Published in final edited form as:

J Control Release. 2012 November 10; 163(3): 368–373. doi:10.1016/j.jconrel.2012.09.015.

Photoexcitation of tumor-targeted corroles induces singlet oxygen-mediated augmentation of cytotoxicity

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Abstract

The tumor-targeted corrole particle, HerGa, displays preferential toxicity to tumors *in vivo* and can be tracked via fluorescence for simultaneous detection, imaging, and treatment. We have recently uncovered an additional feature of HerGa in that its cytotoxicity is enhanced by light

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irradiation. In the present study, we have elucidated the cellular mechanisms for HerGa photoexcitation-mediated cell damage using fluorescence optical imaging. In particular, we found that light irradiation of HerGa produces singlet oxygen, causing mitochondrial damage and cytochrome c release, thus promoting apoptotic cell death. An understanding of the mechanisms of cell death induced by HerGa, particularly under conditions of light-mediated excitation, may direct future efforts in further customizing this nanoparticle for additional therapeutic applications and enhanced potency.

Keywords

Nanoparticles; drug delivery; a sulfonated gallium(III) corrole; HER2+; Tumor targeting; Photoexcitation; Singlet oxygen

Introduction

Nanomedicine, defined as the application of nanotechnology to health care, can facilitate medical interventions [1]. Nanoparticles, which provide unique characteristics in pharmaceutical forms, are crucial for nanomedicine. Cases in point are studies focusing on the development of multifunctional nanoparticles, which offer diagnosis, treatment, and imaging simultaneously for pharmaceutical applications [2]. Recently, sulfonated corrole compounds, which bear similarities to porphyrins, have been investigated for the treatment of malignant diseases [3, 4]. In particular, it has been shown that corroles have therapeutic potential as multifunctional nanoparticles for a number of diseases [5, 6]. While Fe- and Mn-corroles have neuroprotective effects (correlated with superoxide scavenging activity *in vitro* and *in vivo*), Ga-corroles, which are cytotoxic when delivered to cells, exhibit intense red fluorescence that is useful for tracking and tumor detection during cancer treatment [7, 8]. Typically, these corrole-metal complexes are negatively charged, and thus unable to penetrate into cells efficiently without membrane-disrupting agents. Hence, the corrole-metal complexes are safe at pharmacologic doses but can kill cells when they breach the cell membrane [9].

The nanoparticle of interest here, HerGa, is a self-assembled complex between the targeted cell penetration protein, HerPBK10, and a sulfonated gallium(III) corrole (S2Ga) (Fig. S1). We have previously shown that this nanoassembly (forming 10-20 nm diameter round particles; Fig. S2) is highly effective for both breast cancer treatment and detection [5, 10-13]. The HerPBK10 carrier protein specifically binds the human epidermal growth factor receptor (HER), and demonstrates preferential targeting of HER2+ cancer cells [5, 9-11, 14, 15]. Receptor binding triggers receptor-mediated endocytosis, after which endosomal penetration and cytosolic entry are mediated by a specific domain (the penton base domain) engineered into HerPBK10 [5, 10-13]. Hence, when S2Ga is assembled with HerPBK10, S2Ga can be targeted to and internalized by HER2+ tumor cells, resulting in tumor-targeted toxicity *in vitro* and *in vivo* [5, 6]. Importantly, S2Ga emits intense red light when excited with light at ~424nm, and thus tumor targeting by HerGa enables tracking of delivery and visualization of tumor regions optically. It follows that HerGa can be used for both tumor detection and intervention [5, 6].

We have recently elucidated the mechanism of HerGa tumor-toxicity [9]. Our studies showed that HerGa undergoes early endosome escape after cell uptake and induces reactive oxygen species (ROS) that cause oxidative damage to mitochondria (disrupting membrane potential) and the cytoskeleton in MDA-MB-435 cancer cells [9]. In addition, we observed that HerGa-treated tumor cells exhibited the late apoptotic event of chromosomal fragmentation, but did not externalize phosphatidylserine (PS), in support of a mechanism in

which corroles activate intracellular executioners of the apoptotic pathway by directly impacting the mitochondrion [9].

We also have found that light irradiation at specific wavelengths, including within the red light range, augment mitochondrial disruption in HerGa-treated cells. These findings suggest that the combination of the inherent tumor targeted toxicity and additional photoexcitation enhanced cytotoxicity of HerGa may facilitate greater potency and specificity for tumor treatment, yielding a therapeutic with optimized efficacy and safety [16]. However, until now, the mechanism of photo-excitation enhanced cytotoxicity of HerGa has been unknown.

In the current investigation, we show that HerGa receiving light at specific wavelengths compromises the mitochondrial membrane potential by singlet oxygen generation, thereby promoting mitochondrial membrane disruption, which in turn results in membrane potential collapse and cytochrome c release. The centrality of the mitochondrion for basic cellular functions and as a major executioner of apoptosis underscores the impact of HerGa on tumor cell survival. Therefore, identifying the cell death mechanism of HerGa, particularly when excited by light, could promote its eventual translation into the clinic.

Materials and Methods

Materials

MDA-MB-435 cells were prepared and maintained in complete medium [Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin] at 37° C, 5% CO₂. HerGa was complexed by combining S2Ga with HerPBK10 at a molar ratio of 30:1 as previously described [9, 16]. For immunostaining of cytoskeleton, mouse anti α -tubulin IgG, AlexaFluor 488 Goat anti-mouse IgG, 4',6-diamidino-2-phenylindole (DAPI), rhodamine phalloidin, and ProLong Antifade Kit (Invitrogen Corp) were used as previously described [9]. An Alexa fluor 488 cytochrome c apoptosis detection kit and a mitotracker red probe were purchased from Invitrogen. Finally, trans-1-(2'-methoxyvinyl)pyrene (MVP) and sodium azide were purchased from Invitrogen and ACROS for detecting singlet oxygen.

Cell death dose curve

MDA-MB-435 cells were cultured at 10⁴ cells per well in two 96 well dishes for 36 hours, after which the media was replaced with 50uL complete media containing the different concentrations of HerGa (10⁻⁴ ~10⁴nM). The cells in one of the dishes received light at 424nm (1.1J/cm²) after rocking at 37°C for two hours, followed by rocking at 37°C for another two hours, after which an additional 50uL of complete media was added and cells continued incubation at 37°C without rocking. We determined the cell number using crystal violet (CV) assay at 24 hours after the start of the treatment.

Mitochondrial membrane potential measurement

Cells were plated in four delta T chambers (10⁴ MDA-MB-435 cells/chamber) and incubated in complete media at 37°C for 36 hours. The media was replaced with fresh media containing 1 μ M of HerGa, 1 μ M of S2Ga, HerPBK10 (at equivalent protein concentration to HerGa), and PBS, [17]. 24 hours later, the cells were washed with PBS, followed by exposure to 1 μ l of 20nM tetramethylrhodamine methyl ester (TMRM), whose accumulation in the mitochondrion is dependent on intact mitochondrial membrane potential. After TMRM accumulation in the cells reached equilibrium (~ 1 hour), two-photon excited confocal TMRM fluorescence images were acquired with and without light exposure (450nm-490nm, 17 J/cm²) at different z-depths. The results were quantified by calculating the fluorescence intensity ratio of mitochondria to cytoplasm [17, 18]. To calculate this

ratio, we measured the average fluorescence intensity for 10 different mitochondria regions and one mitochondria-free (i.e. cytoplasmic) region per cell, and averaged these ratios for all cells in three independent fields. For comparison, the mean ratios were normalized by each mean value obtained before light irradiation, respectively.

Cytoskeleton immunostaining

10^4 MDA-MB-435 cells were plated on a 12mm round coverslip in each well of a 24 well dish. After the cells were incubated for 36 hours, media was aspirated from each well and replaced with 1.0 mL complete media containing $1\mu\text{M}$ HerGa, $1\mu\text{M}$ S2Ga, HerPBK10 (at equivalent concentration with $1\mu\text{M}$ HerGa), and PBS respectively. After 1 hour, the wells were then exposed to light at 424nm (1.1 J/cm^2). After the light irradiation, all wells were washed with PBS at 24, 4, and 0 hours, and then the treated cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature (RT). Endogenous fluorescence was quenched by agitating in 50mM NH_4Cl diluted with PBS for 5 minutes at RT. The wells were again washed three times by agitating in PBS for 5 minutes, and then cell membranes were permeabilized by agitating in 0.1% TritonX-100 diluted with PBS for 5 minutes at RT followed by PBS washing again. The coverslips were then removed from their wells and placed in blocking buffer containing $1\mu\text{g/mL}$ mouse anti α -tubulin IgG and incubated overnight at 4°C . The coverslips were then washed in PBS before being placed in blocking buffer containing $6.6\mu\text{g/mL}$ AlexaFluor 488 Goat anti-mouse IgG, 137ng/mL DAPI (300nM), and 1 U/mL Rhodamine phalloidin, and incubated at RT for 30 minutes followed by washing, and mounting on slides using Prolong Gold Anti-Fade mounting media. Slides were imaged by epi-fluorescence microscopy.

Singlet oxygen detection

2×10^6 MDA-MB-435 breast cancer cells, pretreated with HerGa for 1 hour, were incubated with $80\mu\text{M}$ MVP for 30 minutes, and then chemiluminescence of MVP exposed to HerGa or control (PBS) before and after light exposure at specific wavelengths (424nm, energy/area: 0.7 J/cm^2) was measured every 20 seconds using a luminometer (Veritas, Turner Biosystems) for singlet oxygen detection. To validate the singlet oxygen production induced by HerGa, the chemiluminescence of MVP in the absence or presence of 10 to 50 mM sodium azide (NaN_3), a singlet oxygen scavenger, was measured every 20 seconds after the light exposure. Note that the MVP probe emits chemiluminescence when it reacts with singlet oxygen, but not when it reacts with other reactive oxygen species such as hydroxyl radical, superoxide or hydrogen peroxide, thus enabling to detect the singlet oxygen specifically [19].

Examination of effects on singlet oxygen on mitochondria

To test whether singlet oxygen induced by HerGa +light directly damaged mitochondria, we investigated mitochondrial membrane potential changes of cells pretreated with $1\mu\text{M}$ HerGa – and + light irradiation in the presence of NaN_3 at the indicated concentrations, 0, 4, 10, 20, and 30 nM. For this experiment, the cells pretreated with $1\mu\text{M}$ HerGa for 4 hours were washed with PBS and incubated in complete media containing NaN_3 at the indicated concentrations for 1 hour. The media was replaced with TMRM solution after washing with PBS. After 1 hour, 2-photon excited fluorescence imaging of TMRM accumulated in the cells was performed – and + light irradiation, followed by the quantitative analysis as described previously [9, 16].

Cytochrome c release from mitochondria

1×10^4 MDA-MB-435 cells were grown on coverslips in a 12-well dish (for 36 hours, followed by exposure to HerGa ($0.5\mu\text{M}$), S2Ga ($1\mu\text{M}$), HerPBK10, and PBS for 24 hours.

The media was then replaced with prewarmed (37°C) staining solution containing the MitoTracker red probe (final concentration: 50nM), and then the cells were incubated at 37°C for 30 minutes. After the staining was complete, we applied 424 nm light on the cells, and then washed the cells in fresh prewarmed PBS before processing the cells for cytochrome c staining using an Alexa fluor 488 cytochrome c apoptosis detection kit (Invitrogen, Life Technologies, Grand Island, NY, USA), following the manufacturer's procedures.

Two-photon excited and confocal fluorescence imaging

Leica TCS SP5 two-photon and SPE confocal imaging microscopes were utilized for these investigations. The Leica two-photon imaging system was utilized to measure mitochondrial membrane potential of live cells, whereas the confocal imaging system was used for both cytoskeleton damage and cytochrome c release experiments. In the two-photon imaging for the mitochondrial membrane potential measurement, 830nm femtosecond pulsed light (100fs) was utilized for excitation of TMRM accumulated in the live cells, and fluorescence from cells within 500-590 nm, which was filtered by an emission acousto-optics tunable filter, was recorded in a photomultiplier tube. During the live cell experiments, a delta T chamber was utilized in order to maintain tightly controlled ~37° C temperature. The temperature around the cells was maintained by use of a temperature-controlled stage integrated with the microscopes. In confocal imaging for the investigation of cytoskeleton disruption, a DAPI, a FITC, and a Cy3 filter channel were utilized in order to acquire nucleus, tubulin, and actin images respectively. In the cytochrome c release experiment, a FITC and a Cy3 filter channel were utilized for the acquisition of cytochrome c and mitochondrial images.

Light irradiation for photoexcitation of HerGa

A mercury lamp was used to irradiate cells during imaging. Specifically, light from the mercury lamp was filtered by excitation band-pass filters in filter-cubes and then delivered to cells through the objective utilized for imaging. For light irradiation during mitochondrial membrane potential measurements using the 2-photon and confocal microscopes, a band-pass filter (450nm-490nm) was utilized for excitation of HerGa in order to image the same field of view before and after the light irradiation. Note that this wavelength range (450nm-490nm), while not perfectly matched with the maximum absorbance of the Ga-corrole (424 nm), nevertheless still excites HerGa. For the remaining experiments, a band-pass filter (414nm-434nm) incorporated into an epi-fluorescence microscope was utilized for excitation of HerGa. Here the energy density for the light irradiation was determined by the equation: Energy density = $P \times t / A$ (unit: J/cm²), where P is light power, t is irradiation time, and A is area receiving light. Here the light power coming from the objective was measured using an optical power meter (ThorLabs). The area receiving light was determined by the field of view of the objective.

Results and Discussion

Light augments HerGa cytotoxicity in MDA-MB-435 cells

Our previous studies have established that submicromolar HerGa doses are sufficient to induce cell death and that the toxic effects can be detectable *in vitro* as early as 24 hours after exposure to the nanoparticle [9]. Moreover, our recent studies showed that light can augment HerGa-mediated cell death [16]. Here, we performed an *in vitro* dosed toxicity analysis +/- light exposure in order to quantify the enhancement of HerGa cytotoxicity by light irradiation. Our cell killing curves indicate that the CD50 of HerGa without light is ~0.1-1 nM (consistent with our previously reported data) [9], whereas irradiation reduces it to ~0.001-0.01 nM (Fig. 1A).

Furthermore, Figure 1(B) shows that the cells that were resistant to initial HerGa treatment without irradiation could still be killed by subsequent light irradiation. Cells receiving both HerGa and light exhibited rounding, and membrane blebbing immediately after irradiation, whereas cells receiving either treatment alone showed no such phenotype (Fig. 1B). These findings indicate that light at specific wavelengths can promote HerGa-mediated cytotoxicity to cancer cells (at > 10 times lower doses compared to nonirradiated cells), and further suggest that light irradiation can be used to eliminate cells that survive initial HerGa treatment.

To investigate the mechanism by which irradiation enhances HerGa-mediated cell death, we first quantified the extent to which irradiation may enhance HerGa-mediated mitochondrial damage. Mitochondrial membrane permeability changes (indicating cellular health status) of MDA-MB-435 cells treated with HerGa +/- light were compared using two-photon excited confocal fluorescence imaging. Our previous studies showed that the mitochondrial membrane potentials in HerGa-treated MDA-MB-435 cells were ~50% less than those in Mock-treated MDA-MB-435 cells [9]. In the present studies, although many HerGa-treated cells exhibited relatively lower mitochondrial membrane potentials than mock-treated cells, some cells exhibited resistance to the HerGa treatment and thus still preserved notable mitochondrial membrane potentials. Therefore, to highlight the mitochondrial damage by photoexcitation-induced HerGa cytotoxicity, HerGa-treated cells still preserving notable mitochondrial membrane potentials were selected.

In Figure 2(A), while mitochondrial and cytosolic patterns are distinguishable by fluorescence intensity before light exposure, TMRM accumulation decreased only in the HerGa-treated cells compared to control (S2Ga alone, HerPBK10 alone, or Saline) cells after the light exposure. The average ratio of mitochondrial:cytosolic TMRM fluorescence in the HerGa-treated cells decreased by about 80% after light irradiation ($P < 0.001$ compared to those without light exposure, as determined by 2-tailed unpaired t-test), indicating the collapse of mitochondrial membrane potential in those cells (Fig. 2B). S2Ga-treated cells also exhibited a compromise in mitochondrial membrane potential after light irradiation, albeit by ~30%. In contrast, control treatments (HerPBK10 alone or saline) had no impact on mitochondrial membrane potential with or without irradiation (Fig. 2B). Our previous studies have shown that while S2Ga alone can be endocytosed with serum albumin, it cannot effectively enter the cytosol without a membrane-penetrating molecule. As cytosolic entry appears to be a requirement for S2Ga toxicity, S2Ga alone is relatively nontoxic to cancer cells unless used at excessive pharmacological concentrations. Here the photoexcitation-induced mitochondrial damage in S2Ga treated cells is likely to result from S2Ga that was partially internalized by serum albumin in complete media. However, it is important to note that the enhanced mitochondrial damage in S2Ga treated cells is much less than that in HerGa treated cells (Fig. 2B). In addition, we measured the actual temperature in the cell chambers during light irradiation, as well as the remaining volume of each chamber after light irradiation, to assess whether irradiation caused heat-induced cytotoxicity. The temperature was slightly increased from 36.7°C to 36.9°C during irradiation at 450-490nm while irradiation at 510-550 nm produced no detectable temperature changes (Fig S3). In addition, we measured the volumes of remaining media in the cell chamber after light irradiation at ~37°C (which was maintained by a temperature controller) and room temperature, respectively. While the volume of remaining media at ~37°C under the temperature control was decreased from 1mL to ~950μL, the volume of the remaining solution at room temperature after the light irradiation was ~996μL, which is almost identical to the volume (1mL) before the light irradiation. These findings indicate that the volume decrease at ~37°C was due to temperature control, and not due to the light irradiation. These findings also indicate that irradiation does not cause well-heating, which could otherwise be a possible contributor to cell damage.

Light irradiation augments HerGa-mediated cytoskeletal damage

Our previous studies revealed that HerGa treatment promotes cytoskeletal disruption[9]; and we hence have now examined the effect of light irradiation on this activity. Figure 3 shows that the light irradiation used here had no detectable effect on untreated cells, which exhibited extended polymerized actin filaments as well as the web-like microtubule pattern typifying resting adherent cells. In contrast, HerGa-treated cells receiving irradiation displayed a diffuse actin distribution, suggestive of disrupted or damaged microfilaments (Fig. 3), as well as a disrupted microtubule distribution reminiscent of nocodazole damage [5]. Importantly, without the light irradiation, we could observe cytoskeletal disruption by 24h after HerGa treatment [9], whereas light irradiation induces an earlier onset of cytoskeletal disruption (within 4h after HerGa treatment).

HerGa + light induces singlet oxygen production

Next, we investigated whether HerGa receiving light at specific wavelengths can generate singlet oxygen, considering that other type of corroles, particularly antimony corroles, as well as other photosensitizers, exhibit singlet oxygen elevation upon irradiation [20, 21]. Trans-1-(2'-methoxyvinyl)pyrene(MVP), which displays chemiluminescence in the presence of singlet oxygen, was utilized for the detection of the oxidant induced by irradiation of HerGa.. Notably, it is well-established that the MVP probe emits chemiluminescence in the presence of singlet oxygen, but not in the presence of other reactive oxygen species such as hydroxyl radical, superoxide or hydrogen peroxide [19]. Figure 4(A) shows that HerGa induced chemiluminescence from MVP after light irradiation that was well over that of the controls (PBS and sodium azide). Furthermore, we examined singlet oxygen generation in the presence of different concentrations of sodium azide, a singlet oxygen scavenger, in order to verify that the oxidant was produced by HerGa-treatment with light irradiation. Figure 4(B) shows that chemiluminescence from MVP decreases as the concentration of sodium azide increases, thereby validating that singlet oxygen is indeed produced from irradiated HerGa. Amphiphilic and lipophilic nanoparticles can undergo self-aggregation in aqueous solutions. For such particles that are capable of photosensitization, undesired aggregation can reduce singlet oxygen generation as well as the targeting capability and intracellular delivery of the nanoparticle payload [22]. The gallium corroles are amphiphilic and water soluble, and therefore have somewhat the potential to be self-aggregated at relative high concentrations. However, our previous studies showed that the aggregation of the corroles is not very significant at $< 10 \mu\text{M}$ [23]. Moreover, the dose of HerGa exhibiting sufficient tumor toxicity in vitro and in vivo is well below this concentration ($1 \mu\text{M}$) [5, 9], and thus likely to avoid the undesired aggregation.

Singlet oxygen directly damages mitochondria

To determine the impact of singlet oxygen induced by HerGa +light on mitochondria, we examined mitochondrial membrane potential changes of cells treated with $1 \mu\text{M}$ HerGa after light irradiation in the presence of NaN_3 at different concentrations. In this experiment, we tested 0 to 30 nM NaN_3 , which is a low enough concentration range to avoid NaN_3 toxicity to cells. The mitochondrial membrane potential of HerGa treated cells was less disrupted by light irradiation as the concentration of NaN_3 increased (Fig. 5), suggesting that singlet oxygen produced by HerGa/light irradiation directly disrupts mitochondria.

Mitochondrial damage by photoexcited HerGa triggers cytochrome c release from mitochondria

The previous results suggest that HerGa photoexcitation promotes mitochondrial damage via singlet oxygen elevation in HerGa-treated cells. As release of mitochondrial proteins can trigger apoptosis, we examined whether photoexcitation of HerGa results in cytochrome c

release from mitochondria. Using confocal fluorescence imaging, we observed that cytochrome c diffused into the cytoplasm of only HerGa treated cells after light irradiation (Fig. 6), indicating that mitochondrial damage induced by HerGa irradiation triggers the release of this heme protein. In contrast, cytochrome c release was not observed in HerGa-treated cells without light irradiation, nor in S2Ga, HerPBK10, and PBS -treated cells after light irradiation. The cytochrome c has typically been observed during the early stages of apoptotic cell death [24]. Once in the cytosol, the cytochrome c results in the processing and activation of pro-caspase-9 in the presence of dATP, thus leading to biochemical and morphological features characteristic of apoptosis [24]. Therefore, the cytochrome c release from mitochondria due to HerGa irradiation here indicates that HerGa photo-excitation augmented cell death pathways are likely to involve the cascade apoptotic events [25].

Conclusions

The tumor-targeted corrole particle, HerGa, ablates cancer growth in mice without light irradiation [5], but recent findings suggest that photoexcitation may be used to augment this effect [16]. In the present study, we have elucidated the mechanism of irradiation-induced toxicity in HerGa-treated cells using fluorescence optical imaging. Singlet oxygen produced by the combination of HerGa and irradiation enhances disruption of the cytoskeleton and mitochondria, causing an 80% decrease in mitochondrial membrane potential and triggering apoptotic events (i.e. cytochrome c release). Previous studies [26, 27] showed that free-base corroles and corrole derivatives generate singlet oxygen when they are excited by light at specific wavelengths. In particular, a certain family of corrole compounds exhibit the potential for DNA photocleavage by singlet oxygen generation [28]. However, it is important to note that in the present study, we have for the first time elucidated the cytotoxic mechanism at the molecular level in cancer cells, and have presented evidence suggesting that the outcomes of this study hold promise for *in vivo* application. With regard to the latter, these studies present additional possible strategies for tumor elimination. This has been demonstrated by irradiation-induced toxicity to cells surviving previous HerGa-treatment without irradiation (as shown in Figure 2), thus suggesting that photoexcitation could be used as a follow-up treatment to eradicate residual cells that may have become HerGa “resistant”. The observations pointing towards prolonged tumor retention of HerGa (up to 30 days after intratumoral injection in comparison to injection of non-tumor tissue, which clears sooner) allow for a generous time window in which follow-up irradiation could be applied (Supplemental Information; Fig. S3). Thus, we trust that the insight on the mechanism of photoexcitation-mediated HerGa cytotoxicity may help direct the translation of HerGa into a feasible option in the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NIH (R01 CA140995, and R01 CA129822). Work at Caltech was supported by NIH DK019038 and the Arnold and Mabel Beckman Foundation. Work at the Technion was supported by The Herbert Irving Cancer and Atherosclerosis Research Fund and The United States-Israel Binational Science Foundation.

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Figure 1. HerGa cell death dose curve $\pm$ light

MDA-MB-435 cells were incubated with HerGa at the indicated doses for 24h before cell survival was assayed by (A) crystal violet (CV) stain, and (B) microscopy. In (A), cell survival is expressed as CV absorbance of each HerGa-treated sample normalized by mock (PBS) treated samples, or CV abs of experimental/control. Error bars represent 1 SD of triplicate treatments. In (B), the same treated cell populations are shown before ($-$ light) and after ($+$ light) irradiation.

Figure 2. Mitochondrial membrane potential variations of MDA-MB-435 cells treated with HerGa, S2Ga, PBS, and HerPBK10 with and without light irradiation

Two-photon excited fluorescence images of TMRM accumulated into MDA-MB-435 cells treated with 1 μ M HerGa, 1 μ M S2Ga, PBS, and HerPBK10 (at equivalent protein concentration as HerGa) were acquired using two-photon excited confocal fluorescence imaging (excitation: 830nm, emission: 500-580nm, 20x objective) with and without light irradiation (wavelength: 450-490nm, energy per square centimeter: 17 J/cm²). (A) TMRM fluorescence images of the treated cells with and without light irradiation. The image at the right side shows the magnified image around the region selected by the dotted rectangular. The circle and arrow indicate cytoplasm and mitochondria, respectively (B) Quantitative analysis of mitochondrial membrane potentials in the treated cells before and after light irradiation. Mean TMRM fluorescence ratios between mitochondria and cytoplasm were calculated and normalized by the mean TMRM fluorescence ratios obtained before light irradiation, respectively.

Figure 3. Effect of light irradiation on HerGa-induced cytoskeletal disruption

MDA-MB-435 cells were treated with HerGa at 1 μ M final corrole concentration, then assessed for cytoskeletal changes at 0, 4, and 24h after treatment with light exposure by fluorescence labeling of actin (red), tubulin (green), and nucleus (blue). Arrows point to structural disruption of actin and tubulin, respectively.

Figure 4. Singlet oxygen is induced by HerGa +light

MDA-MB-435 breast cancer cells (2×10^6 cells) treated with HerGa for 1 hour were incubated with 80 μ M MVP (for singlet oxygen detection) in the absence or presence of 10 to 50 mM NaN₃, and MVP chemiluminescence measured every 20 seconds after light irradiation (excitation: 424nm, energy/area: 0.7 J/cm²). (A) Time course of MVP chemiluminescence induced by HerGa in comparison to control (PBS) and sodium azide (1mM) treatment before and after light irradiation at specific wavelengths (424nm). The small and large arrows indicate the initial light irradiation and singlet oxygen generation by HerGa + light irradiation, respectively. (B) Effect of sodium azide (NaN₃) on singlet oxygen induced by HerGa.

Figure 5. Mitochondrial membrane potentials $\pm$ light irradiation in the presence of NaN_3 at the indicated concentrations (0, 4, 10, 20, and 30 nM)

(A) 2-photon excited TMRM fluorescence images of HerGa treated cells $\pm$ light irradiation at the indicated concentrations of NaN_3 . (B) Quantitative analysis of mitochondrial membrane potential $\pm$ light irradiation (left-panel) and mitochondrial membrane potential versus NaN_3 after light irradiation (right-panel).]

Figure 6. Cytochrome c release from mitochondria of HerGa treated cells with light irradiation
 Panels show immunofluorescence of cytochrome c (green) and mitochondria (red) before (-) and after (+) light irradiation. Arrows indicate cytochrome c release.