A Nonlinear Logistic Regression Model for the Measurement of Drug Potency in Photodynamic Therapy

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Abstract. Medical physics has revolutionized cancer diagnosis and treatment. From imaging to therapy, the principles of physics have shown the inseparable relationship with biological systems. One such example is Photodynamic Therapy (PDT), a therapeutic modality that uses light to kill cancer by means of a photochemical reaction that is initiated when a photosensitizer (PS) molecule absorbs a photon of light to become phototoxic. Like all other therapies, the potency of PDT has to be determined before confirming its usage. Many PSs are available, some are being investigated and yet more will emerge in future. To measure the therapeutic potency of these PSs in PDT therefore, a good model and technique for the accurate measurement of potency is indispensable. Unlike most therapies, where a single drug causes effect, in PDT there are two input variables to produce a response, the PS and the light. A design for accurate estimation of PDT potency was therefore developed in this present investigation, using regression analysis of the inhibition of proliferation of cells treated with PDT. A cancer cell line, SiHa cells, was cultured and treated with serially diluted PS concentrations for treatment at two different laser fluencies. Using nonlinear regression, the dose response curve was fitted and the half growth inhibition (GI50) value was calculated using a Four Parameter Logistic (4PL) Model. This work has since provided guiding principles for the accurate estimation of PDT potency for early stage PDT investigations, and includes theoretical considerations for the accurate estimation of the GI50 value.

1. Introduction

Accurate estimation of drug effectiveness or inhibition capacity is the single most important aspect of any drug discovery exercise in both academics and industrial research. The value of the concentration corresponding to a response midway between the highest and lowest concentrations i.e. IC50 for half Inhibitory concentration or GI50 for Half Growth Inhibition, which basically are the same thing just different terminology, is used in the early stages of drug discovery to determine the activity of a drug, and the value is used to refer to the drugs' potency. These parameters are required for the evaluation of the performance and suitability of a drug being investigated [1]. When measuring the EC50/IC50 on any therapeutic compound, the proposition is based on the assumptions that a monotonic relationships

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exists between the dose of the compound and the response in the assay and secondly, that a consistent definition of a particular response at a given concentration is predictable based on this relationship.

Statistically, this value can therefore be determined from analysis of responses at varying concentrations and different ways of achieving this are available for different applications. In Photodynamic Therapy (PDT) however, this important pharmacological factor is not adequately investigated for the confirmation of the most appropriate method for determination of PDT potency *in vitro,* for *in vitro* experimentation. In PDT, photosensitization of a photoactivatable drug, the Photosensitizer (PS), is what causes cytotoxicity. This photosensitization is induced by a photo-activating probe usually light of a specific wavelength in the excitation range of the PS [2]. Meaning that there are two variable in the process both of which that need optimization and assessment. Unlike mere synergism, the relationship between the PS and light is inseparable because the PS is an inert molecule in its ground state and only upon excitation to an excited triplet state does it become active to devise a reaction, and similarly the light in the absence of PS does not have any therapeutic significance in PDT. This interrelationship is unlike most in pharmacology where addition of a drug shows direct effect and measurement of drug potency is unambiguous and can be achieved by fitting a simple linear or nonlinear regression where concentration is the independent variable and the response is the dependent variable.

In PDT as explained both variables need to be considered using reliable statistical evaluation. This present study therefore sought to investigate the most appropriate way of determining the IC50/GI50 value that is accurate considering the relation between PS and laser and all the important variables in between PS administration and laser exposure, on an *in vitro* setting, using a monolayer of cells. Different approaches can be used, but the Four Parameter Logistic (4PL) Model is a non-linear regression model that is used for fitting dose-response and concentration-response data, and shows a sigmoidal curve to represent the response of cells to a drug [3]. The conventional optimal 4PL model might not be the best for PDT situations hence this investigation reports on a 4PL model design best suitable for PDT, one that not only is accurate, but also cost effective while meeting the requirement for reporting EC50/IC50 values according to the recommendation of the Assay Guidance Manual.

2. Methodology

2.1 Sample Preparation and Cell Culture

A commercially procured PS, Al (III) Phthalocyanine Chloride Tetrasulfonic Acid (Frontier ScientificTM, Cat# AlPcS-834) with a molecular weight of 895.21 and molecular formula of C32H16AlClN8O12S4, was used in this investigation. A stock solution of 100 mM was prepared from the solid dye to a final volume of 5 ml in Phosphate Buffered Saline (PBS) at neutral pH (pH 7.4) using the Genway UV-Vis Spectrophotometer (Lasec). Spec-trophotometric scanning from 400 to 800 nm at 2 nm wavelength intervals was performed and the obtained data was plotted on a line chart. An adherent cell line, SiHa cells (ATCC® HTB35™) suitable for in vitro monolayer experimentation, were procured from Separations Scientific SA (Pty) Ltd. The cells were initially propagated in culture using Dulbecco's Modified Essential Medium (DMEM) (Sigma Aldrich), at 37°C with 5% CO2 and 85% humidity in a cell culture incubator ((Thermofisher Scientific). Upon reaching the required cell volumes, cells were seeded in 3.4 cm2 tissue culture plates (Corning Inc.) at a density of 3 × 105 cells per plate and incu-bated as described for propagation. An attachment window of 8 hours was given before addition of PS after which a serially diluted range of PS concentrations (Supplementary material S1) was added to the monolayer of cells and left for 18 hours to allow for maxi-mum intracellular uptake and internalization. After 18 hours, the media was removed, and the monolayer was washed three times with pre-warmed Hanks Balanced Salt Solu-tion, HBSS (Sigma Aldrich) to remove unabsorbed PS, in preparation for irradiation

2.2 Irradiation

Three control groups and twelve experimental groups of cells were prepared and cultured is 3.4 cm^2 tissue culture plates as shown in table 1 below. Group one cells were not treated with either PS or light, group two cells were treated with light only without addition of PS, group three cells were treated with PS without exposure to light. The experimental groups were treated with a serially diluted concentrations of PS starting with a high concentration of 500 µM down to 7.8 µM using a dilution factor of 0.5. Irradiation was done using a monochromatic semiconductor diode laser with a wavelength of 673.2 nm (Oriel, USA), at 5 and 10 J/cm^2, in 1 ml of media. Exposure times for each fluence was determined using equation 1 below. After irradiation, all groups were left for 12 hours at culture conditions in the incubator for post-treatment cellular assessment.

Table 1. Control and experimental groups

Group	**Description**	
Control Group One	No Photosensitizer, No Irradiation	
Control Group Two	PS Negative Control	
Control Group Three	Laser Negative Control	
Experimental Groups	500 µM+5 J/cm^2	500 µM+10 J/cm^2
	250 µM+5 J/cm^2	250 µM+10 J/cm^2
	125 µM+5 J/cm^2	125 µM+10 J/cm^2
	62.5 µM+5 J/cm^2	62.5 µM+10 J/cm^2
	31.3 µM+5 J/cm^2	31.3 µM+10 J/cm^2
	15.6 µM+5 J/cm^2	15.6 µM+10 J/cm^2
	7.8 µM+5 J/cm^2	7.8 µM+10 J/cm^2

$$\frac{X\,mW \times 4}{(\pi) \times 3.4\ cm^2} \quad (1)$$

Where *X* is the power output in mW measured by a power meter and the 3.4 cm^2 is the pole size diameter which also is equivalent to the diameter of the plates used.

2.3 Cellular Responses

Morphological assessment was done to observe the changes in structure and distribution of cells after treatment. Cells were taken from the incubator and observed directly before washing, using the inverted light microscope (Wirsam, Olympus CKX41) with a built in camera. Images were captured at 200× magnification, where the Olympus cellSens Imaging Software was used for acquisition of images, and ImageJ was used for further analysis of the captured images. Following microscopy, the CellTiter- Glo luminescent cell viability assay (AnaTech: Promega, PRG7571) was used for the determination of proliferation. In this assay luminescent signal is detected from the reaction of luciferase enzyme with ATP, at a signal proportional to the amount of ATP in the cells. Cells were washed once with HBSS and suspended in 200 µl of media. An equal volume the CellTiter-Glo reagent was added to the plates and shook using a rocking shaker for 2 min to induce lysis. The mixer was then immediately pipetted into opaque-walled 96 multi-well plate in duplicate luminescence in relative light units (RLU) was then measured on the Perkin Elmer, VICTOR Nivo Multimode Microplate Reader, Part # HH35000500).

2.4 IC50/GI50 Calculation

To calculate the IC50/GI50, the 4PL model was used as shown in equation 2 below. Experiments were repeated three times (n=3) and statistical analysis was performed using SigmaPlot software version 14.0. Student t-test was performed to determine the statistical difference between the control and experimental groups.

$$Y = d + \frac{(a-d)}{1+\left(\frac{x}{c}\right)^{b}} \tag{2}$$

Where, Y = Response (i.e. the dependent variable)
x = Concentration (i.e. Dose, the independent variable)
a = The Minimum (response at zero dose)
d = The Maximum (response at infinite dose)
c = Point of inflection (i.e. the EC50/IC50)
b = Hill coefficient (i.e. the slope at point c).

3. Results and Discussion

3.1 Morphology and Cell Proliferation

Morphology of cells after treatment indicated alterations as observable under bright field microscopy. Changes in morphology were suggestive of an ongoing cell death mechanism, with some apoptotic and some necrotic features. Terminal cell death as seen in floating cell and cellular debris was also evident in higher doses as seen in figure 1 A and B below. Extent of cells damage was dose dependent with low doses having little no damage, followed by progressing cellular damage until the highest dose. Control groups, not include in the figure, showed no changes in morphology and appeared similar in shape, size and attachment with the standard control.

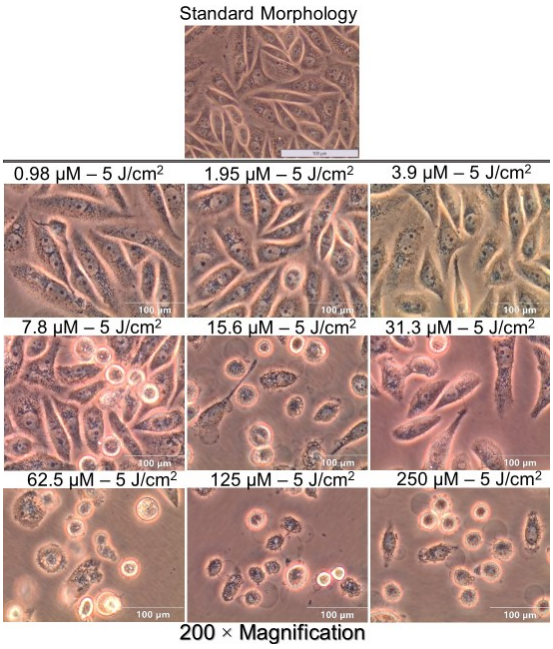

Figure 1 A. Cells irradiated with 5 J/cm^2 showing the morphology of PDT treated cell with characteristic changes in a dose dependent manner

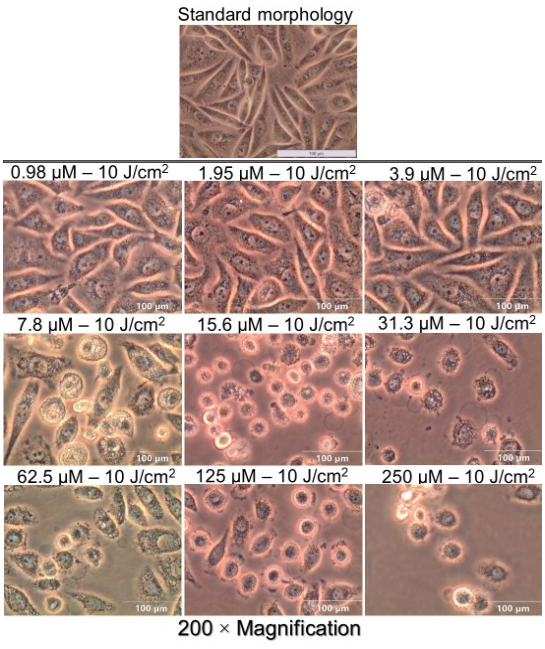

Figure 1 B. Cells irradiated with 10 J/cm^2 showing the morphology of PDT treated cell with characteristic changes in a dose dependent manner

Following this observation, the proliferation rate of cells also indicated similar trends with proportional decrease of proliferation inversely to the concentration of PS, i.e. dose dependent decrease. Similarly. control cells were used to measure the maximum ATP concentration from which all other groups are compared. The untreated standard control showed a high ATP concentration which was used to calculate the 100% proliferation. All control groups i.e. either PS alone or laser alone, had increased proliferation at the end of the experimental period. Experimental groups however showed significant decrease in the concentration of ATP ($p<0.001$), indicated by low luminescence signal. Shown in figure 2 below is the observed dose dependent decrease in proliferation of PDT treated cells plotted using a logarithmic expression of 2.

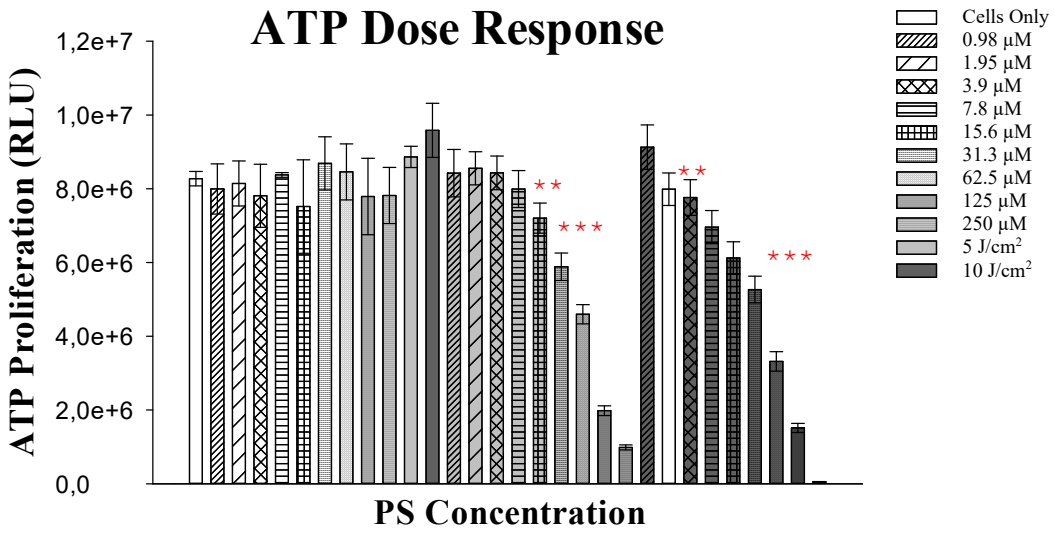

Figure 2. Dose response assessment of cell proliferation using ATP measurement as an indicator of cellular proliferation. Results showing a dose dependent decrease in LDH, inversely proportional to the concentration used. 5 J/cm^2 is plotted in blue and 10 J/cm^2 in red, with significance shown using ** for ($p<0.01$), and *** for ($p<0.001$).

3.2 Calculated IC50/GI50

At the concentration of 500 µM for 5 J/cm^2, it was established that the proliferation response had its maximum inhibition, for both 5 and 10 J/cm^2. Similarly, the minimum inhibition lies at the same concentration as the 0% plate control. Hence, both 0% and 100% values were determined and confirmed to insert the *a* and *d* in equation 2 above. By terminology, it is therefore conceivable to calculate the absolute IC50/GI50 value for this response [4]. Using equation 2 and by plotting a logistic regression curve, the absolute IC50/GI50 was therefore determined at 63.426 µM and 40.813 µM for 5 J/cm^2 and 10 J/cm^2, respectively as shown in figures 3 A and B below. This means that at these respective concentrations, a half response rate is achieved, for each fluence.

This calculation is crucial and attention needs to be given to both accuracy of information since it informs of the therapy's measurable effect and inferences regarding dosing is based on the estimated IC50/GI50. In therapeutic applications, there are many reasons for clinical failures, but the choices made concerning identification and optimization during the discovery phase is one major contributor. As a vital part of drug discovery, *in vitro* experimentation is the stage where drug potency at cellular level is determined and the effectiveness and usability of a drug are ascertained.

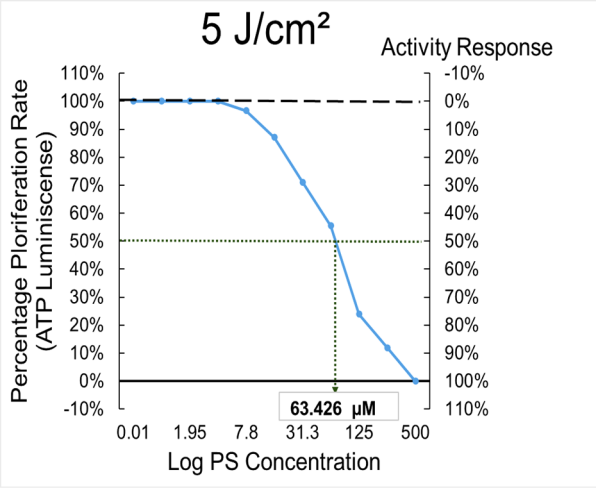

Figure 3 A. The IC50/GI50 of 63.426 in cells irradiation with 5 J/cm^2, plotted using with reference to equation 2

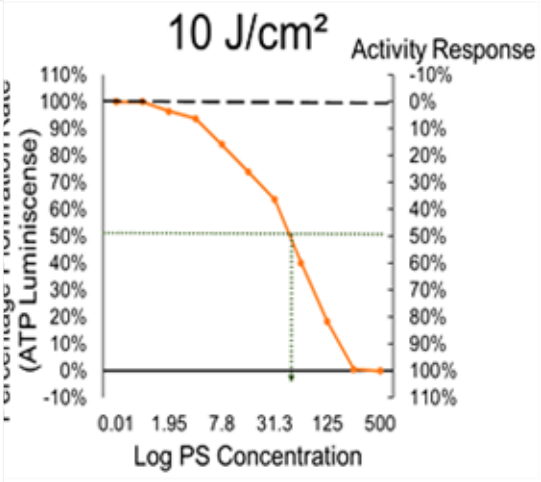

Figure 3 B. The IC50/GI50 of 40.813 in cells irradiation with 10 J/cm^2, plotted using with reference to equation 2

4. Conclusion

For PDT experiments *in vitro*, we described here for the first time using nonlinear logistic regression, how the EC50/IC50 should be calculated for the purpose of reporting an accurate value. This technique is inferable for all current and new PSs, for the determination of working doses and studying cellular responses. Wrongfully reported values are a menace to the process of drug discovery, and should by all means be avoided. This present study was performed *in vitro* using a monolayer cultured cells. The concentration and variables mentioned here are for the purpose of cells treated as a monolayer, to understand the PDT potency at cellular level.

5. Acknowledgements

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