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## Acridine Orange as a Novel Photosensitizer for Photodynamic Therapy in Glioblastoma

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**Abstract**

*Object:* Photodynamic therapy is an exciting treatment modality that combines the effects of a chemical agent with the physical energy from light or radiation to result in lysis of cells of interest. Acridine orange is a molecule with fluorescence properties that was demonstrated to possess photosensitizing properties. The objective of this study was to investigate the photodynamic effect of acridine orange on glioblastoma cell viability and growth.

*Methods:* Glioblastoma cells (n = 8000 cells/well at 0 hours) were exposed to acridine orange followed by white unfiltered light-emitting diodes (LED) light. Cultures were exposed to either 10 or 30 minutes of light. The cell number per well was determined at 0, 24, 48, and 72 hours after exposure.

*Results:* A dramatic cytotoxic effect of acridine orange after exposure to as little as 10 minutes of white light was observed. There was almost complete eradication of the glioblastoma cells over a 72-hour period. Although acridine orange or light alone exhibited some effect on cell growth, it was not as pronounced as the combination of acridine orange and light.

*Conclusions:* This is the first study to demonstrate the photodynamic effect of acridine orange in glioblastoma cells. This data supports the need for further studies to characterize and evaluate whether this striking cytotoxic effect can be achieved in vivo. The combination of acridine orange and exposure to white unfiltered LED light may have potential future applications in management of glioblastoma.

## Introduction

Photodynamic therapy (PDT) is a treatment modality for targeting and treating disease that combines a sensitizing agent and light. The sensitizing agent accumulates in the cells of interest, and when activated by light, results in formation of cytotoxic molecules.<sup>1</sup> The utility of photodynamic therapy in the skin is perhaps one of the most studied applications given the accessibility of skin diseases.<sup>2,3</sup> It is clinically used to treat a range of inflammatory conditions such as acne and neoplastic conditions such as actinic keratosis and squamous cell carcinoma in-situ.<sup>4</sup> The premise of treatments with PDT relies on selective accumulation of the sensitizing agent in the tumor or inflammatory cells.<sup>4</sup>

PDT has been employed with promising results during the surgical resection of primary brain malignancies<sup>5</sup> This application may be invaluable during resection to further optimize the extent of macroscopic and microscopic tumor resection and improve survival.<sup>6</sup>

In this study we propose to evaluate the utility of acridine orange as a photosensitizing agent for PDT of glioblastoma. The photodynamic effect of acridine orange was first noted while studying the effects of acridine on paramecia cultures.<sup>7</sup> Von Tappenier and colleagues continued with testing photodynamic therapy using acridine orange for skin cancers.<sup>8</sup> The effects of acridine orange as a single agent in the inhibition of tumor growth in mice were described in the late 1940s.<sup>9,10</sup>

Interest in acridine orange has since grown as a vital stain and an antitumor agent. The accentuation of toxicity of acridine orange after exposure to light was observed in early studies and has been more recently established in a series of experiments examining the photodynamic effects on cultured cells.<sup>11-13</sup> Most recent reports focused on the utility of acridine orange topical application, although intravenous application of acridine orange has also been proposed.<sup>14,15</sup> Direct application of acridine orange to the tumor bed followed by exposure to light has been proven as an effective adjunct to intramarginal sarcoma resections. Furthermore, direct application of acridine orange during surgery has been shown as a radiation-sensitizing agent enhancing the efficacy of postoperative radiation if applied directly to the tumor bed.<sup>16</sup>

Acridine orange has not yet been studied as a photosensitizing agent in brain tumors. In addition to being a photosensitizing agent, acridine orange is also an excellent gross contrast agent as well as an ideal contrast agent for in-vivo microscopy.<sup>17-20</sup> Its selection for applications in neurosurgical oncology therefore serves as both a contrast agent for macroscopic and microscopic tumor identification as well as for PDT.<sup>21,22</sup> This may therefore maximize its potential use for microscopic tumor eradication in neurosurgery, particularly for glioblastomas.

In addition to a photosensitizing agent, a light source is necessary for the photodynamic therapeutic effect. Light source modalities range from lasers to light-emitting diodes (LED).<sup>23</sup> Recent trends in dermatologic photodynamic therapy demonstrated the creative application of natural daylight in treatment of actinic keratosis, particularly of the scalp.<sup>24,25</sup> The method has been shown to be as effective as conventional photodynamic therapy which uses filtered light with restricted wavelengths.<sup>26,27</sup> For the purpose of this study, we utilized unfiltered white light from an LED with a luminous flux (LUX) similar to that of direct sunlight (100,000 LUX).<sup>24</sup>

## Materials and Methods

*Cell Lines:* To demonstrate the combined effect of acridine orange and light on the glioblastoma cell lines, 8000 cells/well of adherent U-373 MG (Sigma-Aldrich, Cat. # 08061901) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) and supplemented with 10ml, 10% fetal bovine serum. The well plates (TPP, Cat. # 92006) were incubated for 2 days before being exposed to acridine orange and/or light.

At each time point (0-72 hours), three saline control wells with no exposure to light were counted. These wells were always kept in the dark and briefly exposed to low-intensity room light for minutes during counts. The absolute numbers per well and the average for the three wells per time point were recorded (Figure 1a and b show averages). There were five different treatment regimens tested. Acridine orange alone, 10 minutes of white light alone, 30 minutes of white light alone, acridine orange plus 10 minutes of white light and acridine orange plus 30 minutes of white light. Nine wells per treatment were prepared to account for 3 measurements at 24 hours, 48 hours, and 72 hours per treatment. The measurements per well were recorded and average determined for each time point as demonstrated in Figure 1a and b.

*Acridine Orange Preparation:* Acridine orange was prepared by diluting acridine orange base (dye content 75%) (Sigma-Aldrich, CAS # 494-38-2) in saline at a final concentration of 0.001 mg/ml and used at 1ml per well.

*Light Therapy and Intensity:* After the wells were prepared, white unfiltered light from CREE XM-L T6 LED bulbs calibrated to a distance so that light incident at the level of the culture is at 100,000 LUX. The light source and intensity were selected so that no significant change in temperature was detected at 10 minutes and 30 minutes (measured using an infrared thermometer). Also, 100,000 LUX is similar to the intensity of daylight. The temperature of the cultures was measured and the light was then applied for 10 minutes and 30 minutes based on the treatment. The temperature at the end of treatment was measured and confirmed to be constant. Wells were then stored in a dark incubator

and low-light environment was used whenever wells were removed from the incubator for counts. Exposure to light in treatments involving acridine orange was performed within 5 minutes after acridine orange was added to the cultures, and was performed only once at 0-hour mark of the experiment.

*Counting Method:* Trypan blue exclusion of live cells on the Neubaure chamber hemocytometer was counted from the following formulation for each condition. Counting was carried out in three individual experiments (n=3).

$$\text{Total cells/ml} = \frac{\text{Total cells counted} \times \text{dilution factor}^* \times 10,000}{\text{Number of squares counted}}$$

\*In this study trypan blue was used 1:1, therefore, the dilution factor is 2.

Briefly, at the end point of the experiment, cells were detached using 0.5% trypsin (Gibco, Life Technology, Cat. # 25300-054) and collected in 1 ml of IMDM+10% fetal bovine serum (FBS). Next, 10  $\mu$ l of cell suspension was mixed with the same amount of trypan blue and then 10  $\mu$ l of it was placed under the cover slide of a hemocytometer. Finally, 5 squares of the slide were counted under a regular light microscope.

## Results

The results are outlined in Figures 1a-c and Fig 2. Treatment of the cultured glioblastoma cells with acridine orange and light showed impressive cytostatic and cytotoxic effects within 72 hours.

At 24 hours, there was a significant reduction in the counts from wells exposed to both acridine orange and light (PDT wells) [ $p = 0.003$  (10 min) and  $p = 0.0005$  (30 min)]. There was no significant difference between 10-minute and 30-minute light exposure times for these wells. There was a mild, insignificant reduction in the number of cells in the wells with either acridine orange alone or exposure light alone at 24 hours.

At 48 hours and 72 hours, there is marked reduction in the cell counts in wells with photodynamic therapy. There was still no significant difference between the 10-minute and 30-minute light treatments. The wells exposed to either acridine orange or light alone did show a significant reduction in counts compared with control wells ( $p = <0.005$  at 48 hours and  $p = <0.0001$  at 72 hours). However, the wells treated with PDT still exhibited significant reduction in comparison with all other wells ( $p = <0.0001$ ).

One interesting finding was that counts after 72 hours in photodynamically-treated wells were not only reduced in comparison with control wells at 72 hours, but they were lower than the starting point counts at zero hours, with counts approaching 0 (Figures 1c, 2). This demonstrates a sustained cytotoxic effect that increases with time.

## Discussion

The utility of PDT to augment neurosurgical tumor excisions has been an area of interest and study for the past 50 years.<sup>28,29</sup> Early PDT studies demonstrated the utility of hematoporphyrin as an in-vivo and in-vitro photosensitizer for the treatment of glioblastoma.<sup>28,29</sup> Since then; multiple agents in the porphyrin family have been demonstrated as photosensitizers including; porfimer sodium (photfrin), 5-aminolevulinic acid (ALA), boronated porphyrin, hematoporphyrin (HPD), tetrakis (3-hydroxyphenyl) chlorin (mTHPC, temoporfin), photoporphyrin IX and talaporfrin sodium.<sup>30-33</sup> Photodynamic therapy using porphyrin derivatives, however, often results in prolonged systemic side effects, especially if the photosensitizing agent is injected.<sup>34</sup> This led to development of second- and third-generation photosensitizers with more specificity toward tumor cells while sparing normal cells.

Several studies using photosensitizers demonstrate the potential that PDT provides in treating high-grade gliomas. A recent phase II multicenter randomized controlled trial showed an increase in median survival in patients receiving photodynamic therapy for high-grade gliomas in comparison with the control arm.<sup>5</sup>

Acridine orange is a non-porphyrin molecule that is classified as an aniline dye and has different characteristics from other photosensitizers. It has a low molecular weight (265 g/mol) and is a hydrophobic base that is capable of quickly passing the plasma membrane, entering the cytoplasm, and accumulating in lysosomes.<sup>35</sup> This property is essential for success of photosensitization.<sup>36</sup> Acridine orange particularly accumulates in lysosomes with high acidity.<sup>37,38</sup>

High-grade malignancies tend to have highly acidic extracellular conditions secondary to active cell metabolism in hypoxic conditions and therefore preferentially accumulate acridine orange.<sup>37,38</sup> Interestingly, in addition to its accumulation in low pH structures, acridine orange electrostatically binds to DNA and RNA.<sup>39</sup> The excitation spectrum (wavelength ~460–500 nm) of acridine orange depends on its concentration and its location/aggregation state within the cell.<sup>40</sup> At this wavelength, light does not penetrate the tissues efficiently because of increased scattering. This means that to illicit the photodynamic effect, illumination requires direct application to the surface being treated. It also implies that the photodynamic toxicity to the underlying normal tissues is limited, possibly reducing side effects.

This phenomenon is particularly important in neurosurgical procedures and is unlike conventional photosensitizing agents which absorb light in the 600–800nm spectrum with high penetrance. Acridine orange attached to nuclear double-stranded DNA emits green light (wavelength ~525 nm) as it exists as a non-aggregated monomer.<sup>41,42</sup> This form of excitation carries no toxicity and is useful as a fluorescent dye for microscopy.<sup>41-43</sup> Orange

light (wavelength ~590 nm) is emitted when acridine orange is sequestered in acidic compartments such as lysosomes.<sup>42</sup> The latter is regarded as the photosensitizing state of this molecule. At this low pH, acridine orange is more likely to result in oxygen radical formation and cell death.<sup>37,43,44</sup> For this purpose, acridine orange has been used as a marker for apoptotic cells when orange fluorescence is detected as the lysosomal density and activity increases.<sup>45</sup>

Lysosomal accumulation of acridine orange also accounts for prolonged accumulation within malignant cells because it is not readily eliminated from these cells. We demonstrated this lasting effect in our experiment as the number of cultured glioblastoma cells continued to diminish 72 hours after application of the acridine orange. This prolonged accumulation in malignant cells may also account for the apparent selectivity of acridine orange PDT to malignant cells and sparing normal cells in clinical trials.<sup>15,37,44,46</sup>

Several studies documented the success of acridine orange PDT in marginal resections, particularly of sarcomas.<sup>14,16,47-49</sup> Similar to our study, the photodynamic effect of acridine orange has been established in cultured osteosarcoma cell lines and consequently has led to its clinical use in sarcoma surgery.<sup>15</sup> One important advantage of acridine orange is its utility as a gross and microscopic fluorescent contrast agent.<sup>21,22</sup> The topical addition of acridine orange has been used for gross tumor delineation as well as an in- vivo microscopic fluorescence agent.<sup>50</sup> This allows for the possibility to grossly and microscopically use fluorescence-guided resection of gliomas followed by PDT for elimination of residual tumor cells. Acridine orange also exhibits excitability following radiation, enhancing its possible utility as a radiation sensitizing agent in the adjuvant setting.<sup>16</sup>

Future considerations and studies should examine the ideal mode of photosensitizer delivery and light application as they influence the efficacy of treatment. Most photosensitizing agents are injected intravenously, with the therapeutic light applied directly to the tumor resection bed.<sup>51,52</sup> Studies have shown that topical application of photosensitizing agents, such as in dermatologic applications, is also effective.<sup>34</sup> Fluid-filled balloons that distribute the light to the cavity walls have been studied to evenly distribute the therapeutic light throughout.<sup>5</sup> Recently, a few studies have also demonstrated the potential application of interstitial PDT applied via laser fibers for intra-tumor light delivery in non-resectable and recurrent glioblastomas.<sup>53-55</sup>

Reports and studies of intravenous and topical use in humans do not report immediate or long-lasting side effects,<sup>14,15</sup> however, there currently is no substantial literature documenting the toxicity of acridine orange and further studies are needed. Mutagenic effect has been observed in bacteria<sup>56-58</sup>; however, it has not been extensively studied in humans and large animals.

**Conclusions**

We have demonstrated, for the first time, the potentially impressive photodynamic effect of acridine orange on cultured glioblastoma cells. Treatment with acridine orange with as little as 10 minutes of white unfiltered light exhibited a significant cytotoxic effect that increased with time. Acridine orange is a photosensitizing and contrast agent that with further optimization of delivery and light-exposure strategies may hold promise as a treatment modality for glioblastomas. Further studies validating its safety and in-vivo photodynamic effect are warranted.



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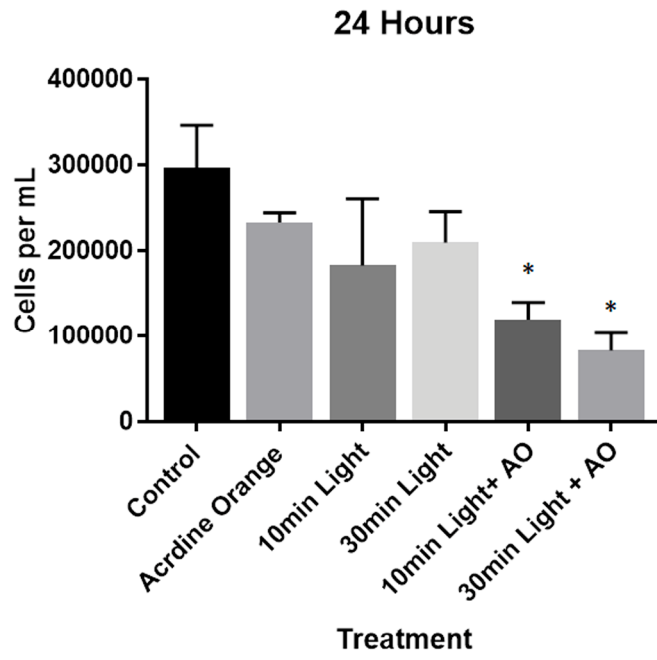
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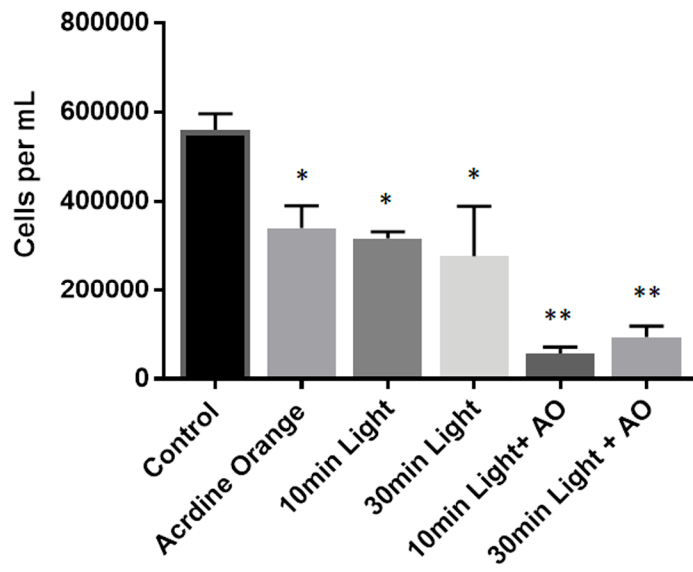
### **Figure Legends**

Figure 1. Graphical representation of cross-sectional cultured cell counts at 24 hours (a), 48 hours (b), and 72 hours (c) demonstrating the enhanced effect of combining acridine orange and light. Wells treated with acridine orange and exposed to light showed statistical reduction in cell counts at 24 hours, 48 hours, and 72 hours. Wells with exposure to either light or acridine orange alone showed significant reduction only after 48 hours. Also, there was a significant reduction in counts in photodynamically treated wells compared with those with either acridine orange or light alone at 48 and 72 hours ( $P < 0.005$ ). Statistical analysis was performed using Tukey's multiple comparison's test. Values shown are means  $\pm$  standard deviation of three measurements. \*  $P < 0.005$ ; \*\*  $P < 0.0001$ . Abbreviations: AO, acridine orange; min, minutes.

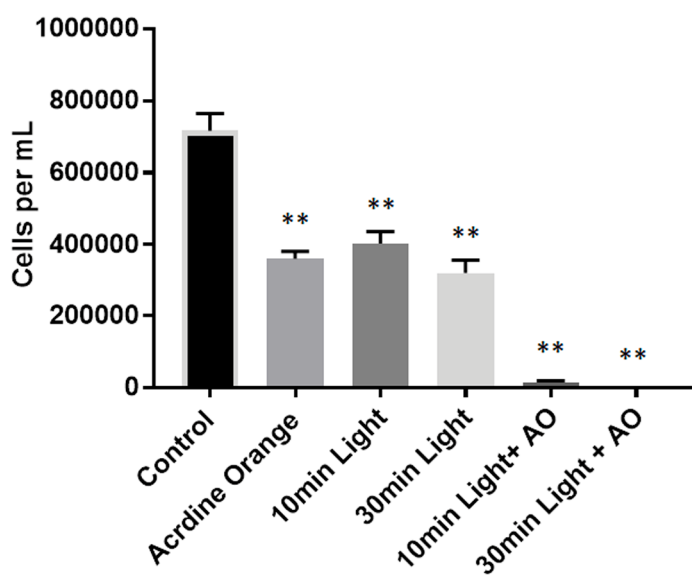
Figure 2. Graphical representation of culture counts showing the dramatic photodynamic effect of acridine orange combined with light. Cultured cells with added acridine orange and exposed to as little as 10 minutes of light demonstrated complete, sustained, and progressive eradication of the cells within 72 hours. Acridine orange and light alone demonstrated mild reduction in the cultured cell counts in comparison with the control. Abbreviations: min, minutes, AO: acridine orange.



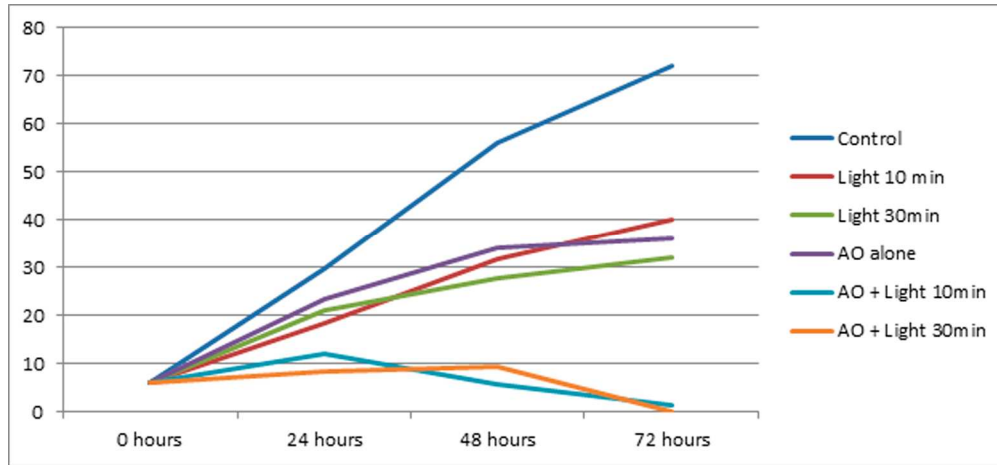
## 48Hour Data



## 72hour Data







### Highlights

- Acridine orange photodynamic effect is demonstrated in glioblastoma cell lines
- Treated glioblastoma cells had lower counts at 72 hours compared with starting point
- 10-minute white light is sufficient to elicit acridine orange photodynamic response
- Further optimization of acridine orange photodynamic therapy in-vivo is required

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February 5, 2018

Edward C. Benzel, MD  
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Dear Dr. Benzel:

The authors have no financial disclosures or conflict of interest in regard to this manuscript, and all ethical standards have been strictly followed.

Sincerely,

Aaron A. Cohen-Gadol, MD, MSc

## Abbreviations

**Acridine Orange as a Novel Photosensitizer for Photodynamic Therapy in Glioblastoma**

AO: acridine orange

ALA: aminolevulinic acid

FBS: fetal bovine serum

HPD: hematoporphyrin

IMDM: Iscove's Modified Dulbecco's Medium

LED: light-emitting diodes

LUX: luminous flux

PDT: photodynamic therapy