm^2 power for 10 sec (0.23–1.38 J/cm^2) in each 15-sec interval of image acquisition. The light dose in a 10-μm diameter spot illuminated for local photosensitization was obtained by measuring the power at the microscope stage using a power meter (OPHIR, Laser Measurement Group, Israel). Time-lapse images of phase-contrast and fluorescence microscopy taken at 15-sec intervals and assembled into QuickTime movies by the MetaMorph imaging system. At least 10 examples were observed in each experiment.

Cell surface blebs, which are protrusions or bulges of the plasma membrane caused by localized decoupling of the actin cytoskeleton from the plasma membrane, are an initial

morphological hallmark of cell injury or cytotoxicity.[26] Using time-lapse phase-contrast microscopic images, we measured time to bleb formation on the cell surface after illumination with 430 nm of light in the presence of XL147 at concentrations ranging from 0 to 10 μM. In addition, ethidium homodimer-1 (EthD-1; Setareh Biothech, Eugene, OR), a membrane-impermeable fluorescent dye that binds DNA, was used to assure cell death. EthD-1 was applied to cells at a final concentration of 2 μM and observed by fluorescence microscopy (excitation 560 nm/emission 630 nm).

Detection of ROS

Generation of ROS was detected using a Total ROS/Superoxide Detection Kit (Enzo Life Sciences, Farmingdale, NY). The oxidative stress detection reagent is a nonfluorescent, cell-permeable total ROS detection dye which reacts directly with a wide range of reactive species and generates fluorescent products in live cells. According to the manufacturer's protocol, cells were loaded with the ROS-responsive fluorescence probe. After 10-min incubation at 37°C, the production of ROS in cells treated with or without 2 μM XL147 was monitored through the ET-EGFP filter set (Chroma Technology) of the fluorescence microscope during photoactivation. The average fluorescence intensity of the ROS-detecting reagent in the cell was measured by MetaMorph software and quantitatively analyzed ($n = 10$ illuminated areas in each condition).

Detection of apoptosis

For relocating cells after photosensitization, cells were grown in a 150-μm grid glass-bottom dish (Iwaki, Shizuoka, Japan), which is marked numerically in one direction and alphabetically in the other. In the absence or presence of 1 μM XL147, cells were illuminated with 430 nm light at 78 mW for 2 min (total light dose = 6.2 J/cm^2) and then returned to the ordinary culture medium in a CO_2 incubator. After 6–24 hr, cell apoptosis and necrosis were detected by Annexin V-Cy3 Apoptosis Detection Kit Plus (BioVision, Inc., Milpitas, CA) according to the manufacturer's protocol. The illuminated areas were relocated by phase-contrast microscopy and examined Annexin V-Cy3 and SYTOX-green staining by fluorescence microscopy. Annexin V-Cy3, which binds to cell surface phosphatidylserine (PS), detects the initiation of apoptosis. SYTOX-green is impermeant to live cells and early apoptotic cells but stains dead cells after necrosis and late apoptosis. For quantitative analysis, increased fluorescence intensity of Annexin V-Cy3 or SYTOX-green in the area illuminated by 430 nm light was calculated by subtracting the average fluorescence intensity of the nonilluminated area from that of the illuminated area using MetaMorph software. To detect chromatin condensation and/or nuclear fragmentation indicative of late apoptosis, Hoechst 33342 (Sigma-Aldrich) staining was performed after 4% paraformaldehyde fixation.

Statistical analysis

The data are expressed as the mean ± standard error (SE) of three independent experiments (>10 cells in each condition). For comparison of two groups, unpaired Student's *t*-test was used to determine the significance of the difference. For multiple comparisons, data sets were assessed with a one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant when the calculated *p* values was <0.05.

Results

Photoactivation of XL147 induces cell injury and death in a dose-dependent manner

XL147 (chemical structure and absorbance spectrum are shown in Supporting Information Figs. S1*a* and S1*b*) is cell membrane-permeant and cyan-fluorescent in PC3 cells when excited by 430-nm wavelength light under a fluorescence microscope (Supporting Information Fig. S1*c*). XL147 is usually applied to culture cells at a concentration of 10 μM as a pan-class I PI3K inhibitor. When we treated PC3 cells with 10 μM XL147 for 30 min, no remarkable change in cell morphology was observed by phase-contrast microscopy (Fig. 1, top panel). However, after 30-min illumination of the 430 nm blue light at 138 mW/cm^2 power (total light dose = 165 J/cm^2) in the presence of 10 μM XL147, these cells exhibited cellular swelling, which is the most universal indicator of cellular injury. When we applied the dead cell indicator EthD-1 to these cells, the nuclei were stained with EthD-1 (Fig. 1, middle panel). Illumination of cells with 430 nm blue light in the absence of XL147 did not induce severe cell injury or cell death (Fig. 1, lower panel). These findings indicate that cell injury or cell death was caused by photosensitized XL147 rather than PI3K inhibition or simple photo damage resulting from light illumination.

Next, we examined the dose-dependent cytotoxic effect of XL147 combined with 430-nm light illumination at a certain power (138 mW/cm^2) by live-cell imaging. Time-lapse microscopy of live PC3 cells (Figs. 2*a–e* and Supporting Information Movies S1–S5) and time measurement of bleb formation (Fig. 1*f*) revealed that 430-nm light illumination of PC3 cells in 0.2 μM XL147 produced small blebs on the cell surface after ~10 min of illumination (Fig. 2*b* and Supporting Information Movie S2), although cells that had not been treated with XL147 did not show bleb formation (Fig. 2*a* Supporting Information Movie S1). The small blebs increase in number and size with time. After increasing the concentration of XL147 to 0.5 μM, the time to bleb formation was shortened to ~4 min (Figs. 2*c* 2*f* and Supporting Information Movie S3). At concentrations of XL147 higher than 1 μM, cellular swelling is more prominent than blebbing, although small bleb formation occurred in ~2 min (Figs. 2*d* and 2e and Supporting Information Movies S4 and S5). In association with cellular swelling, cytoplasmic membrane organelles, possibly the endoplasmic reticulum and/or mitochondria,

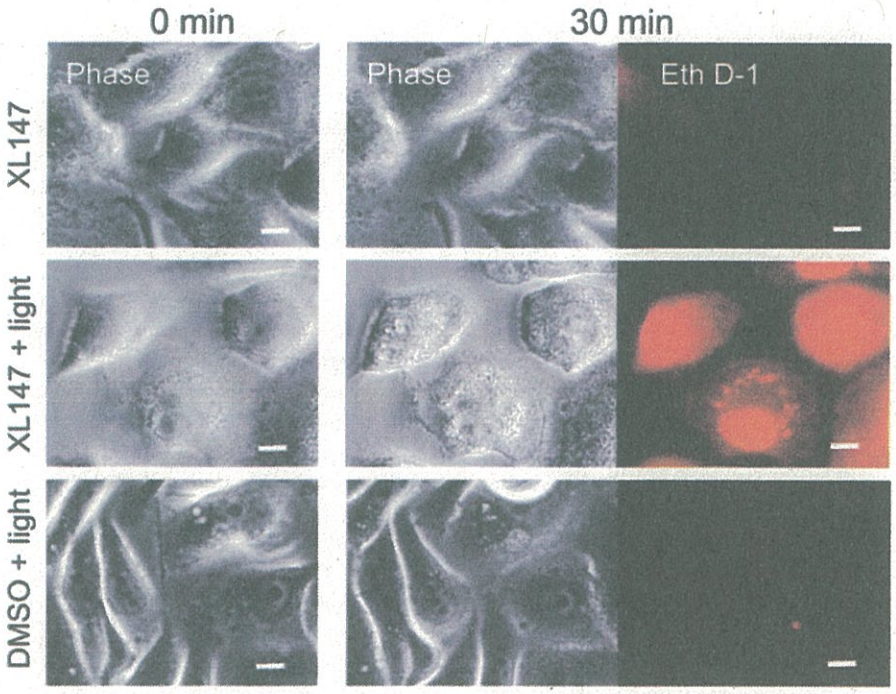

Figure 1. Illumination of PC3 cells in the presence of XL147 causes severe cytotoxicity and cell death. PC3 cells grown on coverslips were incubated with 10 μM XL147 or 0.1% DMSO on the thermo-controlled stage of an inverted microscope. Phase-contrasted images were acquired before (0 min) and after 30-min illumination with 430 nm light at 138 mW/cm^2 power (total light dose = 165 J/cm^2). The cell-impermeant viability indicator ethidium homodimer-1 (EthD-1) was applied to cells so that nucleic acids in dead cells were stained fluorescent red. Scale bars: 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

seemed to be swollen and/or vacuolated. These data suggest that photoactivation of XL147 induces cell injury in a dose-dependent manner.

Similar cytotoxic effects of photoactivated XL147 were observed using other cell lines such as DU145, RAW264, and A431 cells, although changes in morphological features were somewhat different among cell types (Supporting Information Fig. S2).

Next, we compared the photocytotoxic effect of XL147 using illumination with different wavelengths. Neither 500-nm nor 560-nm illumination caused cell surface blebbing, cytoplasmic vacuolation, or EthD-1 staining in XL147-treated PC3 cells, whereas 430 nm illumination under the same conditions induced severe cell injury or death (Fig. 3). This result indicates that illumination with a wavelength of ~430 nm can photosensitize XL147.

Furthermore, to evaluate the light-dose dependency of XL147 photosensitization, we compared the cytotoxicity effect of XL147 using different powers of 430-nm light illumination (Fig. 4). Time-lapse microscopic examination showed that very small bleb formed after ~4.5 min when 1 μM XL147-treated PC3 cells were illuminated with 430 nm light at 46 mW/cm^2 power [1.84 J/(cm^2 min)]. However, large blebs or cellular swelling was not observed. At 78 mW/cm^2 power [3.12 J/(cm^2 min)], bleb formation was observed within a few minutes. Then, the cells became gradually swollen with time (Fig. 4*a*). Increasing the power of 430-nm light illumination significantly shortened the time to bleb formation (Fig. 4*b*). Cellular swelling and cytoplasmic vacuolation were more prominent at the higher power of 430 nm light.

Pretreatment with other competitive PI3K inhibitors reduces cytotoxic effect of XL147 as a photosensitizer

To confirm whether the cytotoxic effect of photosensitizing XL147 is mediated through binding to p110 subunits of class I PI3Ks, we examined whether the cytotoxic effect of XL147 induced by 430-nm light illumination is reduced by other ATP-competitive inhibitors that bind to the p110 subunit. PC3 cells were pretreated with 10 μM LY294002, 100 nM wortmannin, or 0.1% DMSO (vehicle) for 10 min, and then 0.5 μM XL147 was added, followed by illumination with 430 nm light. By time-lapse phase-contrast microscopy, time to bleb onset was measured using 10 cells per each condition. Figure 5 shows that the time to form bleb was significantly prolonged by pretreatment with LY294002 or wortmannin, in comparison with XL147 alone (Fig. 5), indicating that XL147 photocytotoxicity is mediated by its binding to the p110 subunit, which is competitively hindered by other PI3K inhibitors. These data suggest that XL147 is effective as a

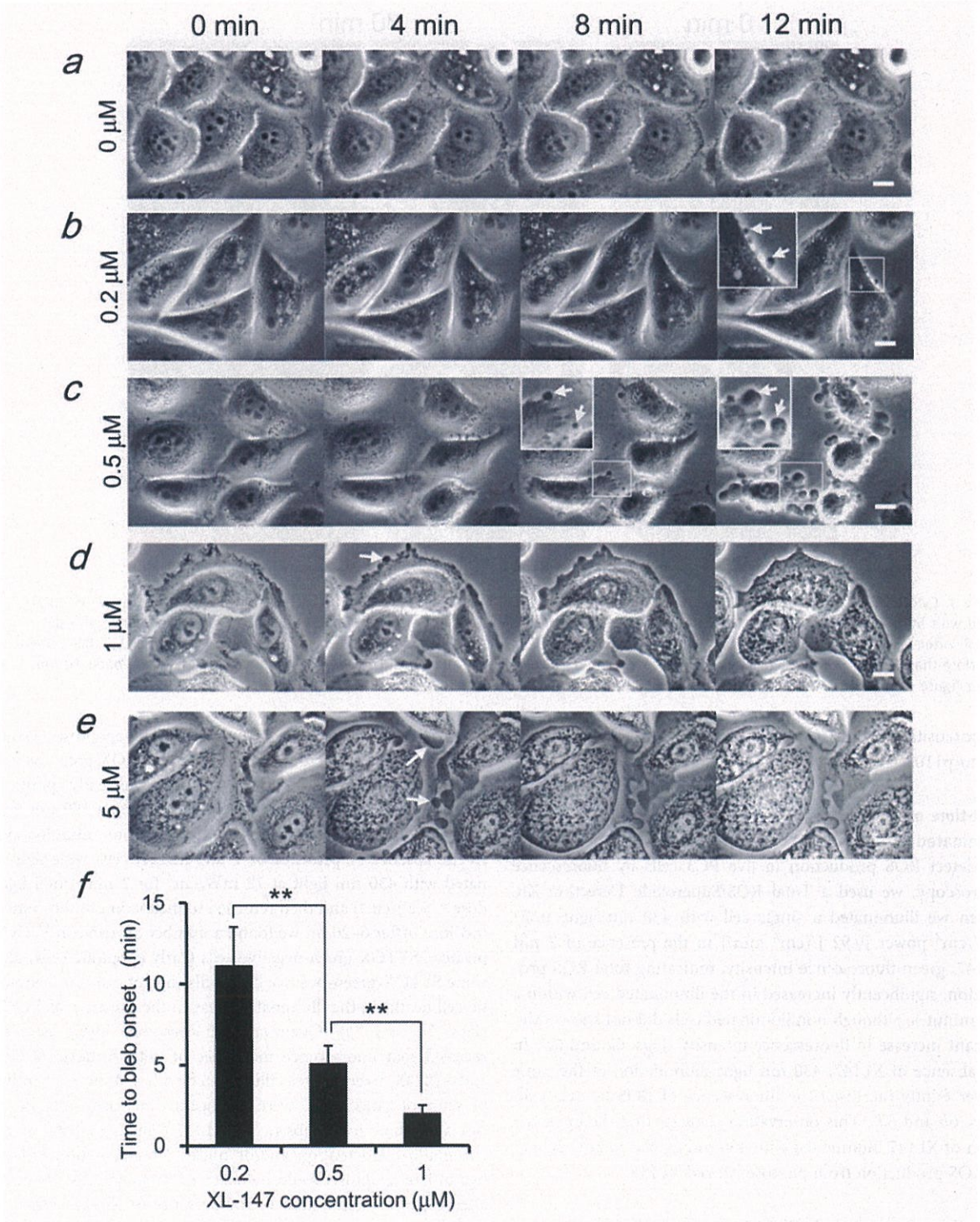

Figure 2. Dose-dependent cytotoxicity of XL147 combined with 430-nm light illumination. (*a–e*) PC3 cells grown on coverslips were observed by phase-contrast microscopy and illuminated with 430 nm light at 138 mW/cm^2 power (8.3 J/cm^2 × min) in the presence of XL147 at different concentrations: (*a*) 0, (*b*) 0.2, (*c*) 0.5, (*d*) 1.0, and (*e*) 5.0 μM. Insets show enlarged images of boxed areas. Arrows point to blebs on cell surface. Scale bars: 10 μm. (*f*) Average time to form small blebs by 430-nm light illumination in the presence of XL147 at the indicated concentrations was quantified. The data are expressed as the mean ± SE, $n = 3$ independent experiments. **Significant difference $p < 0.01$ (one-way ANOVA, Tukey test).

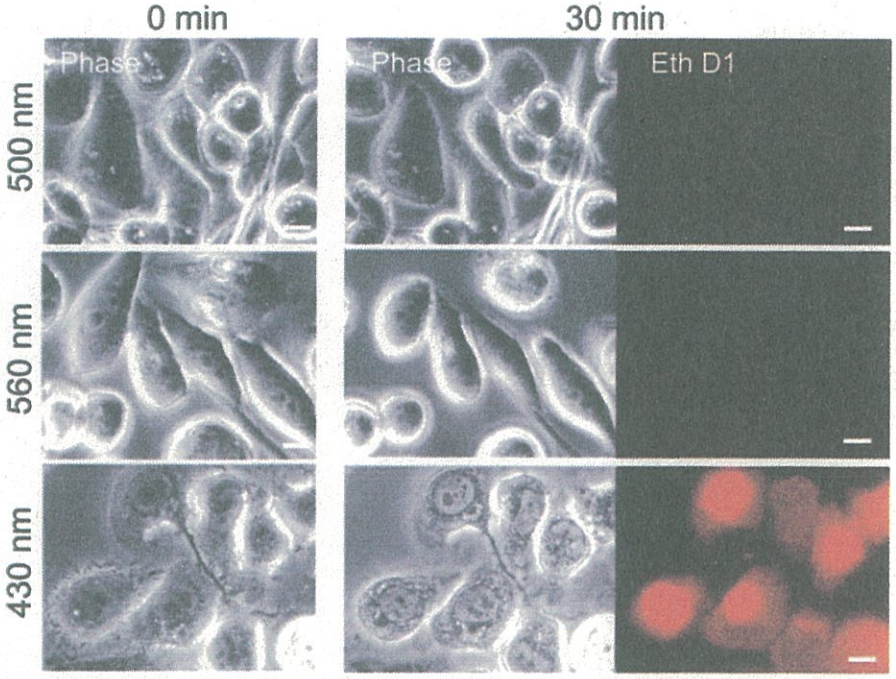

Figure 3. Cytotoxic effect of different wavelength lights on PC3 cells in the presence of XL147. PC3 cells grown on coverslips were illuminated with 500 nm, 560 nm, or 430 nm light at 138 mW/cm^2 power for 30 min (total light dose = 165 J/cm^2) in the presence of 1 μM XL147. After 30 min, cytotoxicity and cell viability were monitored by phase-contrast microscopy and ethidium homodimer-1 (EthD1) staining. Note that 500- or 560-nm light illumination of XL147-treated PC3 cells did not cause severe cell injury or death. Scale bars: 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

photosensitizer where it has accumulated in the cell by binding to p110.

Real-time observation of ROS production after XL147 was illuminated with blue light

To detect ROS production in live PC3 cells by fluorescence microscopy, we used a Total ROS/Superoxide Detection Kit. When we illuminated a single cell with 430 nm light at 23 mW/cm^2 power [0.92 J/(cm^2 min)] in the presence of 2 μM XL147, green fluorescence intensity, indicating total ROS production, significantly increased in the illuminated cell within a few minutes, although nonilluminated cells did not show a significant increase in fluorescence intensity (Figs. 6*a* and 6*c*). In the absence of XL147, 430-nm light illumination at the same power faintly increased the fluorescence of ROS in PC3 cells (Figs. 6*b* and 6*d*). This observation suggests that the cytotoxic effect of XL147 illuminated with 430 nm light is mainly caused by ROS production from photosensitized XL147.

Induction of apoptosis by XL147 photosensitizing with a low-dose light illumination

From the clinical point of view, it is preferable that a putative photosensitizer for PDT should induce apoptotic cell death.[27,28] Therefore, we examined whether cell apoptosis can be induced by XL147 photosensitizing. The appearance of PS residues on the surface of the cell is an early event in apoptosis. Using Annexin V-Cy3, which binds to PS, and SYTOX-green, which stains nucleic acid of dead cells, we distinguished early apoptotic cells from dead cells. PC3 cells were cultured on a 150-μm grid glass-bottom dish for relocating cells after 430 nm illumination. In the absence or presence of 1 μM XL147, cells were illuminated with 430 nm light at 78 mW/cm^2 for 2 min (total light dose = 6.2 J/cm^2) and then returned to incubation in the culture medium. After 6–20 hr, we found a number of Annexin V-Cy3-positive, SYTOX-green-negative cells (early apoptotic cells) and some SYTOX-green-positive dead cells (necrotic and/or apoptotic cell death) in the illuminated areas in the presence of XL147 (Figs. 7*a* and 7*b*). Quantitative fluorescence image analysis revealed that fluorescence intensities of both Annexin V-Cy3 and SYTOX-green in areas illuminated with 430 nm light in the presence of 1 μM XL147 were significantly increased in comparison with those in the absence of XL147 (Fig. 7*c*). Furthermore, chromatin condensation and/or nuclear fragmentation indicative of late apoptosis could be observed in some cells 24–30 hr after 430 nm illumination in the presence of XL147 (Fig. 7*d*). These results indicate that XL147 photosensitizing with a low-dose light can evoke apoptosis.

Discussion

In this study, we revealed the effectiveness of XL147 as a photosensitizing reagent in PDT for cancer treatment using

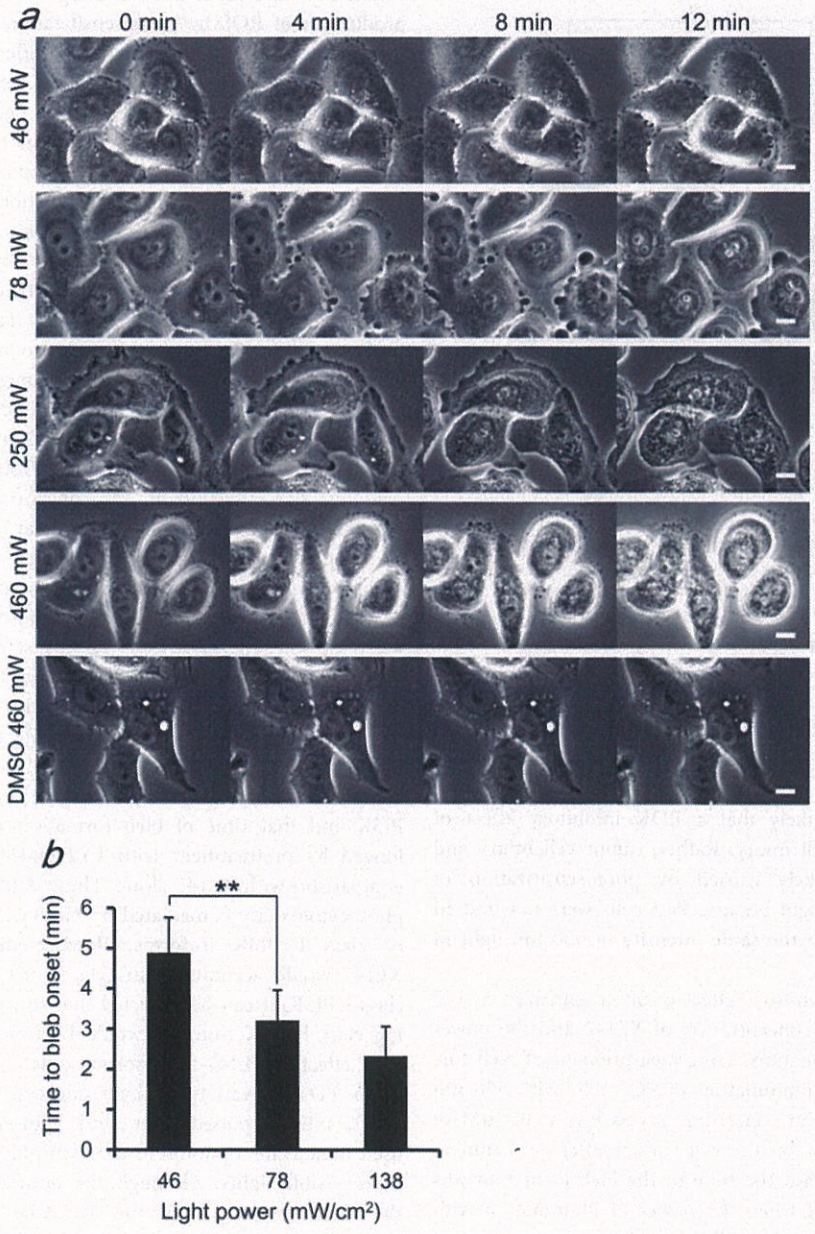

Figure 4. Light power-dependent cytotoxic effect of XL147 as a photosensitizer. (*a*) Time-lapse images of PC3 cells showing phototoxic damage with 430 nm illumination at different powers in the presence of 1 μM XL147. PC3 cells grown on coverslips were imaged by phase-contrast microscopy and illuminated with 430 nm light at different powers: 46, 78, 250, or 460 mW/cm^2. The bottom panel shows PC3 cells without XL147. In the presence of XL147, phototoxicity increases with the illumination power of 430 nm light. Scale bars: 10 μm. (*b*) Average time to form small blebs by 430-nm light illumination at indicated power outputs in the presence of XL147 was quantified using time-lapse phase contrast images. The data are expressed as the mean ± SE, $n = 3$ independent experiments. ** Significant difference $p < 0.01$ (one-way ANOVA, Tukey test).

image-based analysis with an automated photoactivation microscope. XL147 was recently developed as an orally available anticancer drug that inhibits the class I PI3K isoforms.[22,29] XL147 has been used to inhibit PI3K activity in cancer cell lines at concentrations from 2 to 10 μM.[21] In our microscopic observation, PC3 cells treated with 10 μM XL147 showed evidence of cell injury such as marked cell surface blebbing, cytoplasmic vacuolation, and cellular swelling after illumination with 430 nm light. After 30 min of illumination, those same PC3 cells treated with XL147

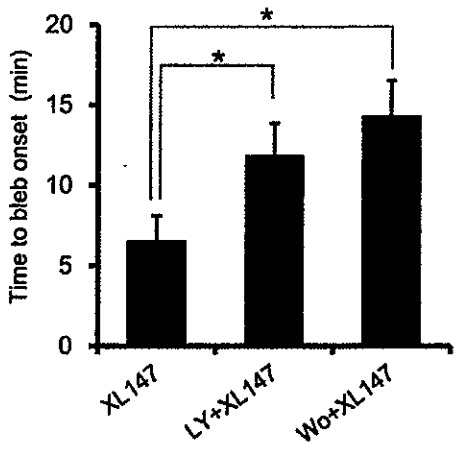

Figure 5. Cytotoxic effect of XL147 combined with 430-nm light illumination was reduced by pretreatment with other PI3K inhibitors that competitively bind to p110 of PI3K. Cells were pretreated with 10 μM LY294002 (LY) or 100 nM wortmannin (Wo) and subjected to 0.5 μM XL147 photosensitization with 430 nm illumination. Time to bleb onset was measured by time-lapse phase-contrast microscopy. The data are expressed as the mean ± SE, $n = 3$ independent experiments. $^*p < 0.05$ significantly higher than XL147 only.

became positive for EthD-1, a dead cell indicator. As PC3 cells treated with 10 μM XL147 did not show any morphological hallmarks of cell injury or death when illumination was absent, it is unlikely that a PI3K inhibitory effect of XL147 causes such cell injury. Rather, tumor cell injury and death were more likely caused by photosensitization of XL147 with 430 nm light because PC3 cells were not injured when illuminated with the same intensity of 430 nm light in the absence of XL147.

Importantly, the cytotoxic effect of photosensitized XL147 depends on both the concentration of XL147 and the power density of light illumination. Time measurement of bleb formation revealed that illumination of PC3 cells with 430 nm light at 138 mW/cm^2 at concentrations as low as 0.2 μM of XL147 produced blebs on the cell surface after ~10 min of illumination. In contrast, the time to the bleb formation was significantly shortened when the power of illumination with 430 nm light was increased. Cellular swelling and cytoplasmic vacuolation were more prominent at the higher power of 430 nm light. Both dose dependencies indicate that the cytotoxic effect is derived from the photosensitization of XL147.

In PDT, it is well known that light-activated photosensitizing reagents cause oxidative stress and tumor cell death by producing ROS in tumor tissues.[30] When photosensitizer that has accumulated in tumor cells is exposed to light of a certain wavelength, it is transformed from its ground state into an excited singlet state. From this state, the drug may decay directly back to its ground state by emitting energy. The ROS produced during this process can kill tumor cells by causing oxidative stress and by inducing apoptosis.[1,31,32] In our observation by fluorescence live-cell imaging, we demonstrated the production of ROS by photosensitization of XL147. Accordingly, it is evident that the cytotoxic effect caused by XL147 during illumination resulted from the oxidation of proteins, lipids, and DNA by ROS. The acute cell damage or death observed in our short-term observations (<30 min) seems to be a nonapoptotic process. However, apoptosis would still be caused later because excessive production of ROS is known to induce both apoptosis and necrosis in tumor cells/tissues.[33,34] Consistently, we could detect early apoptosis of PC3 cells at 20 hr after XL147 photosensitizing with a low-dose light illumination (6.2 J/cm^2; Fig. 7). Taken together, it is likely that XL147 photosensitizing produces excessive ROS, which causes progressive oxidative damages to many cellular components, including mitochondria, endoplasmic reticulum, cell membrane, and DNA, leading ultimately to cell death through apoptosis and/or necrosis. Probably the effects of oxidative stress depend on the concentration of XL147 and the light dose: severe oxidative stress can kill cells as accidental cell death by physiochemical damages in a short time; and even moderate oxidation can trigger apoptosis through signals of caspases, cytochrome *c*, and/or other pathways, whereas more intense stresses may cause necrosis.[35]

Targeting cancer cells with photosensitizers is important in reducing side effects on normal tissues. In this experiment, we demonstrated that the cytotoxic effect of XL147 induced by 430-nm light illumination is reduced by other ATP-competitive inhibitors that bind to the p110 subunit of class I PI3K and that time of bleb formation is significantly prolonged by pretreatment with LY294002 or wortmannin, in comparison with XL147 alone. These data suggest that XL147 photocytotoxicity is mediated by binding to the p110 subunit of class I PI3K isoforms. Because membrane-permeable XL147 would accumulate in cells which abundantly express class I PI3K, it can be expected that cancer cells overexpressing class I PI3K isoforms would be more susceptible to the PDT effect of XL147 than normal cells.

In PDT, a variety of light sources, including low-level lasers, intense pulsed light, and light-emitting diodes, are used to activate a photosensitizer with blue light, red light, or other visible lights. Although the optimal light depends on the ideal wavelength for the particular drug that is being used and the target tissue, activation with a longer wavelength of light is generally advantageous in clinical PDT, because longer wavelengths of activation allow for deeper tissue penetration. For instance, activation at 400 nm is measured at a depth of 1 mm, whereas 630 nm is measured at a depth of 2–3 mm.[36] Actually most clinically used photosensitizers, including Photofrin®, Foscan® (temoporfin), and Photosens® (phthalocyanine), have an activation peak at longer than 630 nm. Therefore, as a clinical PDT device providing light at 630 nm, an argon-pumped dye laser coupled to an optical fiber is frequently used.[37] However, XL147 photosensitization requires a wavelength of ~430 nm, which does not allow for particularly deep penetration. Consequently, the

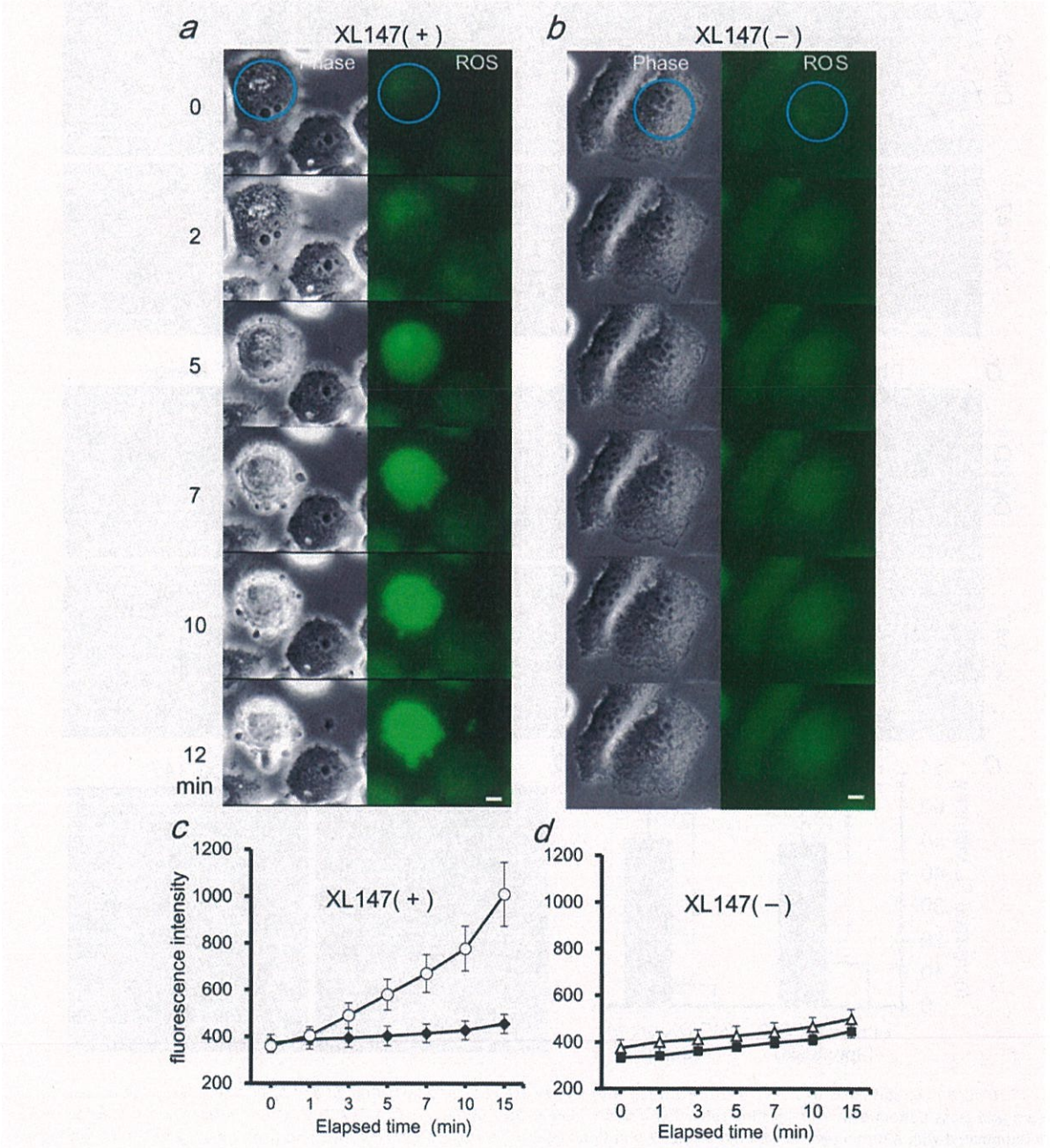

Figure 6. Real time observation of reactive oxygen species (ROS) produced in PC3 cells illuminated with 430 nm light in the presence (*a*) or absence (*b*) of XL147. Live PC3 cells grown on coverslips were treated with a ROS detection reagent and observed by phase-contrast and fluorescence microscopy. Circled areas were locally illuminated with 430 nm light at 23 mW/cm^2 (0.9 J/cm^2 × min). In the presence of XL147 (*a*), green fluorescence indicating ROS production increases with time during 430-nm light illumination; however, ROS production was only faintly detected in the absence of XL147 (*b*). Scale bars: 10 μm. (*c*, *d*) Quantitation of ROS production by fluorescence intensity analysis of PC3 cells in the presence (*c*) or absence (*d*) of XL147. Average fluorescence intensity of the ROS detection reagent in cells illuminated (open symbols) and nonilluminated (filled symbols) was measured by the MetaMorph software. Data are mean ± SE, $n = 10$ cells. Results shown are representatives of three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

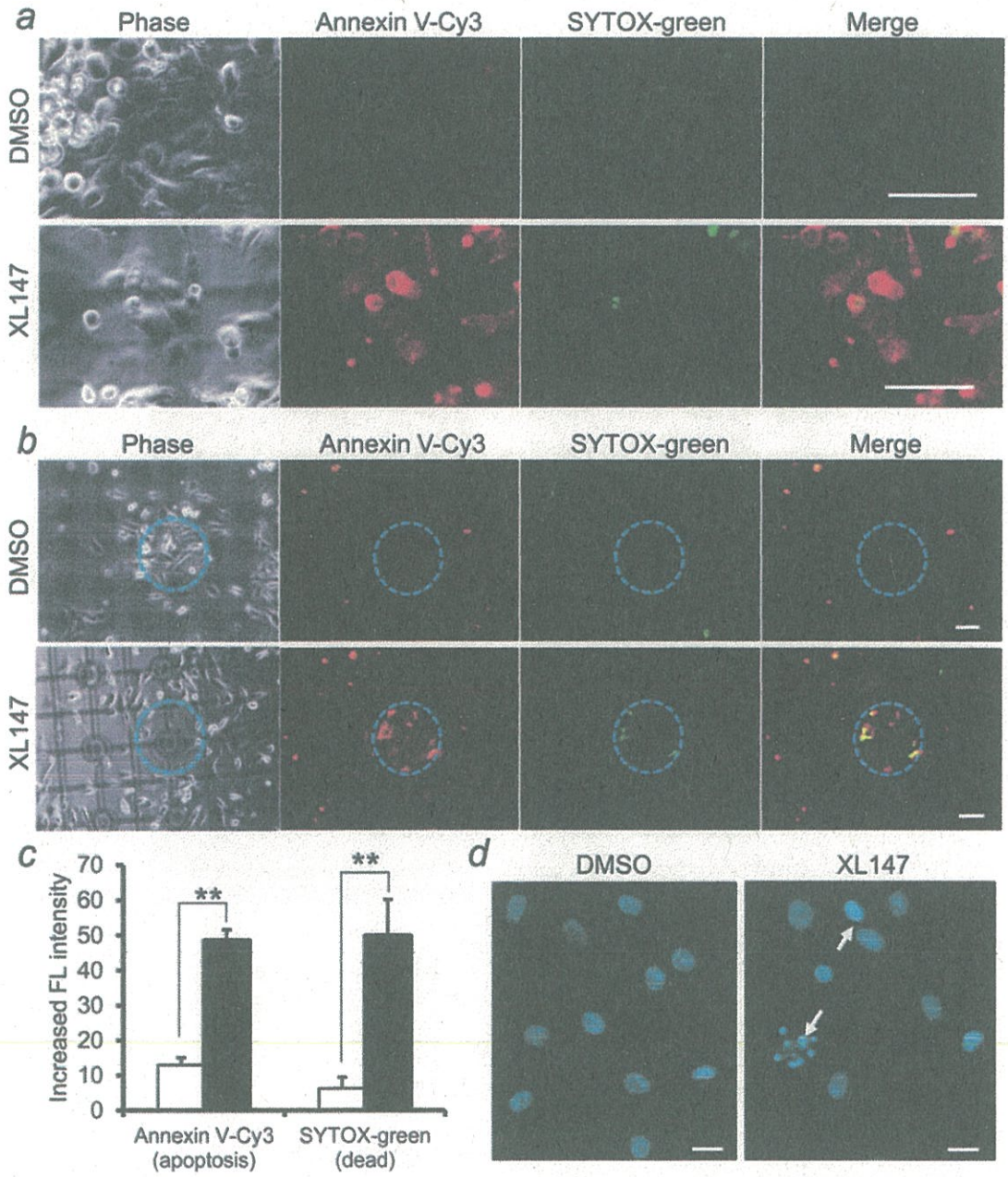

Figure 7. Induction of apoptosis by XL147 photosensitized with a low dose of light illumination. (*a* and *b*) PC3 cells were cultured on a 150-μm grid glass-bottom dish for relocating cells after 430 nm illumination. In the absence (DMSO only) or presence of 1 μM XL147, cells were illuminated with 430 nm light at 78 mW/cm^2 for 2 min (total light dose = 6.2 J/cm^2) and then returned to incubation in the culture medium. After 6 (*a*) or 20 hr (*b*), apoptotic cells and dead cells were detected by Annexin V-Cy3 and SYTOX-green, respectively. The illuminated areas were relocated by phase-contrast images and observed for detecting apoptosis and necrosis. Representative images from 10 illuminated areas in the presence or absence of XL147 are shown. Scale bars: 100 μm. (*c*) Quantitative fluorescence image analysis for apoptosis by Annexin V-Cy3 staining. Increased fluorescence intensity of Annexin V-Cy3 or SYTOX-green in the area illuminated by 430 nm light (broken line circles in *b*) was calculated by subtracting the average fluorescence intensity of the nonilluminated area from that of the illuminated area using MetaMorph software. Fluorescence intensities of both Annexin V-Cy3 and SYTOX-green in areas illuminated with 430 nm light in the presence of XL147 (filled bars) were significantly increased in comparison with those in the absence of XL147 (DMSO only, open bars). The data are expressed as the mean ± SE, $n = 3$ independent experiments. **Significant difference, $p < 0.01$. (*d*) Observation of nuclear morphology of PC3 cells 24 hr after 430 nm illumination in the absence (DMSO) or presence of XL147 by Hoechst 33342 staining. Arrows indicate chromatin condensation and nuclear fragmentation. Scale bars: 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

modification of chemical compounds that can absorb longer wavelengths of light is to be desired. Alternatively, novel illumination devices may overcome this limitation. Two-photon laser microscopy which can observe tissue much deeper than one-photon laser microscopy is used for *in vivo* microscopy. Application of ~700–1000 nm two-photon lasers, which can penetrate deeper than conventional single-photon lasers, might also be useful in PDT.[38,39] Because 700–1000 nm two-photon lasers can excite molecules at half of its wavelength, photosensitizers with an absorbance range of 350–500 nm might be ideal in this case.[40]

One of the important attributes of any photosensitizer is tumor specificity. Because XL147 has been developed as a tumor-targeting drug, XL147 can be expected to accumulate in tumor cells which overexpress class I PI3K. In addition, in our study, XL147 was shown to compete with other PI3K inhibitors, suggesting that the effect of XL147 is specific to PI3K. Another important attribute of photosensitizers is safety. At the present time, the development of photosensitivity in patients is a problematic side effect of photosensitizers. Patients must therefore avoid sunlight after they have been administered these drugs. In phase I clinical trials, XL147 demonstrated an acceptable safety profile in patients with advanced solid tumors.[22] However, skin rash was reported as an adverse effect of XL147.[22] As the photodynamic reaction of XL147 has not so far been studied, we did not pay attention to light exposure after the administration of XL147. Prevention of sunlight exposure may help to reduce skin rash in patients that have been administered XL147.

In summary, based on the above-mentioned results, XL147 is taken into the cells by binding to PI3K, where, on illumination with blue light, it can then produce ROS and induce the injury of tumor cells, depending on its concentration and the dose of illumination. XL147, therefore, has the potential to become a useful new photosensitizer. As our findings in this study were based on *in vitro* cell culture observations with microscopy, it is still difficult to compare the PDT efficacy of XL147 with those of currently available photosensitizers for clinical PDT. However, earlier studies reported that Photofrin, which is one of the most frequently used PDT photosensitizers in oncology, induced apoptosis in carcinoma cell lines including PC3 cells by ~6 J/cm^2, similar to the light dose we used in this study.[41,42] In addition, evidence has been provided for the clinical benefit of XL147 in patients with advanced solid tumors, lymphoma, and chronic lymphocytic leukemia by its pharmacological impact on class I PI3Ks.[22,43] Therefore, we can say that XL147 is a drug that is expected to have both antitumor effects of a PI3K inhibitor and a photosensitizer when illuminated by blue light.

In this study, we have provided experimental evidence for the application of a preexisting anticancer drug to PDT. After using live-cell imaging and administering the test reagent, the method for judging the cell dysfunction caused by photoactivation was relatively simple. Using this method, we are able to conclude that there is a good possibility for discovering new photosensitizers within the existing test reagent. The outcome of this study can be expected to aid the further development of a new generation of photosensitizers for PDT.

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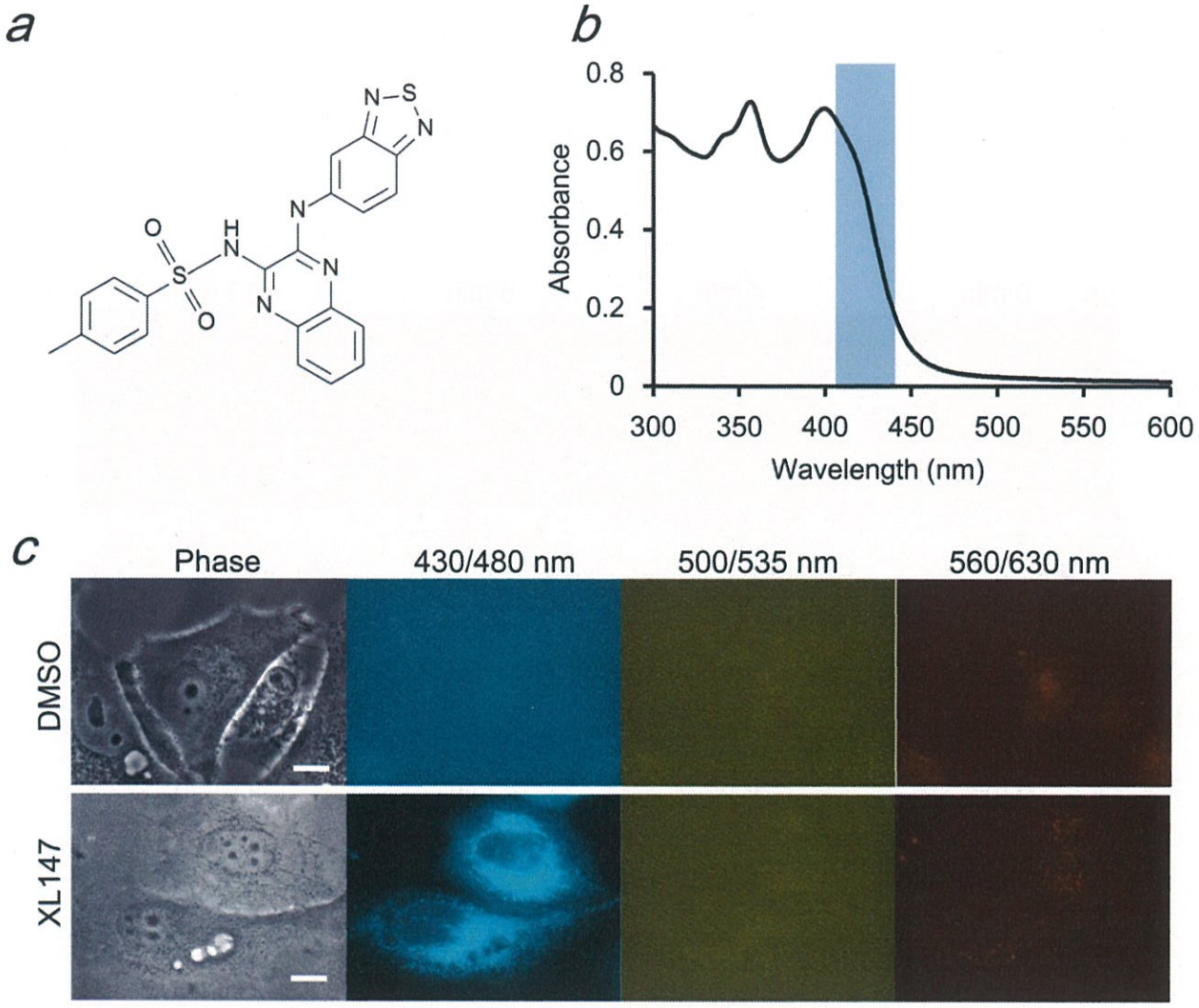
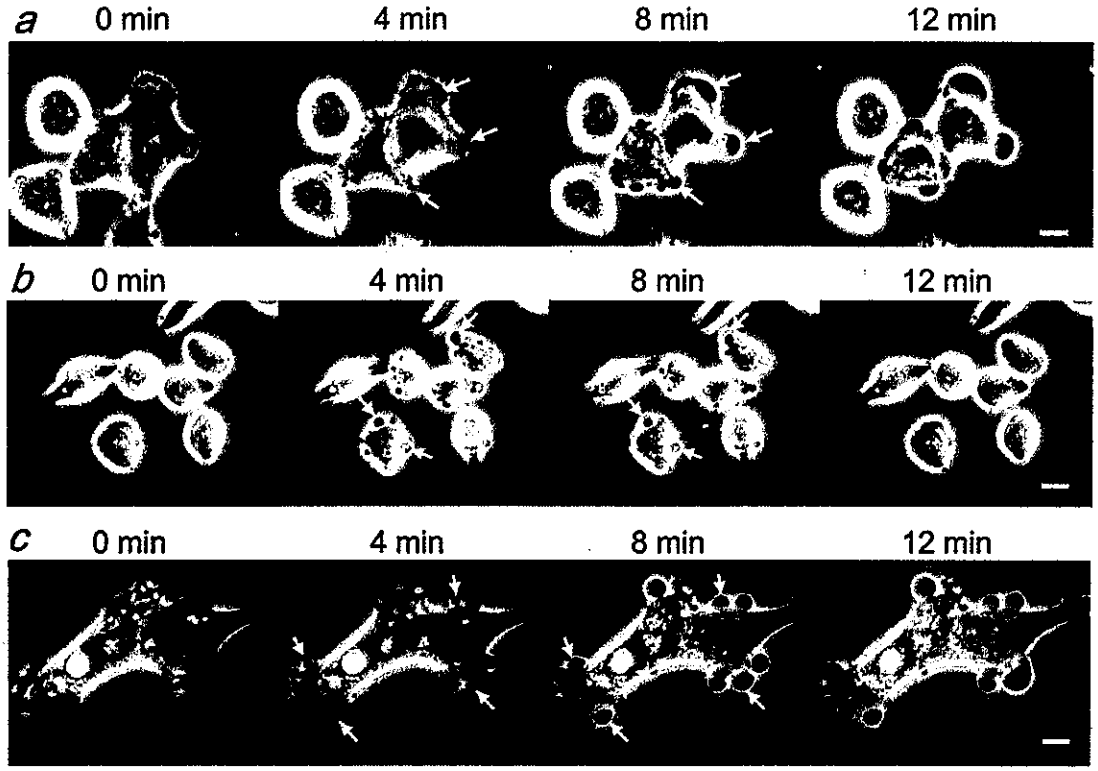


Supplementary Figure S1. (*a*) Chemical structure of XL147. (*b*) Absorbance spectrum of XL147. The excitation wavelength range of filter sets (430 ± 10 nm) we used in this study was shown in blue. (*c*) Fluorescence images of XL147 in PC3 cells in the abcense (DMSO) or presence of 1 μM XL147. PC3 cells were cultured on cover slips. After 0.1% DMSO (vehicle) or 1 μM XL147 addition, phase-contrast and fluorescence images were acquired by indicated excitation/emission filter sets (Em/Ex nm wavelength), Representative images from 10 illuminated areas in each group are shown. Bars: 10 μm.



Supplementary Figure S2. Effect of 1 μM XL147 combined with 430 nm light illumination on different cell types. Cells grown on coverslips were observed by time-lapse phase contrast microscopy. Arrows indicate cell surface blebs. (*a*) DU145 cells, (*b*) RAW264 cells, (*c*) A431 cells. Scale bars: 10 μm.