

OPTIMISATION OF LIGHT DOSE AND PRELIMINARY STUDIES ON THE EFFICACY OF SYMMETRICAL DIIODINATED SQUARAINE FOR PDT APPLICATIONS ON SKIN TUMOR INDUCED MODELS

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ABSTRACT

Photosensitive agents and light have been used for medical purposes for a very long time. Photodynamic therapy (PDT) involves the delivery of photosensitizers to tumor tissues followed by irradiation with light of corresponding wavelength. The present study is focused on the optimization of light dose which can effectively induce tumor regression by PDT using Symmetrical diiodinated squaraine, one of the newly developed photosensitizers, on skin tumor models. Skin tumor was induced in Swiss albino mice by the topical application of DMBA and croton oil. For selecting the dose of light for squaraine PDT which can effectively cause tumor regression the parameters like the number of tumors per mouse and mean tumor volume were analysed before and after treatment. The optimum light dose was found to be 100 J/cm². The hematological parameters were also analysed in all the groups. Significant change was observed in the levels of haematological parameters after squaraine PDT. As a whole, the dye selected for our study, Symmetrical diiodinated squaraine, was found to be a promising agent for PDT applications.

KEYWORDS: Haematological Parameters, Photodynamic Therapy, Swiss Albino Mice, Symmetrical Diiodinated Squaraine, Tumor Statistics

INTRODUCTION

Photodynamic therapy (PDT) is gaining acceptance as a technique for cancer treatment in recent years (Dougherty et al. 1998; Sharman et al. 1999; Brown et al. 2004). Photodynamic therapy (PDT) has emerged as an alternative strategy for treating cancer. PDT consists of three main components: a photosensitizer, light, and oxygen. PDT takes advantage of an appropriate wavelength of light that excites a photosensitizer to its singlet excited state and a subsequent intersystem crossing to reach the triplet energy state (Jurranz et al. 2008; Ortel et al. 2009). In the presence of molecular oxygen, energy is transferred to relax the excited state of the photosensitizer. This energy transfer in turn excites molecular oxygen to form excited, singlet state oxygen. Singlet oxygen induces cell death via damaging oxidation or redox-sensitive cellular signaling pathways, thus mediating the effects of PDT (Jurranz et al. 2008; Huang et al. 2008). Intriguingly, PDT has also been shown to regulate processes beyond tumor cell death including tumor angiogenesis and modulation of the immune system (Jurranz et al. 2008; Dolmans et al. 2003; Gollnick et al. 2010; Reddy et al. 2006). Each photosensitizer is activated by light of a specific wavelength (Dougherty et al. 1998). This wavelength determines how far the light can travel into the body (Vrouenraetes et al. 2003).

An ideal photosensitizer should meet the following criteria that are clinically relevant: a commercially available pure chemical, possessing low dark toxicity but strong photocytotoxicity, good selectivity towards target cells, long-wavelength absorbing, rapid removal from the body and ease of administration through various routes. These criteria provide a general guideline for comparison. Although some photosensitizers satisfy all of or some of these criteria, there are currently only a few photosensitizers that have received official approval around the world. However, PDT with the currently

FDA-approved photosensitizers is not without adverse effects. For example, Photofrin®, the first systemic drug approved, is well known for causing an intense inflammatory and necrotic reaction at the treated site and prolonged widespread photosensitivity for up to several weeks post-PDT, thereby imposing severe limitations on the patient's lifestyle (Dougherty et al. 1990, Moriwaki et al. 2001). Because of this and other drawbacks of Photofrin®, many additional photosensitizers have been synthesized, and a few of them have developed into FDA-approved drugs or are under clinical trial.

The aim of this study was to optimize the light dose and to determine the tumor burden and therapeutic efficacy of Symmetrical diiodinated squaraine on skin tumor induced animal models. Skin tumor was induced in Swiss albino mice (Male, 20-25g) using DMBA and croton seed oil.

METHODS

Skin Tumor-Induction in Mice and Photodynamic Treatment

Swiss albino mice-Male, 6-8 week old, weighing 20-25g were divided into four groups as:

Group I - Control

Group II- DMBA + Croton oil

Group III- DMBA + Croton oil + Squaraine dye (12.5 mg/kg body wt)

Group IV- DMBA + Croton oil + Squaraine dye (12.5 mg/kg body wt) + Light treatment

[Animals Sacrificed after 2 Weeks of Light Treatment]

Skin tumor was induced in the animals of the Groups II, III, and IV using DMBA and croton seed oil by the method of Ewing et al. (Ewing et al. 1998). The body weight of each animal was noted, the animals were observed regularly for any lesion and tumor development and papillomas appearing on the shaved area of the skin were recorded at weekly intervals. Tumor promoting activity of DMBA and croton oil was evaluated by determining both the proportion of tumor-bearing mice and the number of tumors (>2.00 mm in diameter) per mouse. After the completion of the study period (20 weeks), the tumor bearing mice of Groups III and IV were given an intraperitoneal injection with the dye [dissolved in phosphate buffered saline (PBS), pH 7.4] at a concentration of 12.5mg/kg body weight. The mice were kept in dark for 24 hours after injection to eliminate any possible tissue damage due to the exposure to sunlight. The animals in group IV were divided into different groups for optimization of light dose. In Group IV, the treatment with light was done after 24 hours. The tumor area was then exposed to visible light from a 1000 W halogen lamp, kept at a distance of 33cm for different time intervals.

Selection of Optimal Light Dose for *in Vivo* PDT

For the optimization of light dose, skin tumor-induced Swiss albino mice (Male, 20-25g) were divided into different groups and subjected to photodynamic therapy at different light doses after injecting 12.5mg of the dye/kg body weight dissolved in phosphate buffered saline. The mice were kept in dark for 24 hours after injection to eliminate any possible tissue damage due to the exposure to sunlight. Individual light treatment was given for each mouse. The grouping of animals is given as follows.

Group IVa: Tumor induced

Group IVb: Tumor induced+dye (12.5mg /kg body weight) + total light dose of 80 J/cm²

Group IVc: Tumor induced+dye (12.5mg /kg body weight) + total light dose of 100 J/cm²

Group IVd: Tumor induced+dye (12.5mg /kg body weight) + total light dose of 120 J/cm²

Group IVe: Tumor induced + total light dose of 80 J/cm²

Group IVf: Tumor induced + total light dose of 100 J/cm²

Group IVg: Tumor induced + total light dose of 120 J/cm²

The total light dose was adjusted by varying the time duration of illumination. The total light doses and the corresponding time required for illumination is given as:

80 J/cm² : 1000W halogen lamp kept at a distance of 33cm from the tumor; time of illumination-18 min*.

100 J/cm² : 1000W halogen lamp kept at a distance of 33cm from the tumor; time of illumination-22 min*.

120 J/cm² : 1000W halogen lamp kept at a distance of 33cm from the tumor; time of illumination-26 min*.

*The time of illumination for each total light dose was calculated based on the relationship between Joules and Watt (W = J/sec) as follows:

Distance between lamp (1000W) and the specimen = 33 cm

Area illuminated by the lamp when placed at 33cm away = 75cm x 175cm (calculated by measuring the length and breadth of the illuminated rectangular region when the light source was kept at 33 cm) = 13125 cm²

The dose of light incidenting per cm² = 1000 W / 13125 cm² = 0.0762W/cm²

Required light dose = 'L' J/cm², where L=80, 100 or 120 as the requisition may be

$L \text{ J/cm}^2 = 76.2 \times 10^{-3} \text{ W/cm}^2 \times 't' \text{ sec}$, since W = J/sec; J = W x sec

Therefore, $t = L/0.0762 \text{ sec}$, where L=80, 100 or 120 as the requisition may be.

Skin of the mouse except the target tumor was covered with aluminium foil and it was kept in a cooled container prior to PDT treatment. The tumor area was then exposed to visible light from a 1000 W halogen lamp, kept at a distance of 33cm for different time intervals, corresponding to the different total light doses required in each case. A stream of cool air cooled the area under treatment continuously. Two weeks after the treatment, the animals in all the groups were monitored for tumor statistics. The effect of Squaraine PDT on hematological parameters were also analysed in the mice of all the groups.

RESULTS AND DISCUSSIONS

Skin tumor was induced in Swiss albino mice (Male, 20-25g) using DMBA and croton seed oil. In the tumor induced groups, lesions were observed in the skin at the 5th week of the experiment. The lesions observed were later developed into tumors. The appearance of tumor for the first time was noted at the 8th week after DMBA application. In the tumor bearing animals treated with DMBA, there was a sharp drop in body weight. This may be due to cancer cachexia. Cancer cachexia results in progressive loss of body weight, which is mainly accounted by wasting of host body compartments such as skeletal muscle and adipose tissue. Weight loss and tissue wasting are observed in cancer patients (Khan and Tisdale. 1999). The increase in body weight compared to the cancer induced group at two weeks after PDT treatment indicates the counteractive property of the treatment on tumor progression.

Selection of Optimal Light Dose and Tumor Regression for Squaraine PDT

For selecting the dose of light for squaraine PDT which can effectively cause tumor regression, the parameters like the number of tumors per mouse and mean tumor volume were analysed before and after treatment. The tumor statistics of different groups of animals were analysed and shown in the following table (Table 1).

Table 1: Effect of PDT on Tumor Statistics

Parameters	Group I	Group II	Group III	Group IV						
				IVa	IVb	IVc	IVd	IVe	IVf	IVg
Number of tumors per mouse	Nil	5.1±0.40 ^a	5±0.44 ^a	5.05±0.41 ^a	4.5±0.45 ^b	4.1±0.30 ^c	4±0.27 ^c	4.98±.32 ^a	4.88±.041 ^a	4.91±.053 ^a
Percentage Tumor incidence	Nil	90±2 ^a	89±5 ^a	91±1 ^a	85±4 ^b	82±2 ^c	81±2 ^c	89±1 ^a	90±2 ^a	89±2 ^a
Mean tumor volume	Nil	24.42±2.12 ^a	23.59±1.19 ^a	24.11±0.14 ^a	15.32±3.21 ^b	9.24±1.01 ^c	9.01±2.12 ^c	22.23±4.12 ^a	23.45±1.1 ^a	23.15±3.10 ^a

Values are Mean of Seven Estimations. Comparison is between Groups.

Different Alphabets Indicate Significant Difference at p<0.05

The tumor statistics of different groups of mice are shown in the table. Tumor incidence was found to be decreased significantly in the groups that received the PDT treatment. These results clearly indicate the potency of the symmetrical diiodinated squaraine dye as a novel therapeutic agent against skin cancer models. The optimal dose selected is 100 J/cm² to minimize the thermal effects.

Effects of PDT on Haematological Parameters

The effect of Squaraine PDT on hematological parameters were analysed in all the groups and the results are shown in the Table 2.

Table 2: Effects of PDT on Haematological Parameters

Groups	Hb	Total WBC/mm ³	RBC X 10 ⁶	PCV/dl	ESR
I	12.1±0.13 ^a	4600±34.02 ^a	7.74±0.04 ^a	40.6±0.22 ^a	5±0.07 ^a
II	9.7±0.11 ^b	5160±34.77 ^b	6.34±0.03 ^b	36.8±0.07 ^b	2±0.05 ^b
III	9.8±0.11 ^b	5220±35.52 ^b	6.51±0.03 ^c	37.0±0.07 ^b	2±0.05 ^b
IVc	10.9±0.11 ^c	4790±34.02 ^c	6.871±0.03 ^d	38.7±0.08 ^c	3±0.06 ^c

Values are mean of six estimations. Comparison is between groups.

Different alphabets indicate significant difference at p<0.05

In the tumor induced mice, the hematological parameters showed significant alterations from the normal animals. The values reverted to the near normal levels two weeks after PDT. In cancer chemotherapy the major problem are myelosuppression and anemia. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions. The PDT treatment induced significant changes in the levels of the parameters compared to mice without treatment. This indicates the therapeutic action of Squaraine on the heamato-poietic system.

CONCLUSIONS

From the present study, it can be concluded that the dye selected for our study, Symmetrical diiodinated squaraine, possesses photodynamic therapeutic efficacy and the optimum light dose for *in vivo* applications is 100 J/cm².

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