In vitro cytotoxicity assessment of second-generation photosensitizers for photodynamic therapy

E. STOYKOVA*, K. NEDKOVA, O. SABOTINOV, R. ION[a], R. ALEXANDROVA[b]
Central Laboratory for Optical Storage and Processing of Information, Acad. G. Bonchev Str., Bl.101, P.O. Box 95, 1113 Sofia, Bulgaria
[a]*Institute for Chemical research - ICECHIM, Chemical Analysis Dept., Splaiul Independentei 202, Bucharest-77208, Romania*
[b]*Institute of Experimental Pathology and Parasitology, Acad. G. Bonchev Str., Bl. 25, 1113 Sofia, Bulgaria*

In this study we evaluate the cytotoxic activity of two phthalocyanines (metallo-complexes) with absorption bands in the red part of the spectrum: zinc-tri-sulphonated phthalocyanine (ZnS_3Pc) and zinc-tetrasulphonated phthalocyanine (ZnS_4Pc) on human (8 MG BA and MCF-7) and animal (LSCC-SF-Mc29 and LSR-SF-SR) tumor cell lines using a neutral red uptake cytotoxicity test. The light source is a laser diode emitting at 672 nm. The experiments are made at fluences in the range (2 – 100 J/cm^2) at fluence rate of 120 mW/cm^2 chosen after a series of test measurements. The efficacy of both phthalocyanines is evaluated from light dose response curves obtained for drug concentrations from 1 up to 10 μg/ml. Both studied phthalocyanines exhibit effective treatment of the animal cell line LSCC-SF-Mc29 and the human cell line 8MG-BA. In the case of ZnS_3Pc, the cytotoxic effect is most distinguished for the tumor line 8MG-BA whereas for ZnS_4Pc better results are obtained for LSCC-SF-Mc29. The animal cell line LSR-SF-SR and the human line MCF-7 exhibit much greater resistance to both photosensitizers at low concentration. Phthalocyanines (free-base or metallo-complexes), which are synthetic porphyrin-like dyes are promising candidates among the second-generation PSs due to very large extinction coefficients in the far red (660-700 nm).

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1. Introduction

More of three decades of systematic preclinical and clinical studies showed the potential of the photodynamic therapy (PDT) among therapies selective for tumour tissues. Its curing ability for treatment of non-neoplastic diseases is also well known [1-4]. PDT is a treatment modality that relies on combined action of a photosensitizer (PS), light and molecular oxygen to attack pathological tissues. Over the years, a lot of PSs have been synthesized, but only a few of them are widely used in clinic [5-7]. Implementation of new advanced agents continues to be a pressing task. Recently, second-generation agents with improved photodynamic properties have been designed. To ensure the desirable therapeutic effect of the PDT, the "photodynamic dose" described as the number of photons absorbed by the PS per gram of tissue should exceed some threshold value at the location of the tumour. The threshold value depends on all steps involved in the production of cytotoxic reactive oxygen species by the photochemical interaction as absorption of light by the PS, formation of the PS's triplet state, trapping of the triplet state by molecular oxygen within its lifetime, energy transfer from the triplet state to molecular oxygen etc. However, the practically exponential decrease of the light fluence within the absorbing human tissues as well as the strong wavelength dependence of this decrease make crucial the search of PSs with absorption peaks shifted to the so-called therapeutic window in human tissues between 600 and 1200 nm, where penetration of the PS activating light is sufficiently high, and deeper treatment of a tumor can be achieved [8]. Promising candidates among the second-generation PSs are phthalocyanines (free-base or metallo-complexes), which are synthetic porphyrin-like dyes with very large extinction coefficients in the far red and near infrared regions of the visible spectrum [9-12]. Availability of powerful laser diodes emitting within the absorption peaks of phthalocyanines also inspires the research efforts towards their clinical implementation. This study reports on in vitro cytotoxicity assessment of two phthalocyanines (metallo-complexes) – zinc-tri-sulphonated phthalocyanine (ZnS_3Pc) and zinc-tetrasulphonated phthalocyanine (ZnS_4Pc) on two human and two animal tumor cell lines using a neutral red uptake (NRU) cytotoxicity test.

2. Materials and methods

The phthalocyanines have been prepared in the laboratory, and puriffied by a thin layer chromatography. Their purity have been checked by X-ray diffractometry, FTIR spectrometry and UV-Vis spectrophotometry. Their normalized absorption spectra, measured with a UV-Vis-NIR-spectrophotometer Cary-5E-Varian, are shown in Fig.1. As it can be seen, both ZnS_3Pc and ZnS_4Pc can be activated at wavelengths between 600 and 700 nm.

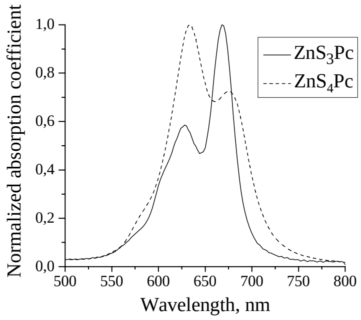

Fig. 1. Normalized absorption spectra of zinc-tri-sulphonated phthalo cyanine (ZnS_3Pc) and zinc-tetrasulphonated phthalocyanine (ZnS_4Pc).

Cells were routinely grown as monolayer cultures in a combination of E-199 and Iscove's modified Dulbecco's medium (IMDM) supplemented with 5 to 10% calf serum (NCIPD, Bulgaria), penicillin (100 U/ml) and streptomycin. The cells were seeded in microtitre tissue culture 96-well plates (Nunclon) at 2 x 10^4 cells per well. We chose this concentration as optimal after test measurements. At higher cell concentration we observed rather large fluctuations of data for the different wells. At the 24th hour the cells from the monolayers were washed and covered with media modified with one of the PSs. After 24 hours period of incubation the cells were exposed to different light doses. The viability of cells was determined by the NUR cytotoxicity assay with respect to the control. The neutral red was also added to a row of blank wells in order to evaluate the background. During irradiation the micro-plate was kept in a thermostatic device, which maintained a temperature of 37^oC. 24 hours after the irradiation, the cells were washed with phosphate-saline buffer (PBS) and 100 µl of culture medium containing 0.0075% neutral red was added. The cells were incubated for 3 hours at 37^oC in a humidified atmosphere of 5% CO_2, washed with PBS and 150 µl ethanol/water/acetic acid (50:49:1) solution was added. The plates were shaken on a micro-titer plate-shaker for 10 min. The absorption of the resulting coloured solution was measured at 540 nm in a micro-plate reader. To avoid the unwanted activation of the PS, all procedures during the experiments were performed in a darken room. Samples of cells grown in non-modified medium served as a control.

Irradiation was performed with a 360 mW laser diode emitting at 672 nm with a bandwidth of emission 10 nm. The laser wavelength corresponded well to the absorption bands of both PSs. The laser spot had a rectangular shape that covered uniformly four adjacent wells. The light doses were chosen depending on the drug concentration to ensure preferably at least three data points between 10% and 90% relative viability for each light dose response curve.

The cytotoxicity assessment of both phthalocyanines was performed using a NRU cytotoxicity test. Most frequently, the toxicity of a PS is assessed by survival doses IC50 and E50 which are defined respectively as the drug concentration at a given light dose or the fluence at a given drug concentration that inflict damage on 50% of incubated cells. In our case, we built light-dose response curves. To obtain a reliable estimate of E50, we process the raw absorbance data from the micro-plate reader as follows:

1) correction of the raw absorbance data by substracting the background determined as a mean value for the blank row;
2) removal of outliers after applying the outlier test;
3) calculation of the mean viability at each delivered light dose *E*;
4) calculation of the relative mean viability, $\hat{\upsilon}(E)$, of the treated cells with respect to the control at each delivered light dose,
5) plotting of the light dose-response curve $\hat{\upsilon}(E) \pm \hat{\sigma}_{\upsilon}(E)$, where the standard deviation $\hat{\sigma}_{\upsilon}$ is depicted as vertical bars on both sides of the relative viability value;
6) a four parameter logistic mathematical model relating the delivered fluence to the relative viability in a sigmoidal shape is used for fitting and evaluation of the survival dose E50.

3. Results

To build each light dose response curve, totally six light doses were delivered to a single plate to alternating columns with control and treated cells, ensuring the same dose per 7 wells. The relative viability values were calculated according the procedure described above.

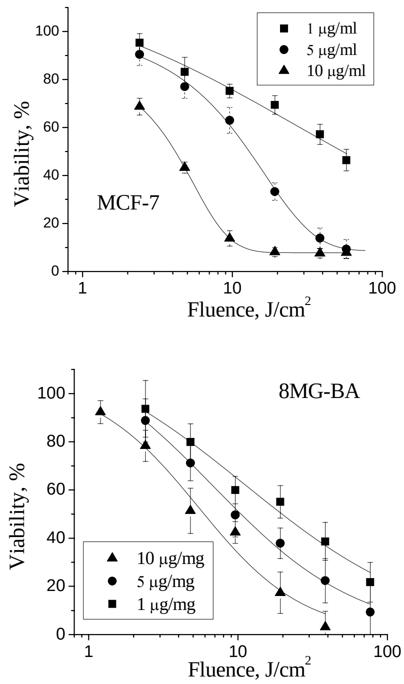

Fig. 2. Light-dose response curves at different PS concentration for the zinc – tri – sulphonated phthalocyanine (ZnS_3Pc) for human cell lines.

Figs. 2 and 3 present the light dose response curves obtained for ZnS_3Pc whereas Figs. 4 and 5 - for ZnS_4Pc. Both data points and the fitted curves are shown. For most of the curves Hill function or Boltzmann sigmoidal distribution were used as fitting models. For some of the cell lines reliable results were obtained for the concentration of 0.5 μg/ml. Both investigated phthalocyanines exhibit less than 5% decrease in viability at concentrations up to 10 μg/ml without irradiation.

As it should be expected, in vitro sensitivity to the photodynamic treatment strongly depends on the cell line. Both studied PSs exhibit effective treatment of the animal cell line LSCC-SF-Mc29 and the human cell line 8MG-BA. In the case of ZnS_3Pc, the cytotoxic effect is most distinguished for the tumour line 8MG-BA whereas for ZnS_4Pc better results are obtained for LSCC-SF-Mc29. The animal cell line LSR-SF-SR and the human line MCF-7 exhibit much greater resistance to both PSs at low concentration. This conclusion concerns drug concentrations between 1 and 10 μg/ml. Increase of the drug concentration from 1 to 10 μg/ml strongly reduces the survival light dose.

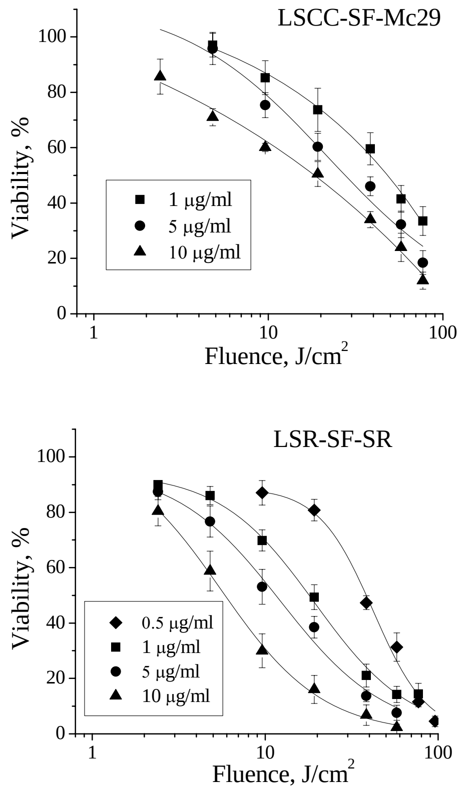

Fig. 3. Light-dose response curves at different PS concentration for the zinc - tri - sulphonated phthalocyanine (ZnS_3Pc) for animal cell lines.

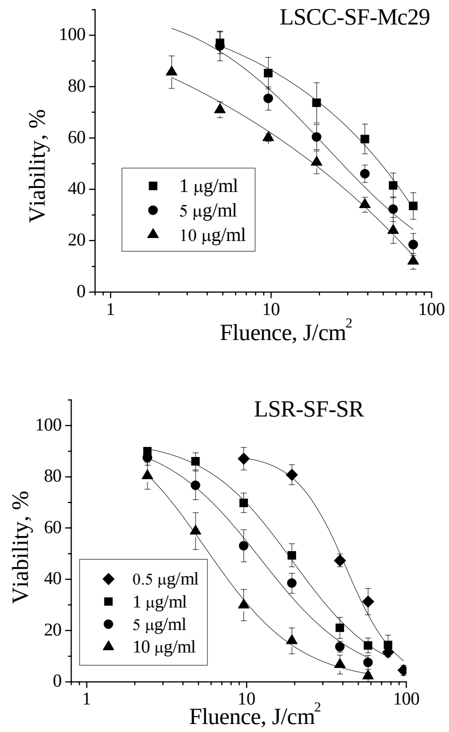

Fig. 4. Light-dose response curves at different PS concentration for the zinc – tri – sulphonated phthalocyanine (ZnS_4Pc) for animal cell lines.

It should be mentioned that comparatively large difference has been registered in viability values obtained at 0.5 μg/ml and 1 μg/ml, and therefore further measurements are required to confirm this result. The efficiency of ZnS_4Pc could be even better if one uses the larger absorption peak in its spectrum. Survival doses at 10 μg/ml with exception of LSR-SF-SR are about 5 J/cm^2. The viability data presented in Figs.2-5, within some interval of light doses, may be used for determination of survival concentration IC50.

4. Conclusion

The cytotoxicity tests play important role in generation of high quality *in vitro* databases that could be used to evaluate the accuracy in prediction for the starting dose in *in vivo* testing and to compare the potency of the tested drugs. Validation study made in this work proved that reliable survival doses can be derived from a single 96-wells micro-plate. To ensure good quality of the light dose response curves we recommend to acquire the data points that build a given curve from the same micro-plate.

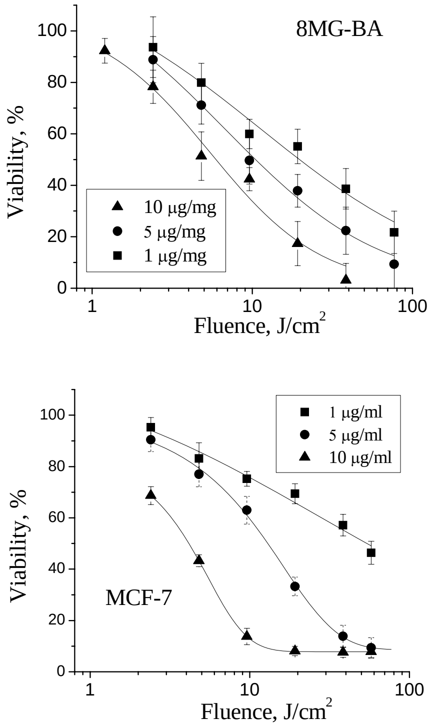

Fig. 5. Light-dose response curves at different PS concentration for the zinc - tri - sulphonated phthalocyanine (ZnS_4Pc) for human cell lines.

Acknowledgements

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References

[1] T. J. Dougherty, C. J. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan, Q. Peng, J. Natl. Cancer Inst. **90**, 889 (1998).
[2] L. Dalla Via, S. M. Marciani, Curr Med Chem. **8**, 1405 (2001).
[3] J. S. McCaughan Jr. Drugs Aging **15**, 49 (1999).
[4] C. H. Sibata , V. C. Colussi, N. L. Oleinick, T.J. Kinsella, Expert Opin Pharmacother **2**, 1 (2001).
[5] R-M. Ion, Curr Top Biophys **24**, 21 (2000).
[6] R. Allison, T. Mang, G. Hewson, W. Snider, D. Dougherty, Cancer **91**, 1. (2001).
[7] H. Barr, Drugs Aging **2**, 81 (2000).
[8] C. Allen, W. Sharman, J. Van Lier,. J Porphyrins Phthalocyanines **5**, 161 (2001).
[9] A. C. Tedesco, J. C. Rotta, C. N. Lunardi, Curr Org Chem.**7**, 1 (2003).

*Corresponding author: elena@optics.bas.bg