Published in final edited form as:
Photochem Photobiol. 2011 May ; 87(3): 707–713. doi:10.1111/j.1751-1097.2011.00902.x.

Analysis of the Bacterial Heat Shock Response to Photodynamic Therapy-Mediated Oxidative Stress

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Abstract

Antimicrobial photodynamic therapy (PDT) has recently emerged as an effective modality for the selective destruction of bacteria and other pathogenic microorganisms. We investigated whether PDT induced protective responses such as heat shock proteins in bacteria. Using the photosensitizer Toluidine Blue O (TBO) at sub-lethal PDT conditions, a 7-fold increase in bacterial heat shock protein GroEL and a 3-fold increase in heat shock protein DnaK were observed in *Escherichia coli* post PDT. Pretreatment with 50o C heat for 30 minutes reduced PDT killing in both *E. coli* and in *Enterococcus faecalis*, with the most pronounced inhibition occurring at 50-μM TBO with 5-J/cm^2 635 nm light, where *E. coli* killing was reduced by 2- $\log_{10}$ and *E. faecalis* killing was reduced by 4-$\log_{10}$. Finally, inhibition of the highly conserved chaperone DnaK using a small molecule benzylidene lactam heat shock protein inhibitor potentiated (but not significantly) the effect of PDT at a TBO concentration of 2.5 μM in *E. faecalis*; however, this effect was not observed in *E. coli* presumably because inhibitor could not gain access due to Gram-negative permeability barrier. Induction of heat shock proteins may be a mechanism whereby bacteria could become resistant to PDT and warrants the need for further study in the application of dual PDT-heat shock protein-inhibition therapies.

Introduction

The need for novel antimicrobial techniques has become critical for a number of reasons. Excessive prescription and misuse of antibiotics accelerates the emergence of resistant strains and existing antimicrobials work poorly in chronic infections even when susceptibility is tested and confirmed *in vitro* (1,2). The issue of resistance is best underscored by the recent and formidable emergence of vancomycin-intermediate and vancomycin-resistant, methicillin-resistant *Staphylococcus aureus*, that are considered highly virulent and lethal strains (3). Additionally, antibiotics are incapable of eradicating bacterial biofilms and spores, such as those found in *Staphylococcus spp.* and soil *Bacillus spp.*, respectively (4,5). As the end of the antibiotic age approaches, it becomes vital to develop novel solutions for the treatment of infections.

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Photodynamic therapy (PDT) was accidentally discovered in 1900 when Raab *et al.* noted the antimicrobial action of acridine and light on *Paramecium spp.* (6). In the latter part of the twentieth-century, PDT emerged as a therapeutic clinical modality, receiving regulatory approval for the treatment of neoplastic and ophthalmological conditions, and has demonstrated efficient eradication of bacteria both *in vitro* and *in vivo* (7–9). During PDT, hyperproliferating cells take up non-toxic, light-sensitive dyes known as photosensitizers (PS). Cells are then irradiated with the appropriate visible wavelength, causing the PS to transition to an excited singlet state. Through intersystem crossing, the PS then reaches a triplet state with a sufficiently long lifetime to react with molecular oxygen. Such interactions result in the formation of reactive oxygen species (ROS) through either a Type I or Type II photochemical pathway (10).

The Type I pathway involves electron-transfer reactions from the triplet state photosensitizer, generating toxic ROS, including superoxide, hydroxyl radicals, and hydrogen peroxide. The Type II pathway involves an energy transfer from the triplet state photosensitizer to ground state molecular oxygen, yielding the highly reactive and transient singlet oxygen ($^1O_2$). $^1O_2$ and ROS are capable of oxidizing nucleic acids, lipids, and proteins, ultimately causing cellular inactivation and death (10).

Heat shock proteins (HSPs) are a group of ubiquitous chaperone proteins responsible for the refolding, repair and recycling of damaged proteins and stabilization of lipid membranes during cellular stress (11–13). In microbial cells, the heat shock proteome has best been characterized in *Escherichia coli*, and it has been found that two major HSP families– GroEL/GroES and DnaK/DnaJ/GrpE – are chiefly responsible for protecting against stress in both Gram-negative and Gram-positive bacteria (14–18). Upregulation of HSPs in *E. coli* occurs several minutes after stress, increasing up to 50-fold the original concentration, only to subsequently stabilize until a given stress diminishes (19). Moreover, upregulation of HSPs during oxidative, antibiotic, osmotic, and acid stress is associated with resistance to these stresses, and upregulation of HSPs prior to subsequent stress enables bacterial cells to acquire “tolerance” to the particular stress (18).

DnaK/DnaJ/GrpE and GroEL/GroES protein repair works in an ATP-dependent process whereby DnaK and GroEL bind stretches of exposed hydrophobic residues on partially denatured proteins. GroEL folding occurs when a non-native polypeptide is encapsulated in a GroEL-GroES complex, driving the peptide into a cavity where folding occurs (20). By an unknown mechanism in the DnaK system, refolding occurs in concert with co-chaperones GrpE and DnaJ in conjunction with DnaK-mediated ATP hydrolysis (21). Moreover, HSPs are recognized as major targets during oxidative stress and it has been suggested that the chaperone DnaK acts as a “shield,” protecting proteins against oxidative stress (22). Previously, it has been demonstrated that the DnaK and GroEL families confer resistance to a wide array of stresses including antibiotic stress (23,18). A study by Ziegelhoffer *et al.* showed that in *Rhodobacter sphaeroides*, a Gram-negative “purple bacterium,” synthesis of the alternative sigma factor E (σ^E) increased post $^1O_2$ stress; σ^E is one member of the major HSP transcription factors and initiates sigma factor 32 (σ^{32}), the transcription factor of DnaK and GroEL in *E. coli* and other Gram-negative bacteria (15,24,25). HSP genes, specifically *dnaK*, are associated with carotenoid biosynthesis in *S. aureus*, where carotenoids are pigments capable of quenching ROS and $^1O_2$ (18). It has also been demonstrated that increased HSP expression of eukaryotic homologs of GroEL and DnaK, the 60 kDa heat shock protein HSP60 and the 70 kDa heat shock protein HSP70, respectively, occurs in murine and human cancer cells post PDT-mediated oxidative stress (26–28,22).

To date, the relationship between bacterial HSPs and PDT-mediated stress in bacteria has yet to be extensively elucidated. There is only one report by Bolean and Paulino *et al.* who demonstrated that GroEL levels increased following Rose Bengal-mediated PDT of the Gram-positive *Streptococcus mutans* (29). Consequently, the goals of this study were threefold: to determine if expression of DnaK and GroEL increases post PDT; to determine if heat pretreatment provides increased stress tolerance to PDT; and to analyze the efficacy of dual HSP-inhibitor, antimicrobial PDT.

Materials and Methods

Bacterial strains

The microorganisms used in this study were *Escherichia coli* ATCC 33780 and *Enterococcus faecalis* ATCC 29212, chosen as representatives of Gram-negative and Gram-positive bacteria, respectively. Samples were routinely suspended in brain heart infusion (BHI) (Becton, Dickinson, and Company, Franklin Lakes, NJ) broth and grown overnight, aerobically in a 37o C shaker incubator (New Brunswick Scientific, Edison, NJ). Cells were removed at an optical density (OD) of 1.0 at 600 nm, approximately equivalent to $10^{8–9}$ colony forming units (CFU)/mL as determined by spectrophotometry (Thermo Scientific, Waltham, MA)

Photosensitizer

A stock solution of 1 mM Toluidine Blue O (TBO) (Sigma-Aldrich, St. Louis, MO) was prepared in distilled water and stored at 4o C in the dark for no more than two weeks prior to use. For photodynamic inactivation, TBO was diluted to the desired concentrations in phosphate buffered saline (PBS).

Photodynamic inactivation minimum inhibitory concentration (MIC) determination

To determine the effects of sub-lethal PDT stress on bacterial HSP expression, the minimal inhibitory concentration (MIC) of TBO in the light and dark was first established. MIC is defined as the lowest concentration of a drug that will inhibit visible growth after overnight incubation, where growth is defined as the presence of turbidity in wells (30). 100 μL of diluted TBO (1000–0.50 μM) was added to 100 μL *E. coli* samples of $10^{8–9}$ CFU/mL suspended in BHI broth. PBS was used for control. Samples were placed in 96-well plates (Becton, Dickinson, and Company) and light samples were exposed to a custom built white LED illuminator device designed to homogenously illuminate a 96-well plate. Cells were irradiated for 30 min at a power density of 15.0 mW/cm^2 as measured by a power meter (Thorlabs, Newton, NJ) to deliver a total fluence of 13 J /cm^2. Spectrophotometry was then performed using the SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA) where optical density of bacterial samples in BHI broth with varying TBO concentrations was compared to broth with varying TBO concentrations alone. The point at which the treated culture OD_{600} was greater than the OD_{600} of broth and TBO alone was considered the highest concentration that did not induce lethal effects.

Photodynamic stress conditions

Results of the MIC test demonstrated that whereas 62.5 μM TBO in concert with 13 J/cm^2 light did not permit visible growth, 31.25 μM TBO in concert with 13 J/cm^2 white light allowed for visible growth. Accordingly, for sub-lethal PDT, 100 μL of 31.25 μM TBO was added to 100 μL PBS cell suspensions ($10^{8–9}$ CFU/mL) of *E. coli*. Samples were irradiated for 30 min as described above. Following stress, cells were prepared for SDS-PAGE.

For lethal PDT (killing studies), cells were illuminated with 5 J/cm^2 of 635-nm light using a Lumacare LC-122 lamp (Lumacare, Newport Beach, CA) at concentrations of 50, 25, 5, and

2.5 μM TBO. The TBO absorption peak is 633 nm, such that excitation of this PS at this wavelength resulted in greater ROS production as opposed to broad-band white light (31). Samples were incubated with PS in the dark for 5 min and PDT was performed. Samples were then plated following methods by Jett *et al.* (32).

SDS-PAGE

Following PDT, cell lysis was performed using the CelLytic™ B Plus kit (Sigma-Aldrich). To ensure equal loading of protein concentration, each sample was harvested at an $OD_{600} \approx 1.0$ (concentration of 10^{8-9} CFU/mL) and the bichinchoninic acid (BCA) protein quantification method was used. Cell lysates were then boiled at 90o C and treated with reducing Laemmli buffer containing 10% SDS (w/v) and 5% β-mercaptoethanol (w/v). 11–15 μL of cell lysates (corresponding to a protein concentration ≈ 7 μg) and 6 μL prestained SDS-PAGE standard solution weight ladder (Sigma-Aldrich), were loaded and separated *via* electrophoresis using the XCell II Surelock Mini-Cell (Invitrogen, Calsbad, CA). A NuPAGE^{R} 4–12% Bis-Tris (pH 8.5) Gel (Invitrogen, Carlsbrad, CA) was used and SDS-PAGE was performed at 100 V for 2 hours. The gel was then stained with 0.1 % Coomassie^{R} stain (Thermo Scientific) to check loading, destained in distilled water, and prepared for immunoblotting.

Western blot

Protein samples were transferred onto a 0.2 μm nitrocellulose membrane (Invitrogen) using the XCell II Surelock Blot Module (Invitrogen) for 1.5 h and membranes were blocked with Tris-buffered saline containing 5% (w/v) skim milk and washed three times in TBS containing 0.1% (v/v) Tween (TBS-T). Washing was performed for 5 min and membranes were incubated with mouse IgG mAb against *E. coli* HSPs GroEL and DnaK (Enzo Life Sciences, Farmingdale, NY) overnight at 4° C at dilutions of 1:2,000 and 1:10,000, respectively, following the manufacturer's recommendations. After rinsing in TBS-Tween, membranes were then incubated with horseradish peroxidase-conjugated secondary antibody goat anti-mouse IgG (Fab) mAb (Enzo Life Sciences, Inc.), diluted at 1:5,000 as recommended by the manufacturer. Finally, membranes were washed twice with TBS-T and once with TBS and developed using Novex^{R} ECL Chemiluminescent Substrate Kit (Invitrogen) which was imaged using the Kodak Image Station 4000R (Carestream Health, Rochester, NY). Note that no loading control was used, consistent with previous bacterial HSP studies that incorporate immunoblotting (33,34). Accordingly, for accurate comparison of experimental and control groups, all cells were harvested at the same time at an OD_{600} of 1.0, and the BCA assay was used to ensure equal protein loading, similar to procedures of other works that did not incorporate loading controls (35,36).

Dot blots

To allow for rapid determination of relative HSP expression values, a BCA assay was used and lysates were boiled at 90o C and treated with reducing Laemmli buffer containing equal protein concentration (7 μg) were manually loaded on nitrocellulose membranes. Membranes were allowed to dry and blocking, antibody incubation, and detection procedures were performed as described above. Densitometry was performed on the Kodak Image Station 4000R to compare relative mean net luminescence intensities, given in arbitrary units. Blotting was performed in triplicate and the positive and negative error bars are given by the standard deviation of samples from the mean value.

Heat pretreatment and tolerance studies

To determine if pretreatment with 50o C heat (a known upregulator of HSPs) would reduce cell killing by PDT, cells were first harvested at an OD_{600} of 1.0. Samples were then

centrifuged at 12,000 rpm for 3 min and resuspended in PBS and incubated with desired TBO concentrations for 5 min. Lethal PDT was performed as described earlier.

HSP-inhibitor and PDT combination therapy

To determine if inhibiting bacterial heat shock protein DnaK would enable photodynamic effects at low concentrations of TBO and light, 100 mM stock solution of heat shock protein inhibitor I (KNK437, *N*-Formyl-3,4-methylenedioxy-benzylidene-γ-butyrolactam) (Calbiochem, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO, $(CH_3)_2SO$) (Sigma). First, a 100 mM KNK437 solution was dissolved in DMSO, and the MIC was determined, as stated earlier. The final concentration of DMSO in cells was < 0.5% (v/v). Once the MIC was determined, samples were exposed to 100 μL 500 μM KNK437 in conjunction with 2.5 μM TBO and 5 J/cm^2 635 nm light. Samples were then serially diluted on agar and colonies were counted.

Statistical Analysis

One-way Analysis of Variance (1-way ANOVA) was used to compare survival fractions of bacterial samples following combination therapy. Analysis was performed using Microsoft Excel Statistical Package (Microsoft, Redmond, Washington) and results were considered significant when $p < 0.05$.

Results and Discussion

Upregulation of GroEL and DnaK in E. coli following PDT

GroEL expression was analyzed immediately after 30 minutes of sub-lethal photodynamic inactivation. Compared to cells grown in BHI and cells exposed to 31.25 μM TBO without light, there was an observed approximate 3-fold increase in GroEL expression as determined by dot blot (Fig 2). To confirm the specificity of antibody binding and to demonstrate positive upregulation of GroEL to 50o C heat, a conventional western blot was performed. Western blot revealed approximate kDa weights of GroEL and DnaK to be 65 and 75, respectively (Fig 3A and 3B). These results are consistent with previous findings (37). It is not possible to compare intensities of bands between Figs 3A or 3B and those in Fig 3C because the images were captured from different gels using different magnifications and exposure times.

This PS concentration and light dose corresponded to less than 1 $\log_{10}$ loss in cell viability (data not shown), indicating that sub-lethal PDT stress was, indeed, occurring inside cells. Moreover, if increased HSP expression is used as a marker for internal stress, then TBO in the dark at a concentration of 31.25 μM was not sufficient enough to induce toxic conditions that warrant upregulation of GroEL (Fig 2 and Fig 3C).

Immediately after PDT, there was an observed 3-fold increase in intracellular DnaK concentrations (Fig 4). Similar to GroEL, DnaK may act as a marker for intracellular stress, such that the slight increase in DnaK observed in the dark may indicate stress from TBO localization and subsequent irregularities in the *E. coli* cell wall. Heat at 50o C demonstrated a significant increase in DnaK concentrations, as determined by the western blot (Fig 3C).

It was surprising that GroEL had a greater increase after PDT than was found for DnaK as detected by immunoblotting. Although DnaK is generally considered more responsive to heat stress, this may, perhaps, indicate that GroEL is predominately involved in stabilizing membranes and participating in post-ribosomal synthesis folding of proteins, a ubiquitous feature of chaperonin protein family members (38). Augmentation of internal GroEL levels is, thus, consistent with reports of TBO localization in bacterial cells: TBO, a cationic

phenothiazinium dye (Fig 1), of the same chemical class as methylene blue, is known to directly bind to the negatively charged lipopolysaccharide (LPS) present on the outer cell envelope of Gram-negative bacteria and to the anionic teichuronic acid residues of the Gram-positive peptidoglycan cell walls, such that when TBO is excited, the predominate form of bacterial destruction is *via* membrane stress (39,31,40). Furthermore, a report by Fredriksson *et al.* demonstrated that in *E. coli*, the GroEL/GroES chaperone complex was more important than the DnaK/DnaJ/GrpE in protecting against protein carbonylation; it is thought that protein carbonylation is a major oxidative byproduct of PDT (16,41)

Although DnaK levels increased 3-fold, it is entirely possible that some DnaK was not detected. This may be explained by considering that HSPs, including DnaK and the homologous HSP70 of eukaryotes, are significantly upregulated in an intracellular manner in response to oxidative stress (26,22). Several studies of bacteria exposed to H_2O_2 stress have shown that the DnaK molecule itself is significantly oxidized, as determined by 2,4-dinitrophenylhydrazine (DNPH) immunoblotting for protein carbonyl groups on DnaK, a marker for oxidative stress (42). After oxidation of amino acids, protein aggregation may occur, preventing effective detection *via* immunoblotting methods.

Prior heat pretreatment confers resistance to PDT in E. coli and E. faecalis

To determine if upregulation of HSPs may limit the effectiveness of PDT for the inactivation of microbes, cell samples were preheated for 30 min at 50o C, a known positive inducer of HSPs (Fig 3). We found that previous heat treatment led to ≈ 2 $\log_{10}$ less killing than of *E. coli* at a TBO concentration of 50 μM and a reduction in killing of about 0.5 $\log_{10}$ at a TBO concentration of 5 μM as compared to killing without heat pretreatment (Fig 5).

Unlike *E. coli*, *E. faecalis* had an extremely pronounced reduction in cell death when preheated before PDT. Whereas 2 $\log_{10}$ less killing was observed in *E. coli* post PDT at 50 μM, heat pretreatment-induced more resistance (approaching 4 $\log_{10}$ less killing) in *E. faecalis* (Fig 6).

It is interesting that *E. faecalis* acquired more tolerance to PDT stress, compared to *E. coli*, considering Gram-positive bacteria are known to be significantly more susceptible to the effects of PDT (7,8). But this is not unexpected, as *E. faecalis*, and most *Enterococci* are known to be able to express intense stress resistance (43,44,17). Most notably, a study by Boutibonnes *et al.* demonstrated that pretreatment with 50o C heat for 30 min conferred 95% survival of bacterial cultures in otherwise lethal, 60o C heat (43). It is also known that HSPs do not discriminate between the types of stress that leads to their upregulation, and that there may be cross-induction of stress protection.

TBO-PDT, KNK437 combination therapy

After demonstrating that pretreatment with 50o C heat conferred stress tolerance to PDT, inhibition of DnaK by a HSP-inhibitor, a benzylidene lactam called KNK437, in conjunction with PDT was explored. After incubating cells with 500 μM KNK437 and subjecting them to PDT at a TBO concentration of 2.5 μM, it was found that KNK437 did indeed result in an enhancement of TBO-PDT killing of *E. faecalis* by ≈ 53%, although not statistically significant, $p = 0.299$ (Fig 7). On the contrary, combination therapy of HSP-inhibitor and TBO-PDT had no noticeable effect on *E. coli* susceptibility compared to low-level PDT (Fig 8). It is important to note that incubation with 500 μM KNK437 had no effect on *E. faecalis* or *E. coli* viability (Fig 7 & Fig 8).

The difference in KNK437-mediated combination therapy amongst the two species tested may relate to the composition of Gram-negative and Gram-positive cell structures. It is well accepted that the presence of anionic LPS and the Gram-negative double membrane reduces

binding and subsequent uptake of hydrophobic compounds (45). Considering KNK437 is hydrophobic and requires dissolution in DMSO, it is possible that the KNK437 molecules bound less to *E. coli*, as the LPS would repel the hydrophobic molecule. This hypothesis is consistent with the observation whereby Gram-negative bacteria are inherently resistant to hydrophobic antibiotics (46). Moreover, hydrophobic molecules have been employed for the destruction of Gram-positive bacteria, including *Bacillus subtilis*, explaining the efficacy of dual therapy in *E. faecalis* (47).

Thus, future research is needed to evaluate the efficacy of combination therapy consisting of PDT and HSP inhibitors. Currently, the supply of HSP inhibitors is limited, and thus there are obvious limitations with this aspect of the work. An HSP inhibitor that selectively targets bacterial DnaK would be ideal and has been investigated by Credito *et al.*, where application of CHP-105, a synthetic peptide, allowed for levofloxacin-mediated killing of otherwise levofloxacin-resistant Gram-negative bacilli (23). At the time this study was conducted, CHP-105 was not commercially available.

Conclusion

This study effectively demonstrated that the expression of two major bacterial HSPs, GroEL and DnaK, increased 7-fold and 3-fold in *E. coli*, respectively, as determined by immunoblotting. Heat pretreatment allowed for a reduction of *E. coli* killing by 2 $\log_{10}$ and *E. faecalis* killing by 4 $\log_{10}$ at a TBO concentration of 50 μM. Finally, combination therapy demonstrated some potentiation of killing in *E. faecalis* and no noticeable potentiation in *E. coli*. It has been suggested that microbes are incapable of developing resistance to PDT due to the non- site-specific killing of bacteria by photosensitizers (48). Previous studies have attempted to induce resistance to PDT, by repeatedly killing and re-growing bacterial samples (49). However it is well accepted that it is more likely that bacteria will develop resistance by growing in the presence of a low level stressor, best exemplified by the emergence of resistant organisms in antibiotic-fed livestock (50). PDT is usually designed to be a relatively short procedure that would last somewhere around 5 min (51). In our study, sub-lethal PDT was performed using white light over an extended period of time (30 min).

As PDT emerges as a potent approach for the inactivation of localized microbial infections and sterilization of wounds and surgical sites, it is crucial to evaluate PDT efficacy impartially. This study has demonstrated that following sub-lethal PDT, there is a significant upregulation of bacterial HSPs, and that upregulation prior to PDT may confer stress tolerance. Although combination therapy did not prove to be statistically significant, this study underscores a need for novel combination therapies, thus warranting the exploration of HSP inhibition in concert with PDT.

Acknowledgments

The authors would like to thank Ursula Jakob and the Jakob Lab of the Department of Molecular, Cellular, and Developmental Biology at the University of Michigan for advice on immunoblotting techniques. The authors would also like to thank Gitika Kharkwal and Sulbha K. Sharma of the Wellman Center for Photomedicine for assistance with SDS-PAGE. Research in the Hamblin laboratory is supported by the US-NIH (grants R01AI50875, R01CA/AI838801 to MRH and R01CA137108 to Long Y Chiang), the Center for Integration of Medicine and Innovative Technology (DAMD17-02-2-0006), the CDMRP Program in TBI (W81XWH-09-1-0514) and the Air Force Office of Scientific Research (F9950-04-1-0079).

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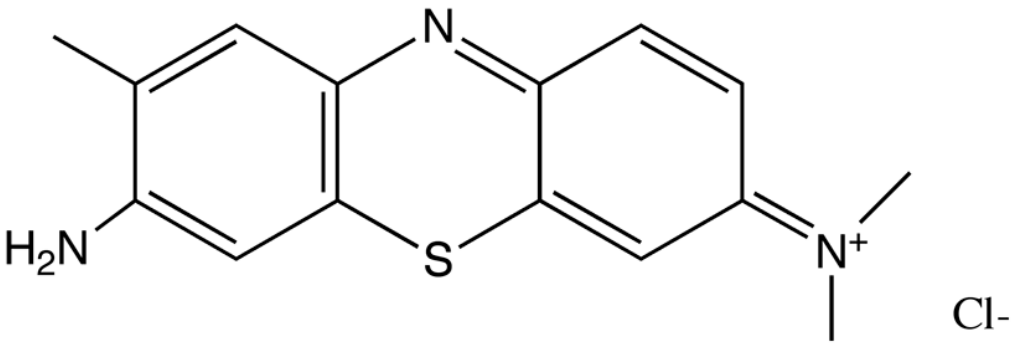

Fig 1.
Molecular structure of Toluidine blue O (TBO).

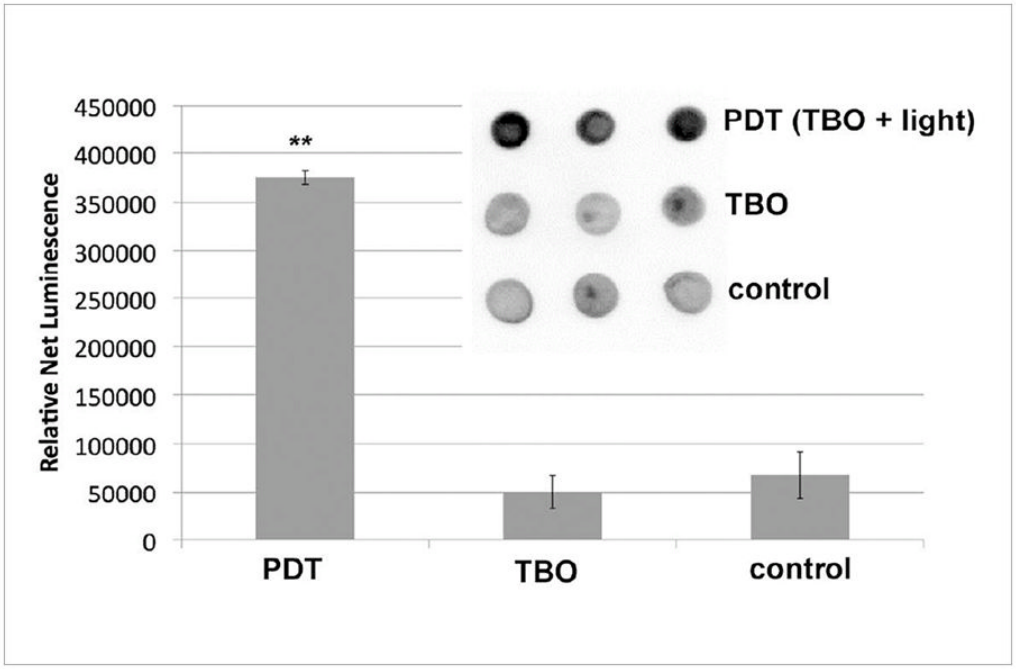

Fig 2.

GroEL expression in *E. coli* 30 min after PDT. PDT consisted of 31.5 μM TBO and 13 J/cm^2 of white light. 1) Results from triplicate dot blots of cell lysate with standard immunoblotting technique collected from a single experiment. 7 μg protein per dot, as determined by BCA assay. (2) Mean relative expression values as determined by densitometry; bars are SD; ** P <0.01 vs TBO and vs control (ANOVA).

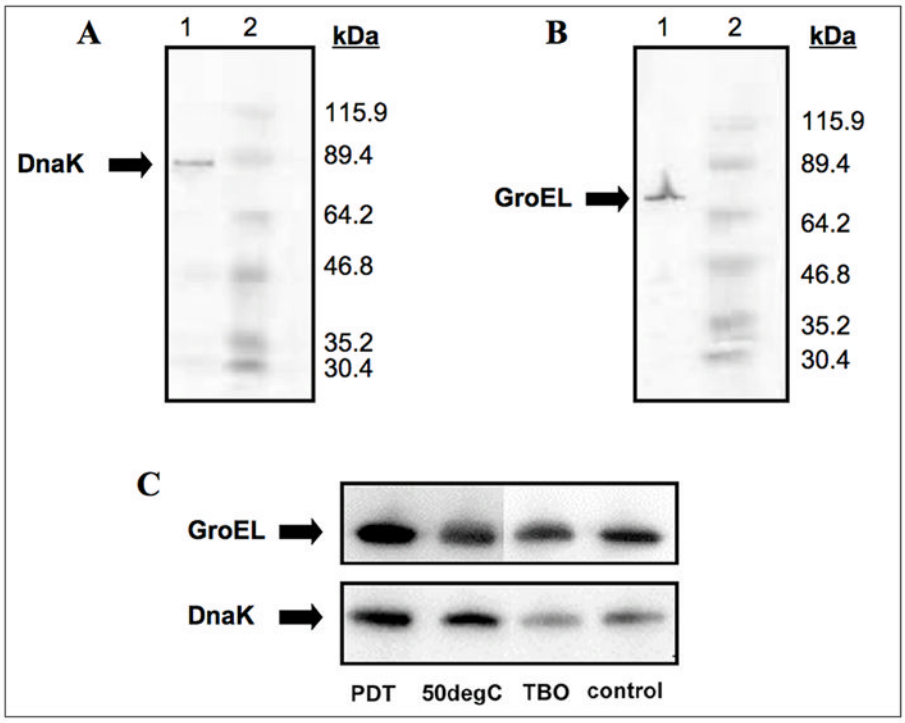

Fig 3.

Western blot analyses of major bacterial HSP family members. (A) Western analysis of DnaK. Lane 1, *E. coli* cell extract, samples were detected with mouse mAb anti-*E. coli* DnaK; lane 2 molecular weight ladder. (B) Western analysis of GroEL. Lane 2, *E. coli* cell extract; lane 2 molecular weight ladder, samples were detected with mouse mAb anti-*E. coli* GroEL. For both experiments, *E. coli* cells were harvested after growth overnight and lysed. Lysates were then subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and subsequently detected. Molecular weight ladders are as follows: -galactosidase from *E. coli* (115,900), lactoferrin from human milk (89,400), pyruvate kinase from chicken muscle (64,200), ovalbumin from chicken egg (46,800), lactic dehydrogenase from rabbit muscle (35,200), triosephosphate isomerase from rabbit muscle (30,400). (C) Comparison was made between PDT (31.25 μM TBO + 13 J/cm^2 of white light, 50° C heat shock, 31.25 μM TBO in the dark, and control *E. coli* cells. For all blots, 7 μg protein was loaded in each well. To ensure equal loading of protein, a BCA assay was performed.

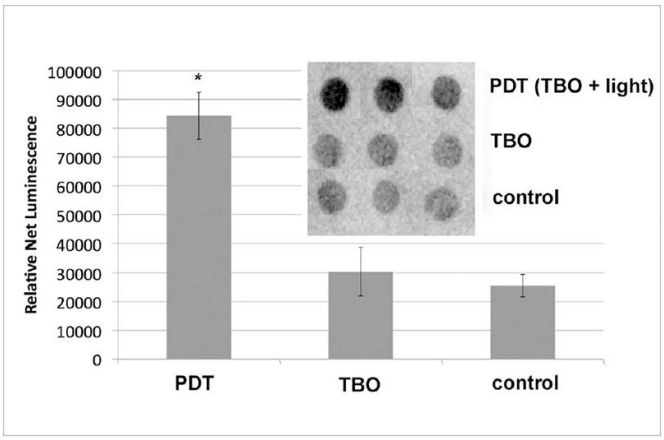

Fig 4.

DnaK expression in *E. coli* 30 min after PDT. PDT consisted of 31.5 μM TBO and 13 J/cm^2 of white light. (1) Results from triplicate dot blots of cell lysate with standard immunoblotting technique collected from a single experiment. 7 μg protein per dot, as determined by BCA assay. (2) Mean relative expression values as determined by densitometry; bars are SD; * P <0.05 vs TBO and vs control (ANOVA).

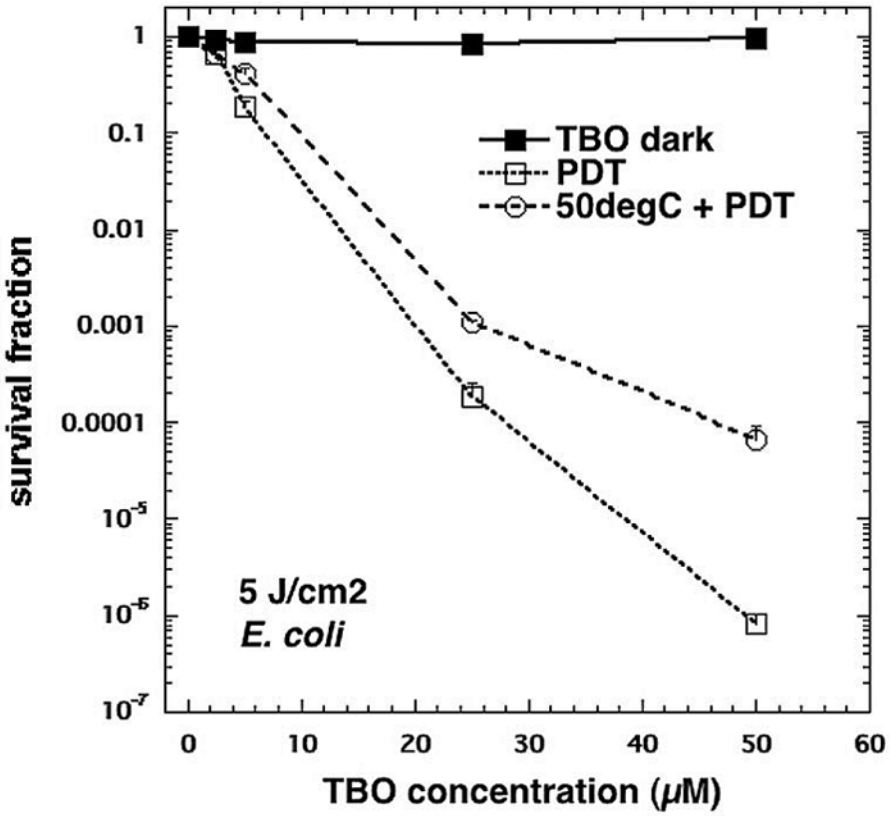

Fig 5.

The effect of heat pretreatment on *E. coli* susceptibility to TBO-PDT lethal stress. Following pretreatment at 50° C or 37° C (control) for 30 min, samples were immediately incubated with various TBO concentrations and subjected or not to 5 J/cm^2 of 635-nm light. TBO dark = cells exposed to varying TBO concentrations without light, PDT = TBO + 5J/cm^2, 50degC + PDT = 30 min 50° C followed by immediate PDT with varying concentrations of TBO + 5 J/cm^2. Cell death is expressed in terms of survival fraction relative to absolutecontrol; bars are SD; * P <0.05 vs PDT alone (ANOVA).

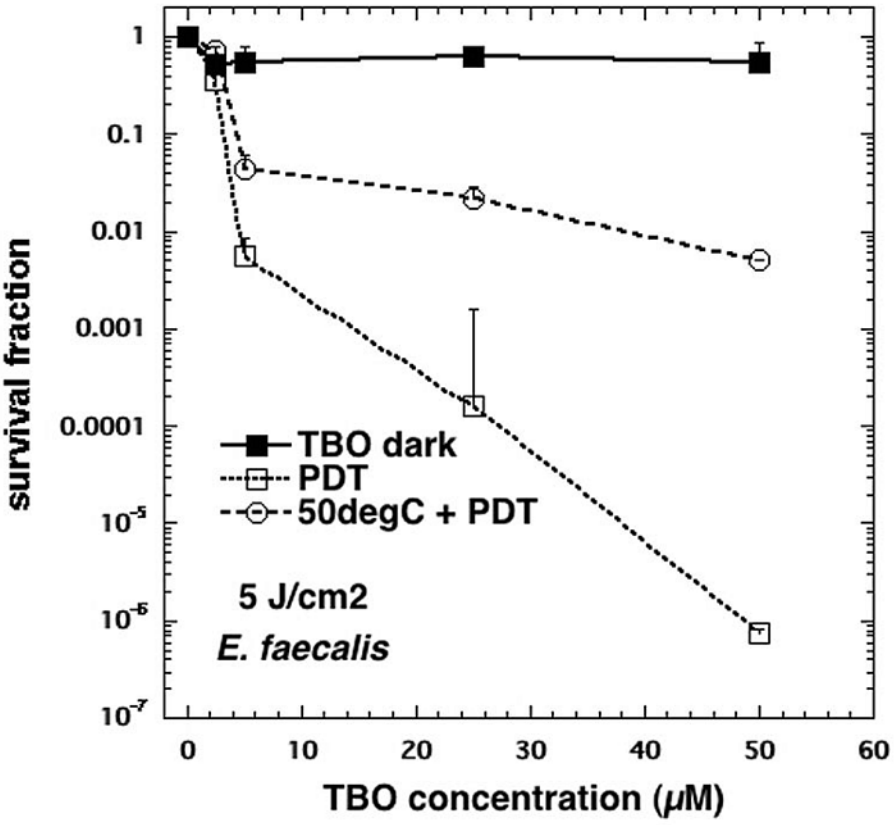

Fig 6.

The effect of heat pretreatment on *E. faecalis* susceptibility to TBO-PDT lethal stress. Following pretreatment or not at 50° C at or 37° C (control) 30 min, samples were immediately incubated with various TBO concentrations and subjected or not to 5 J/cm^2 of 635-nm light. TBO dark = cells exposed to varying TBO concentrations without light, PDT = TBO + 5J/cm^2, 50degC + PDT = 30 min 50° C followed by immediate PDT with varying concentrations of TBO + 5 J/cm^2. Cell death is expressed in terms of survival fraction relative to absolute control; bars are SD; ** P <0.01 vs PDT alone (ANOVA).

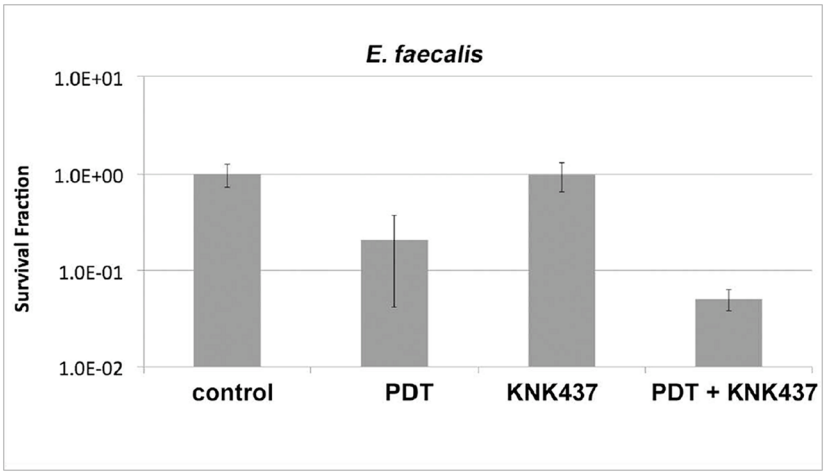

Fig 7.

The effect of pretreatment with KNK437 on *E. faecalis* susceptibility to 2.5 μM TBO + 5 J/cm^2 of 635-nm lig**ht.** After a 1 h incubation with 500 μM KNK437, samples were resuspended in fresh PBS and incubated with 2.5 μM TBO for 5 min. Irradiation reached 5 J/cm^2.

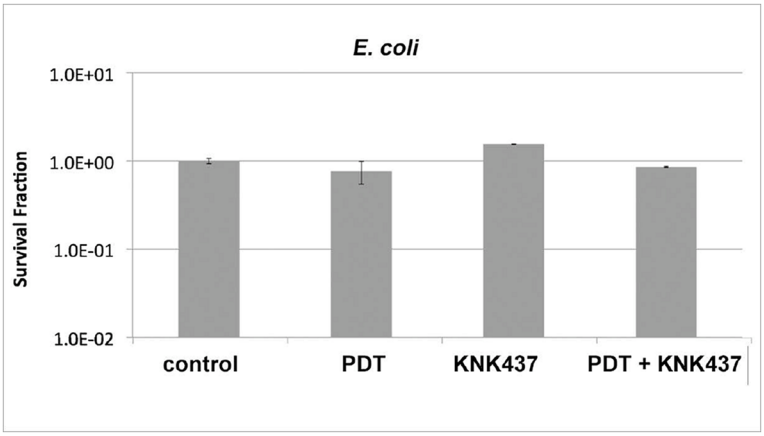

Fig 8.

The effect of pretreatment with KNK437 on *E. coli* susceptibility to 2.5 μM TBO + 5 J/cm^2 of 635-nm light. After a 1 h incubation with 500 μM KNK437, samples were resuspended in fresh PBS and incubated with 2.5 μM TBO for 5 min. Irradiation reached 5 J/cm^2.