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# Comparing the photodynamic treatment of OSCC between natural and artificial photosensitizers

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**Abstract. Purpose:** Since Verteporfin is the generally accepted photosensitizer for OSCC and other forms of skin cancer. Its synthetic structure and broad range in absorption has made it harmful to both tumor and healthy cells and tissues. The study aims to see whether a natural and herbal extract like Chlorophyllin, used in primarily bladder cancer, can become an alternative to Verteporfin. **Methods:** This study uses HSC-2 cell lines and mice models. The cancer cells in vitro and in vivo will be treated with increasing amounts and various durations of Chlorophyllin and Verteporfin. Killing in vitro is measured by MTT assay and in vivo is measured by xenograft tumor size. Positive control is Verteporfin, and negative control is DMSO in vitro or saline solution for the xenograft experiment. **Possible results:** The three main possible results: (1) Chlorophyllin kills more cancer cells in vitro and in vivo and it has less killed normal tissue. (5) Verteporfin kills more cancer cells but also has more damage to normal tissues when compared to Chlorophyllin. (8) Verteporfin kills more cancer cells in vitro and in vivo and it has less killed normal tissue. **Conclusion:** The results of the study will determine if natural alternatives are worth investing research into as they can be low-cost extraction, low toxicity, and high selectivity of cells to kill. Chlorophyllin can pave the way to more intensive research of natural photosensitizers in different types of cancers.

**Keywords:** Oral squamous cell carcinoma, Yes-associated protein, human papillomavirus, Hippo pathway, photosensitizer, Photodynamic therapy, Verteporfin, Chlorophyllin.

## 1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant epithelial neoplasm affecting the oral cavity. It represents more than 90% of head and neck cancers [1]. There are around 300,000 cases of OSCC every year, making it among the 10 most common cancers worldwide. Major etiological and predisposing factors for OSCC includes smoking and drinking, but also human papillomavirus (HPV), nutritional deficiencies and genetic predisposition have been said to be associated as well [1]. The Yes-associated protein (YAP) is a downstream effector of the Hippo pathway and is involved in the tumorigenesis of OSCC [4]. YAP acts as a key transcription cofactor to regulate cell migration, proliferation, and survival. Dysregulation and heterogeneity of the Hippo pathway can cause an upregulation of YAP which then induces tumor initiation, progression and metastasis. With increasing evidence that suggests the accumulation of YAP is related to oral cancer treatment resistance, targeting YAP could represent a key opportunity in mitigating tumor progression and metastasis of OSCC.

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Photodynamic therapy (PDT) is a relatively new method that can target YAP through tagging it with Verteporfin, a photosensitizing dye or photosensitizer. The photosensitizer must have a chromophore, a set of conjugated unsaturated bonds which absorb visible light at a particular wavelength [2]. Then by shining a light on the lesion matching the wavelength and absorption spectrum of the photosensitizers, the photosensitizers exert a cytotoxic effect. Choosing the most appropriate photosensitizer is highly important when considering effective PDT. Verteporfin (VP) is a benzoporphyrin derivative, and a more commonly used artificial photosensitizer, that has also been proven to be a suppressor of YAP protein [1]. VP serves as an inhibitor between YAP and TEAD transcription factors which blocks transcription activation of targets downstream of YAP. VP has a max absorption of 690 nm and incubation time of 30-150 minutes. A natural photosensitizer made from herbal extracts with a similar absorption spectrum and potentially less toxicity could serve as a more effective alternative for VP. The natural photosensitizer that will be examined and compared to Verteporfin is Chlorophyllin. Chlorophyllin is a promising photosensitizer extracted from cyanobacteria and the chloroplasts of algae and plants [3]. Bladder cancer studies involving PDT and Chlorophyllin found that it can localize in lysosomes suggesting the major mechanisms of chlorophyllin PDT in cancer cells is autophagy and apoptosis [5]. Chlorophyllin has a very similar and optimal absorption wavelength of 600-670 nm compared to Verteporfin [3]. Factors to be compared include the quantity of cancer cells destroyed in vitro and in vivo as well as the selectivity of the photosensitizer and impact on healthy tissue and organs. All these factors will be accounted for to determine the effectiveness and quality of artificial and natural photosensitizers. I predict that increasing concentrations and treatment durations of chlorophyllin with HSC-2 OSCC cells leads to increased killing in vitro and reduced tumor size in HSC2 xenografts in vivo with lower toxicity compared to Verteporfin due to absorption of shorter wavelengths.

## 2. Methods

### *2.1. Materials*

Verteporfin and Chlorophyllin. HSC-2 Cell line. Antibodies against YAP. DMSO water as a negative control. Healthy female and male mice (19-21 grams in weight).

### *2.2. Cell line & culture*

Human oral squamous cell carcinoma cells were purchased. 4 6 well plates were dosed and used at the 0, 1, 3, 6, 12, and 24 hour points in treatment duration . In every plate, 2 wells were treated with only DMSO and treated as controlled. 2 wells were treated with 20μL of a 5, 10, 25, 50μM Verteporfin solution. 2 wells were treated with 20μL of a 5, 10, 25, 50μM Chlorophyllin solution.

### *2.3. MTT assay*

Inoculate HSC-2 cells of 0, 1, 3, 6, 12, and 24 hour points in treatment into separate 96-well plate at 2,500-5,000 cells/well in 100 μL volume and incubate overnight to allow cell adhesion. Gently remove the media, and then introduce 50 μL of serum-free media and 50 μL of MTT solution into each well. Allow the plate to incubate at 37°C for 3 hours. Following incubation, supplement each well with 150 μL of MTT solvent. Cover the plate with foil and agitate it on an orbital shaker for 15 minutes. If needed, occasionally pipette the liquid to ensure complete dissolution of the MTT formazan. Measure the absorbance at OD=590 nm, and perform the reading within 1 hour.

### *2.4. Cell Proliferation assay*

In the MTT assay, calculate the average of duplicate readings for each sample and then subtract the background absorbance from the culture medium. The absorbance value correlates with the cell number.

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### *2.5. Cell Cytotoxic assay*

In the MTT assay, calculate the average of duplicate readings for each sample and then subtract the background absorbance from the culture medium. Determine the percentage of cytotoxicity using the formula: % cytotoxicity = (100 x (control - sample)).

### *2.6. Xenograft assay*

Prepare 4-5 million HC2 cells in 100 μL of PBS. Select 10–12-week-old female/male homozygote athymic nude mice, preferably with a body weight of around 20 grams (g). Anesthetize the mice with isoflurane during the procedure. Inject 100 μL of the cells in PBS into the mouse dorsal region using a 27-G needle. Allow HSC-2 to engraft in mice for 11 weeks. Then using intravenous injections, inject 35uL of 0.1, 0.5, 1 and 2 mg/mL of saline, Verterporfin and Chlorophyllin solution into different mice for a duration of 0, 1, 3, 6, 12, and 24 days.

### *2.7. PCR*

Determine the annealing temperature for each primer. Formulate a PCR reaction mix by blending 50 μL of GoTaq Green, 2 μL of the Right Primer, and 2 μL of the Left Primer. Thoroughly mix the components through vortexing and a brief centrifugation. Label PCR tubes with initials and sample numbers. Prepare individual PCR tubes for all cDNA samples by combining 9.5 μL of Nuclease-free water, 13.5 μL of PCR Reaction mix, and 2 μL of cDNA. Place all labeled tubes in a sample rack kept in an ice bucket on the center bench. Organize the samples in a block within the incubator, adjust the incubator settings accordingly, and patiently await the designated incubation period.

## 3. Results

**Table 1.** Combination of Possible Results.

| Possible Observations | CR1 | CR2 | CR3 | CR4 | CR5 | CR6 | CR7 | CR8 |
|---|---|---|---|---|---|---|---|---|
| Increase cancer cell killing after light? (in vitro) | + | - | + | + | - | + | - | - |
| Increase cancer cell killing after light? (in vivo) | + | + | - | + | - | - | + | - |
| Decrease killing of normal tissue by confocal microscopy after light (in vivo) | + | + | + | - | + | - | - | - |
| Supporting Hypothesis | Yes | Partially | Partially | Partially | Partially | Partially | Partially | No |

*Note*. "+" represents a positive result (chlorophyllin works better than Verteporfin). "-" represents a negative result (chlorophyllin works worse than Verteporfin)

**Possible Result 1:** Chlorophyllin kills more cancer cells in vitro and in vivo. It has less killing of normal tissue.

**Possible Result 2:** Verteporfin kills more in vitro but Chlorophyllin kills more cancer cells in vivo and has less damage to normal tissue.

**Possible Result 3:** Verteporfin kills more in vivo but Chlorophyllin kills more cancer cells in vitro and has less damage to normal tissue.

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**Possible Result 4:** Chlorophyllin kills more cancer cells but also has more damage to normal tissues when compared to Verteporfin.

**Possible Result 5:** Verteporfin kills more cancer cells but also has more damage to normal tissues when compared to Chlorophyllin.

**Possible Result 6:** Chlorophyllin kills more cancer cells in vitro but Verteporfin kills more cancer cells in vivo and has less damage to normal tissues.

**Possible Result 7:** Chlorophyllin kills more cancer cells in vivo but Verteporfin kills more cancer cells in vitro and has less damage to normal tissues.

**Possible Result 8:** Verteporfin kills more cancer cells in vitro and in vivo. It has less killing of normal tissue.

**Treatment Concentration and Duration Possible Results:**

Both the increase in treatment concentration and duration of Chlorphyllin in the cultured plates and xenografts will result in more, less or no change in cancer cell killing. The amount of normal tissue damaged could increase, decrease, or not change with the increase of treatment concentration and duration.

## 4. Discussion

Previous studies have frequently demonstrated Chlorophyllin's ability as a photosensitizer. Li et al. has investigated chlorophyllin as a novel option in PDT against human bladder cancer cells. They used T24 and 5637 bladder cancer cell lines and they were incubated with Chlorophyllin and irradiated with a 650 nm laser light [6]. PDT with chlorophyllin exhibited significant phototoxicity in both T24 and 5637 cells, resulting in 82.43 and 85.06% cell death [6]. Moreover, Chlorophyllin has indicated its high solubility in aqueous solutions, easy and low cost extraction process, and low toxicity [6]. All necessary and important factors to consider when choosing a photosensitizer. With similar wavelengths to Verteporfins, Chlorophyllin is a strong candidate for a more effective Verteporfin alternative for photodynamic therapy in OSCC [6].

Combination result 1 indicates that Chlorophyllin is the more selective and effective photosensitizer compared to Verteporfin in OSCC. The smaller range in wavelength allowed Chlorophyllin to target cancer cells and avoid wasting its efforts on healthy tissue cells. After proving that natural photosensitizers are a viable alternative to synthetic photosensitizers, compare and find other existing natural photosensitizers with properties similar to or better than Chlorophyllin.

Combination result 2 reveals that Chlorophyllin is more effective and selective in vivo but not in the culture plates. The plates are cultured to have specifically HSC-2 cells. Chlorophyllin may be better at identifying cells and then going for the cancer cells, but Verteporfin is better at killing cells. A part of this result has to do with the range of wavelengths that Verteporfin and Chlorophyllin function at, with Verteporfin having a larger range. Future experiments from this result could be investigating what makes Verteporfin kill more intensely with the same concentration as a natural photosensitizer. Then, measuring exactly at what concentration can Chlorophyllin produce the same intensity of cancer cell killing as Verteporfin in the culture plates.

Combination result 3 says that Chlorophyll is more effective in vitro than Verteporfin, but still has a lower killing of healthy tissues. If this result is true, then the explanation of wavelength selectivity can be eliminated. A possible explanation for this result is Verteporfin's additional ability to inhibit YAP transcription factors. There is no YAP or hippo pathway in culture plates so although Chlorophyll eliminates more cancer cells directly through PDT, Verteporfin can both directly eliminate cancer cells through Reactive Oxygen Species and inhibition of YAP in mice. Further experiments can compare Verteporfin in vitro with Verteporfin in vivo to see how much of its anti-cancer properties is due to its ability to react with oxygen to generate ROS or its ability to inhibit YAP.

Combination result 4 indicates that Chlorophyllin is more effective in killing cancer but not as selective in the cells it kills. This result is possible if the hypothesis that the importance of the wavelength on selectivity is not as significant as hypothesized. The smaller range in wavelength doesn't have a huge effect on the cells Chlorophyllin targets and doesn't stop it from killing healthy tissues any less than

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Verteporfin. In fact, in this case, Chlorophyllin kills more cells compared to Verteporfin. Future experiments can look at multiple photosensitizers and exactly how large or small the range of wavelength should be to decrease the damage in healthy tissues for a certain amount.

Combination result 5 indicates that Chlorophyllin is less effective in killing cancer cells but more selective in the cells it does kill. This result complies with the idea that the natural components in Chlorophyllin are less toxic to organisms than synthetic photosensitizers like Verteporfin. Also, this result could be a false negative. The concentration needed for Chlorophyllin to take full effect is not reached. Future experiments can look at increasing concentration of Chlorophyllin to produce the same intensity as a set concentration of Verteporfin. Then compare if, at that point, the damage to healthy tissues from Chlorophyllin is still less than Verteporfin.

Combination result 6 reveals that Chlorophyllin is more effective in killing cancer cells in vitro but not in vivo, and it damages healthy tissues more than Verteporfin. This result is possible with the explanation from CR4 and CR3. The range of wavelength is not as significant as hypothesized when looking at its relationship to selectivity of cells killed. Also, Verteporfin has its ability to generate ROS and also inhibit YAP transcription factors in the hippo pathway. This combination of cancer cell killing makes Verteporfin more effective in vivo. Future experiments can focus on finding similar photosensitizers like Chlorophyllin with a decreased killing in healthy tissues. Then, slowly increase the concentration to produce the same intensity as a set concentration of Verteporfin. Finally, compare if, at that point, the damage to healthy tissues from this photosensitizer is still less than Verteporfin.

Combination result 7 reveals Chlorophyllin is more effective in killing cancer cells in vivo but not in vitro, and it damages healthy tissues more than Verteporfin. This result is possible if Chlorophyllin has some sort of inhibition of a protein or pathway like Verteporfin with YAP. Although Chlorophyllin will lose out in effectiveness in a cultured plate of cancer cells, its potential ability to inhibit a mechanism and cause apoptosis is greater than Verteporfin in vivo. Future experiments can be dedicated to finding out exactly what that mechanism is in vivo.

Combination result 8 indicates that Chlorophyllin is less selective and effective as a photosensitizer when compared to Verteporfin. This result would reiterate the fact, in CR4, that smaller range in wavelength doesn't have a huge effect on the cells Chlorophyllin targets and doesn't stop it from killing healthy tissues any less than Verteporfin. This result will also confirm that Verteporfin is better at killing with both its direct ROS approach in vitro and its ROS production and YAP inhibition in vivo. It would also prove the opposite of the hypothesis, demonstrating that Verteporfin is the foremost choice in OSCC photodynamic therapy. Future experiments can focus on finding other natural photosensitizers to compare to Verteporfin with.

## 5. Conclusion

In conclusion, this study explores the effects Chlorophyllin will have on OSCC by comparing it to a generally used synthetic photosensitizer in the same treatment, Verteporfin. The multiple result possibilities of the study will determine if Chlorophyllin is a more, similar, or less effective alternative in OSCC photodynamic therapy. The observations in this study can provide incentives to research more natural alternatives to the popular synthetic photosensitizers, and its usages in other types of cancer. Verteporfin has proven that photosensitizers not only can kill tumor cells through generating ROS but through other mechanisms as well. By exploring the multitude of possible natural photosensitizers, there would be more options in photodynamic therapy and cancer treatment.

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