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Chemical Synthesis in the Development of Therapeutics: Approach through analogies to natural structures and processes

António Manuel d'Albuquerque Rocha Gonsalves

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Abstract

A short story of the academic and the track which has taken him to became a researcher in chemistry. His central line of research in the area of synthetic porphyrin chemistry leading to structures adapted to be exploited as photodynamic therapy sensitizers is presented.

SÍNTESE QUÍMICA NO DESENVOLVIMENTO DE TERAPÊUTICAS: APROXIMAÇÃO POR ANALOGIAS A ESTRUTURAS E PROCESSOS NATURAIS

Sumário

Apresentação sumária do académico e do percurso que o levou a tornar-se um investigador em química. Apresenta-se o seu tema central de investigação na área de química sintética de porfirinas e a forma como conduziu a estruturas adaptadas a serem exploradas como sensibilizadores de terapia fotodinâmica.

Coming here as a chemist, may be interesting to remember that in youth I apparently intended to be an architect, an idea naturally accepted by kin and friends. Truly from early youth I felt a significant disgust by the juridical involving atmosphere and higher affinity for building and creative activities. Before primary school I had a singular curriculum certainly as a result of peculiar neighbors. I learned cutting and stitching, farming and herding, pharmacist, wood joinery, something of ironmonger, mechanics, electric circuits technician, plumber, …

With such a background I come often to think about probably useful education curricula models in contrasting to some stereotypes whose results are more or less well documented, however obfuscated by "blindness". And, from the apparent inadequacy of the scholar curricula to answer the fundamental requisites of social and civic background originates the poor capacity of schools to shape and educate as required. With consequent chronic society diseases we loudly regret presently.

We do not intend bringing to here a comprehensive receipt to solve the general problems of society. First of all because we think that such receipt, simply, does not exist. We only intend to emphasize that the best humane and humanist person shaping has to come from the free and strict observation of our environment and from the capacities and expertise of the human being. Meanwhile, attempts to impose models originating from abstract thinking being interesting and having the potential to insert horizons breaking does not lead to good consequences when taken as single matrix. Let us pay attention to the degradation of education results, to the inconsequent social disturbance, to degradation of political and economical structures originating more chaos than stability, showing difficulty in finding timely and healthy answers to problems.

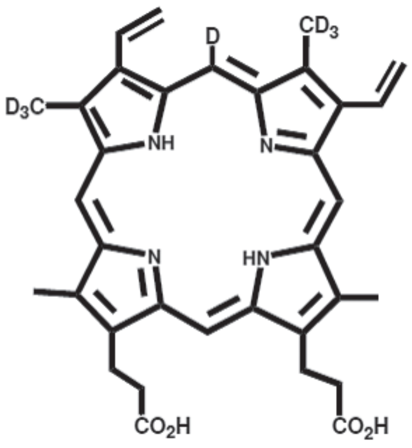
Reasoning about a matrix for a basic scholarity, let us remember that recent experiences raised evidence that learning of the language and of mathematics was deficient. An old known fact only recently acknowledged. Actually good control of spoken and written language is essential to communicate to all fields of knowledge, mathematics logic a basic cultural matrix. However, an approach only supported by these two pillars is too short to handle the problems of the world we live in. Actually it is a physical-chemical reality inhabited by living creatures. Everything having impact in our physical existence is immersed in such reality. The language and mathematics are fundamentals such as are physics, chemistry and biology. So, all these have to be the pillars which must be offered by the basic schools certainly crowned by fundamentals of humanities, ethics, moral, history of religions. There is nothing in our existence or affecting it, outside of these areas.

It must be taken into account that the insertion of these topics has to be done understanding that the majority of the students attending the basic school are not going to become writers, mathematics, neither physicists, chemists, biologists, … Essential for them is pick up the basics to the understanding of human environment. In the production and preservation of food, in the technical activities to promote the well being nothing exists out of the range of some of the preceding basic areas to which the school should have given proper understanding. If the principles of humanism, ethics and moral to which the school must pay attention are not forgotten, then it safeguarded its basic contribution to the building of society.

Huge progress has had the society due to the contribution of schools having highly diversified structures. We are suspicious about attempts to standardize based on plain "theoretical" models divorced of reality. Perhaps, many problems we complain about today are the consequences from inappropriate scholarship models. Youth maladaptation to tackle ordinary difficulties of life are certainly an example, the fruit of yuppie hysteria is here to be seen.

As a chemist, our interest by chemical synthesis was led from the beginning towards molecular structures having important natural functions. In Coimbra still as an undergraduate we started in the area of synthesis searching for a process to make a naphthoquinone having important pharmacological interest but whose natural source was poor and too expensive.
Selecting a topic for a PhD dissertation study we balanced our interest between protein, nucleic acids and tetrapyrrolic macrocycles synthesis. By that time any of these deserved relevant research interest and subject of strong attention by top chemists. We chose tetrapyrrolic macrocycles having the opportunity to have as supervisor in Liverpool Prof. George Kenner who, interestingly addressed his interest in two of those areas, peptide and tetrapyrrolic macrocycles synthesis.

We started our PhD project developing synthetic methods to generate selectively deuterated molecules of protoporphyrin-IX in the different methyl groups and in the *meso*-hydrogens, I.



These molecules were designed to replace the natural ones in the structure of myoglobin and hemoglobin for unequivocal identification of the NMR resonance peaks of the macromolecules using the equipments then available and obtain information about the electronic structure of the macrocycles an the distortions induced in that by different axial ligands, particularly oxygen. The interest in such study was related with the importance of such molecules, particularly on the mechanism of internal breathing.[1]

The labeling project originally thought as a relatively short task ended up occupying all the PhD project period because it became necessary to develop and optimize methods for labeling of precursors, but also to establish a new synthetic methodology to build the molecule of protoporphyrin-IX and the required precursors, Fig.1, to work in different amount scales assuring adequate yields to handle deuterium and also tritium labeled samples, the last ones interesting for the study of biosynthetic sequences, a topic of study of broad interest at the time. Interest in processes to label the different hydrogen atoms of tetrapyrrolic macrocycles would come to extend for over 20 years in the sequence of our studies originating many works promoted by Kevin Smith firstly in Liverpool and later in the USA.

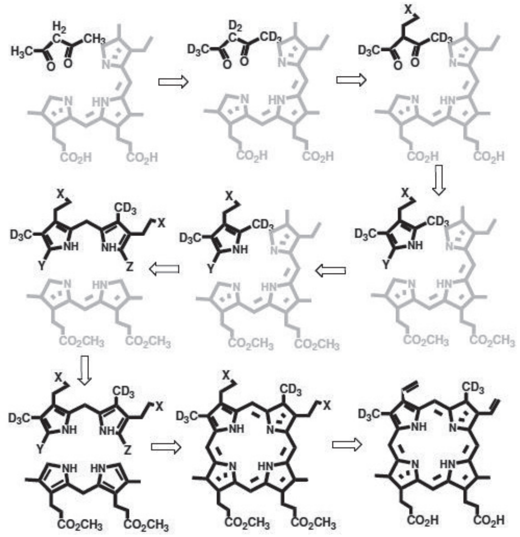


FIGURE 1
Simplified scheme of the required steps to build a deuterated molecule of protoporphyrin-IX.

In Portugal we decided to continue studies in the area of tetrapyrrolic macrocycles structuring our interests following up-to-dated scientific interests but adjusted to the potential of local development and existing working conditions.

Our plan was based in taking as model natural molecules having important and diversified biological roles, namely porphyrins, but exploiting simpler structures than the natural ones, incorporating functionalities able to assure the properties required to mimic the natural activity or conceivable variations. In a presentation brought to this Academy by the late honorable academic António Jorge Andrade de Gouveia[2], we sketched the plan we intended to develop exploiting synthetic processes whose study we had started before. We intended to study harnessing energy using photovoltaic cells, bio-mimetic catalysts, and colorimetric detectors, diagnostic and therapeutic agents. Overall conditions led to begin by the study of catalysts, methods for chemical and photochemical oxidation using non pollutant reagents and further photosensitizers for photodynamic therapy, PDT, photodynamic inactivation, PDI, and photodiagnostic, PD.

Without discarding the possibility of keeping interest for the synthesis of tetrapyrrolic macrocycles having the structural complexity of natural compounds a field where we have had contributions of some significance we focused in simpler structures.

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[1] «Synthetic Porphyrins for Haemoprotein Studies», A.M.d'A. Rocha Gonsalves, Ph. D. Thesis, University of Liverpool, August 1972.)

[2] «Porfirinas em problemas energéticos», A.M.d'A. Rocha Gonsalves, Comunicação na Academia das Ciências de Lisboa em 4 de Novembro de 1982; Memórias da Academia das Ciências de Lisboa, Classe das Ciências, 1981-1982, Tomo XXIV, 353-368

Working with tetrapyrrolic macrocycles allowed overcoming difficulties resultant form degradation of the working conditions brought about by revolutionary disturbances. From the equipment and necessary working conditions, not much more than a visible spectrometer was under good operation conditions, but this machine allowed us to take advantage from the beautiful colors of those compounds and from the so extensive information which can be gathered from a simple spectrum in the visible range.

Chose the synthesis of simple structures does not mean necessarily an easy task implying smaller effort. Typical are the difficulties attached to what is simple. However, let us remember that natural processes are also usually simple when finally we understand them well. In the specific field under consideration, the multitude of natural tetrapyrrolic macrocycles have as precursor the single pyrrol porphobilinogen, PBG, for analogous but so different structures such as hemin, the red iron complex of blood, chlorophyll c, the green magnesium complex of plants, and vitamin B12, a pale indigo cobalt complex, Fig 2.

The synthetic route to generate a porphyrin ring starting from to single compounds, a pyrrol and an aldehyde, Fig. 3, though firstly reported in 1935[3] took more than forty years until conditions allowing for some general applicability and easy work-up were established.

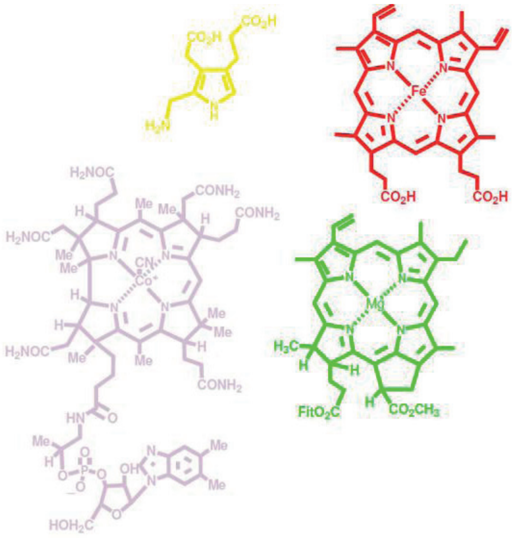

FIGURE 2
PBG the single biosynthetic pyrrol precursor of hemin, chlorophyll c and cobalamin.

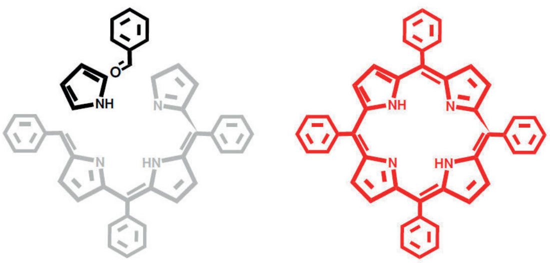

FIGURE 3
The Rothemund process allowed to get directly teraphenylporphyrin, TPP.

The straightforward application of Rothemund synthesis only allows for the synthesis of simple symmetry structures. This, and the fact that the original version only actually allowed for the preparation of a single compound, tetraphenylporphyrin, TPP, encouraged prejudices which certainly shrank the interest in this type of compounds for many years. Somebody highly respected and whom we respectfully remember hearing to talk about TPP used to say “that is not even a porphyrin”. When in 1980 we firstly submitted a paper describing an efficient synthetic method for tetraalkyl porphyrins, one of the referees refused publication on the grounds that “tetraalkylporphyrins are structures which will hardly be of interest to chemists”.

Insisting in the publication and, above all pursuing the study, we apparently won something. We had the opportunity to develop new synthetic approaches which made available, in terms of easy handling and obtaining high yield and purity many meso-substituted porphyrins using easily available starting materials. The established synthetic conditions allow for the preparation of a broad range of porphyrins of quaternary symmetry and opened the

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[3] 3. P. Rothemund, J. Am. Chem. Soc. 57,2010,1935, A.D. Adler, L. Sklar, F.R. Longo, J.D. Finarelli, anda M.G. Finarelli, J. Heterocycl. Chem., 1968, 5, 669

way for synthetic routes of structures of lower symmetry. Our work also originated synthetic methods for tetrapyrrolic macrocycles in different oxidation states, chlorins and bacteriochlorins, either through a novel direct synthesis of chlorins or via reduction of the corresponding porphyrin Fig. 4.[4]

Having easy approach to a significant diversity of structures of macrocycles of the required type we exploited the possibility to increase the range using new reactions over those compounds. Between these it is relevant direct chlorosulphonation with chlorosulphonic acid.[5]

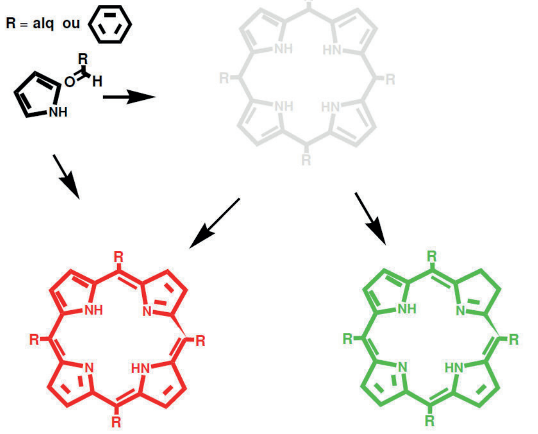

FIGURA 4
As aproximações à síntese de Rothemund introduzidas em Coimbra permitiram introduzir grande versatilidade, altos rendimentos e pureza elevada dos produtos.

Surprisingly, the classical reaction of chlorosulphonation had never been exploited on tetrapyrrolic macrocycles. Actually in the literature we could only find sulphonation with sulphuric acid, a reaction having very pure performance high difficulty of purification and extremely poor yields. Chlorosulphonated derivatives were obtained with extra hardship from the hardly firstly prepared sulphonic acid.[6] Proving that the direct chlorosulphonation of meso-aryl porphyrins was possible we opened the way to the preparation of a very wide range of new derivatives. Contrary to the case of direct sulphonation, chlorosulphonation allows for the preparation of the chlorosulphonated derivative in a quantitative yield, to prepare amines and esters in high yield, an efficient attachment to polymers having appropriate functionalities. Chlorosulphonation is selective on the arylic *meso*-substituents opening the way to purification of mixtures having different aryl substituents.

Access to chlorosulphonation in an high yield allows for the preparation of thiols, structures of potential interest in different applications of porphyrins.

Being easier to have the conditions to promote application studies, we exploited firstly model compounds of biomimetic reactivity such as oxidation catalysts and catalytic systems particularly adjusted for using non polluting oxidants. In that area , our more interesting results were addressed to: development of catalysts and reaction conditions for oxidation reactions using hydrogen peroxide occurring in the interface of a biphasic system liquid-liquid, Fig. 5[7].

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[4] a) «A New Look into the Rothemund meso-Tetraalkyl and Tetraarylporphyrin Synthesis», A.M.d'A. Rocha Gonsalves and Mariette Pereira; J. Heterocyclic Chem., 1985, 22, 931; b) «Some New Aspects Related to the Synthesis of meso-Substituted Porphrins», A.M.d'A. Rocha Gonsalves, J.M.T.B. Varejão, Mariette M. Pereira, J. Het. Chem. 1991, 28, 635; c) «Improved Synthesis of 5,10,15,20-Tetrakisaryl and Tetrakis-alkylporphyrins», A.M.d'A. Rocha Gonsalves, Mariette M. Pereira, A.C.Serra, .R.A.W. Johnstone, M.L.P.G. Nunes, Heterocycles, 1996, 43, 1423; d) «Microwave – assisted synthesis of porphyrins and metalloporphyrins», M. Pineiro, B. F. O. Nascimento, A. M. d'A. Rocha Gonsalves, J. of Porphyrins and Phthalocyanines, 2006, 10, 821; e) «Controlled porphyrinogen oxidation for the selective synthesis of meso-tetraarylchlorins», Arménio C. Serra, António M. d'A. Rocha Gonsalves, Tetrahedron Letters 51 (2010) 4192–4194; f) «MnO2 instead of quinones as selective oxidant of tetrapyrrolic macrocycles», Bruno F.O. Nascimento, António M.d'A. Rocha Gonsalves, Marta Pineiro, Inorganic Chemistry Communications 13, 2010, 395–398)

[5] a) «Porfirinas para preparação de catalisadores», A. M. d'A. Rocha Gonsalves, Abílio J. N. Sobral, Jorge M. T. B. Varejão, Mariete M. Pereira, Paula I. R. R. Proença e Cunha, 12ª Encontro da Sociedade Portuguesa de Química 10-13 Março, Resumos, 308, 1991; b) «New Procedures for Synthesis and Analysis of 5,10,15,20-Tetrakis-(sulphophenyl) porphyrins and Derivatives Through Chlorosulphonation», A.M.d'A. Rocha Gonsalves, Mariette M. Pereira, Abílio J. F. N. Sobral, Arménio C. Serra, P. Stocks, A.M.P. de Santana, Heterocycles, 1996, 43, 829

[6] a) E. B. Fleisher, J. M. Palmer T. S. Srivastava, and A. Chatterjee, J. Am. Chem. Soc., 1971, 93, 3162; b) C. A. Busby, R. K. Di Nello, and D. Dolphin, Can. L. Chem, 1975, 53, 1554

[7] a) «Sulphonamide Porphyrins in the Biometic Oxidation by H2O2. An Efficient Two Phase System», A.M.d'A. Rocha Gonsalves, M. M. Pereira, A.C. Serra, Annales de Quimica, Intern. Edit. 1996, 92, 375; b) «Metalloporphyrin Catalytic Oxidations of Hydrocarbons by H_2O_2», António M. d'A. Rocha Gonsalves and Arménio C. Serra, J. Porphyrins Phtalocyanines, 2000, 4, 599-604

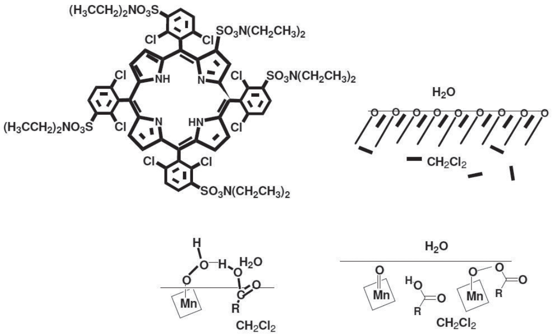

Figure 5
Efficient catalytic oxidation of alkanes and alkenes with H2O2 in the interface water-dichloromethane.

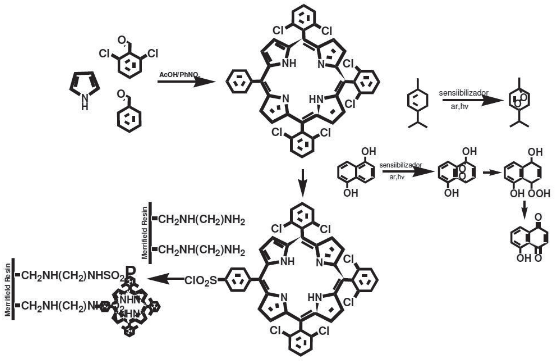

Figure 6
Catalysis of photooxidations using molecular oxygen.

We also developed catalysts for photooxidations by molecular oxygen[8] exploiting the chlorosulphonation reaction for a convenient attachment of the catalyst to a polymer matrix making easier the recovery of the catalyst and its recycling, Fig. 6[9].

Preceding examples illustrate the potential of our synthetic developments giving access to a significant diversity of porphyrins out of simple and cheap starting materials while incorporating functionalities which allow the modulation of properties to provide appropriate properties for each specific application. The possibility to activate and grant stability of the catalyst to reaction conditions allowing recycling, orientation in the liquid-liquid interface, fixation to a solid support.

While promoting catalytic the studies briefly described, we manage to establish a partnership allowing to start studies for the development of sensitizers for photodynamic therapeutics and photodiagnostic an area in which we were not able to find partnership in Portugal. Tierry Patrice, a surgeon oncologist from Nantes also a researcher of the activity of photosensitizers in cells and small animals sowed interest in collaborating with us. We sent him first a porphyrin to which we had attached compounds having an amine function through the corresponding chlorosulphonated derivative. We expected that such structures could have some capacity to internalize in the cancer cells with advantage to their activity as sensitizers of photodynamic therapy, PDT, Fig. 7. Results with these compounds, namely those incorporating amino acid unities demonstrated their efficiency and were later published.[10]

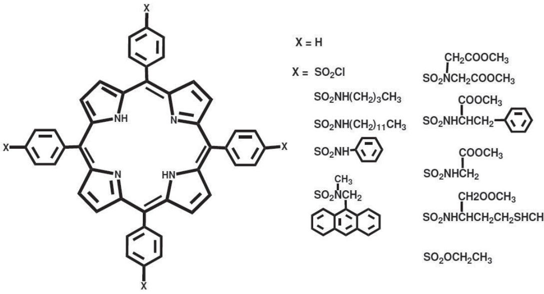

Figure 7
The first tetraphenyl-porphyrin derivatives incorporating sulphonamide side chains tested as PDT sensitizers.

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[8] «Novel porphyrins and a chlorin as efficient Singlet Oxygen Photosensitizers for Photooxidation of Naphtol or Phenols to Quinones», D. Murtinho, M. Pineiro, M. M. Pereira, A. M. d'A. Rocha Gonsalves, L. G. Arnaut, M. Graça Miguel, H. Burros, J. Chem. Soc. , Perkin Trans. 2, 2000, 2441-2447

[9] «Covalently Immobilized Porphyrins as Photooxidation Catalysts», Sonia M. Ribeiro, A. C. Serra, A. M. d'A. Rocha Gonsalves, Tetrahedron, 63, 7885-7891, 2007

[10] «New Sulphonamide and Sulphonic Ester Porphyrins as Sensitizers for Photodynamic Therapy», Abílio J. F. N. Sobral, Sabine Eléouet, Nathalie Rousset, António M. d'A. Rocha Gonsalves, Olivier Le Meur, Ludovic Bourré, Thierry Patrice, Journal of Porphyrins and Phthalocyanines, 2002, 6, 456-462

It is appropriate discussing in here the reason of our interest in developing compounds designed to be PDT sensitizers making a short introduction to the fundamentals and perspectives of this therapy. The importance of light as therapeutic agent is actually known since ancient times. Egyptians, Chinese and Indians used light as a therapeutic agent for the treatment of diseases such as psoriasis, rickets, vitiligo, psychosis and skin cancer. Greeks deepened that knowledge as clinical science, Herodotus (V AC) demonstrated the benefit of solar light to the bone growth, Hippocrates (IV AC) defined Heliotherapy as the therapy with solar light. But, the scientific approach to a therapeutic where a photochemical sensitizer having the capacity to generate reactive oxygen species when activated by light and able to concentrate into the cells of the tissue to be treated, is relatively recent. In 1913, Meyer-Betz was the first to promote patient treatments using this system and porphyrins making the first experiments in his own skin. He got evidence of severe inflammatory condition and pain in zones exposed to sunlight. In 1993, Photofrin®, a product prepared from a natural porphyrin, was approved as the first legal sensitizer for the preventive treatment of bladder cancer recurrence after transurethral resection.

It must be emphasized that photodynamic therapy is useful not only in the field of oncology but also in different areas such as the treatment of age related macular degeneracy, for skin rejuvenation and acne treatment, in depilation, in the treatment of surgical wounds and skin ulcers. The last actually involve a mechanism of photoinactivation of multiresistant microorganisms (PDI). Related is also the technique of photodiagnostic (PD) benefiting from the fluorescence of photosensitizers to define the detail and precise localization of the cells were they concentrate.

Specificities of photodynamic therapy are: allowing the selective destruction of tissues, particularly tumor masse on shining o light following the administration of the sensitizer through intermediacy of oxygen; not having significant secondary effects; the possibility of being used in conjunction with other therapeutics not interfering namely with other oncologic treatments, surgery, radiotherapy and chemotherapy; treatments can be repeated without creating resistance neither loss of efficiency in the areas previously treated; show some evidence of inducing immune response in oncology treatments.

The clinical application of PDT involves the local or systemic administration of the sensitizer which is selectively captures by the cells to be treated, Fig. 8, followed by irradiation of the place of action with a source of light either externally or internally using optical fibers. The treatment can be applied directly to kill the desired tissue, but can also be used simultaneously during a surgical intervention to eliminate random cells in the operative field. More recent are applications of the technique in the treatment of the virus of the Human Papilloma Virus and pre-cancer[11]. Of high potential are the applications for microbial inactivation[12].

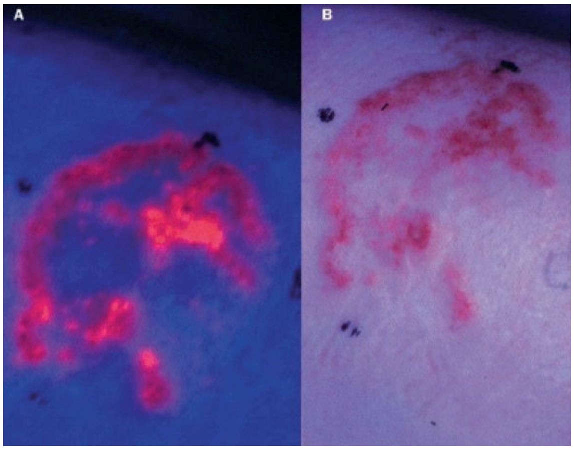

FIGURE 8
Evidence of accumulation of the sensitizer shown by fluorescence

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[11] Clarence Chew , Ondine

[12] Antimicrobial application of it, Ondine

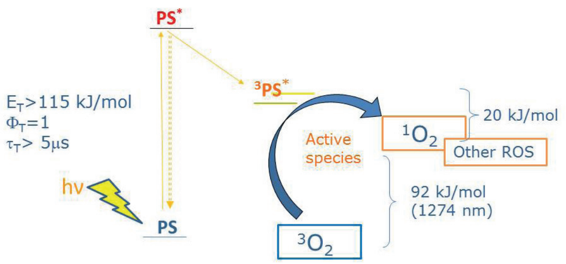

FIGURE 9
Photochemistry of the photodynamic process.

Photochemistry of PDT is based on the capacity of the sensitizer to be excited by absorption of light and become able, through its triplet state, to transfer energy forming reactive oxygen species, ROS, Fig. 9. The triplet state is able to react with other molecules, namely oxygen through one of two mechanisms: Type I or Type II reactions. Type I reactions involve direct exchange, donation or uptake of an electron or hydrogen atom of the substrate molecule which is converted in a free radical , an highly reactive species able to attack different targets in the cell structure.

Type II reactions involve the direct interaction of the sensitizer excimer with oxygen in the triplet state to generate singlet oxygen. Singlet oxygen is highly reactive namely to lipids and proteins.

Aiming to get photosensitizers having high singlet oxygen quantum yield, the oxygen active species apparently more efficient to destroy harmful cells, it was reckoned by that time that it was better to have a sensitizer having an high potential to generate singlet oxygen. On that rational, we started, in collaboration with Sebastião Formosinho a project involving then PhD student Marta Piñeiro to perform a study intended to optimize this parameter, Fig. 9. The hipothesis to exploit considered the possibility of incorporating heavy atoms in the structure of the sensitizer that, being a porphyrin would allow placing a heavy metal forming a complex or the inclusion of peripheral halogen atoms. In any case they were structures we had previously used in our catalysis studies. The study demonstrated that the presence of a number of halogen atoms in specific positions in the porphyrin structure can raise the quantum yield of singlet oxygen of the sensitizer, however, the observed increases were always not very significant, Fig. 10[13].

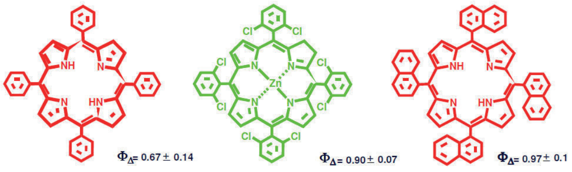

FIGURE 10
Quantum yields of singlet oxygen of tetrapyrrolic macrocycles. Heavy atom effect.

Having a significant number of samples of porphyrins with chlorine, bromine or iodine atoms, which have been prepared to the catalysis studies by the then PhD student Arménio Serra, we selected a sample of tetrakis(2-bromo-5-hydroxiphenil)porphyrin, Br4THPP, to be screened by Patrice as PDT sensitizers over *C6* rat *glioma* cell line D31. This sensitizer was selected on the grounds that it was an halogenated porphyrin whose other characteristics were analogous to one of the sensitizers already in the market, the chlorin Foscan®.

The idea was to choose a catalyst having structural characteristics favoring the transport and internalization to the cells and the good photochemical performance. The hypothesis to exploit, a tetrapyrrolic macrocycle of the type TPP but having for hydroxyl groups in the *meso*-phenyls and four halogen atoms placed in the same phenyls.

This simple approach proved to be interesting. First studies on cells and xenotransplants with C6 cells of tumor rat glioma D31 proved, in Patrice words, that the activity was has high as that of some chlorins. These first results justified the interest for an industrial property protection involving a

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[13] «Photoacoustic Measurements of Porphyrin Triplet State Quantum Yields and Singlet Oxygen Efficiencies», Pineiro M., Carvalho, A. L., Pereira M. M., Rocha Gonsalves A. M. d'A., Arnaut L. G., Formosinho S. J., Chem. Eur. J., 1998, 4, 2299

pharmaceutical company deposit of a patent[14], and later the application for a project later subsidized by the European Program Prime[15].

In project Prime we developed a systematic study screening sensitizer structures designed through rationalization of our analysis of biological studies obtained with porphyrin structures incorporating sulphonamide, and others having hydroxyl and halogen atoms attached to the meso-phenyls[16]. Fig. 11 shows an overview of the tenths of structures we prepared to screen the photodynamic activity, a study we were able to start in Coimbra through a partnership established with Filomena Botelho from the Faculty of Medicine. Porphyrins studied include tetrakis(2-bromo-5-hydroxiphenil)porphyrin, Br_4THPP, firstly studied by Patric, other similar porphyrins having different halogens in the same and other positions, without halogens, having sulphonamide and halogen groups, porphyrins of less symmetry, including more than one hydroxyl group in some of the *meso*-phenyls and other substituents in those phenyls, bearing substituents similar to those of the natural porphyrins in the β pyrrol positions having *meso*-free positions, and also chlorins and bacteriochlorins correspondent to some of the former porphyrins.

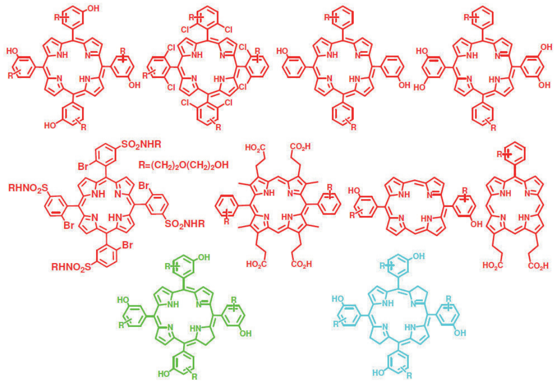

FIGURE 11
Models of structures screened as PDT sensitizers.

The photochemical properties of some of the above mentioned was studied and their phototoxicity at the cellular level was accessed in experiments following the scheme presented in Fig. 12.[17]

The photochemical properties of some of our model sensitizers are presented in Fig. 13.

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[14] «Tetrapyrrolic macrocycles as photodynamic Agents», Gonsalves AMDR, Serra, AC, Pineiro M., EP 1 472 259 B1, Priority: 01.02.2002 PT 10272102

[15] POE/Prime/Proj 3/293/CLARO

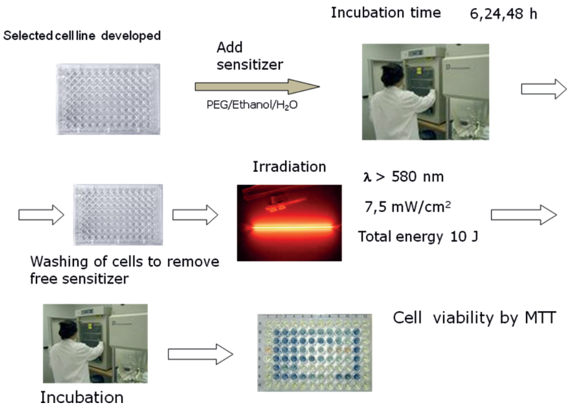


FIGURE 12
Scheme of the study of activity of the sensitizers at cell level

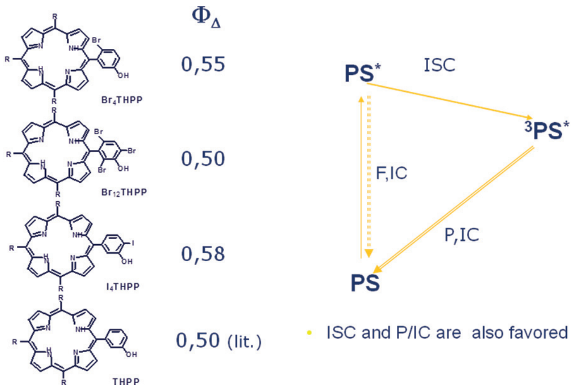


FIGURE 13
Photochemical properties of some of our model PDT sensitizers.

[16] unpublished results

[17] «Halogen atom effect on photophysical and photodynamic characteristics of derivatives of 5,10,15,20-tetrakis(3-hydroxyphenyl)porphyrin», A.C. Serra, M. Pineiro, A.M.d'A. Rocha Gonsalves, M. Abrantes, M. Laranjo, A.C. Santos, M.F. Botelho, Journal of Photochemistry and Photobiology B: Biology 92 (2008) 61-67

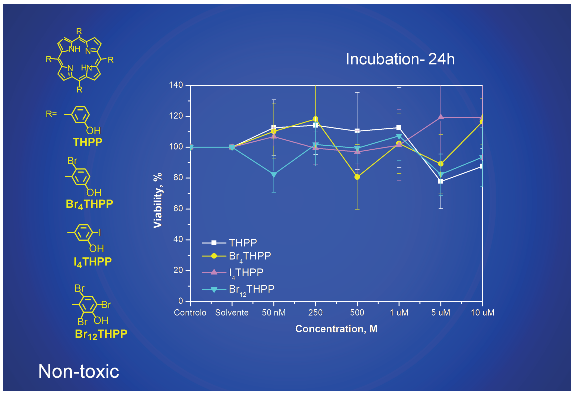
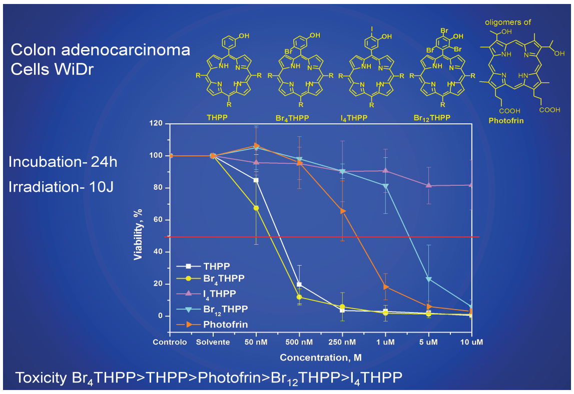


FIGURE 14
Demonstration of non toxicity in absence of light.

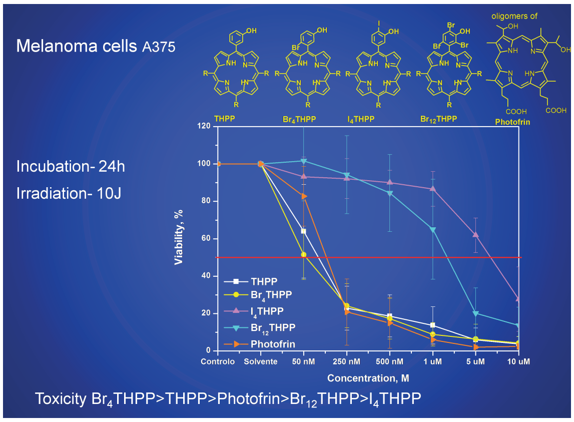


Toxicity Br$_4$THPP>THPP>Photofrin>Br$_{12}$THPP>I$_4$THPP

FIGURE 15
Phototoxicity over cells of colon adenocarcinoma WiDr of some halogenated photosensitizers comparatively to that of Photofrin®.

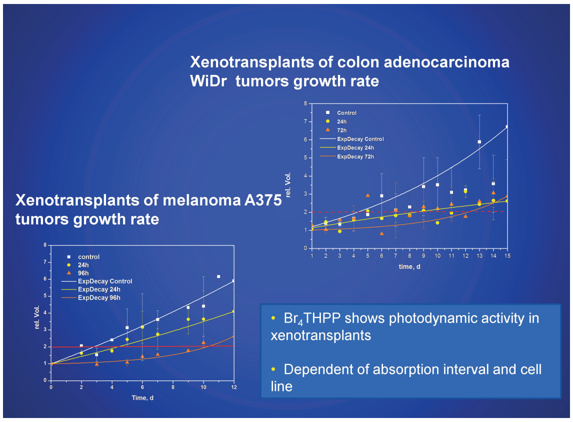
First of all absence of toxicity in absence of light, was demonstrated, Fig. 14, and Photofrin® the sensitizer approved for clinical use was taken as reference. From Fig. 15 and 16, corresponding to experiments made respectively with incubations of colon adenocarcinoma WiDr and melanoma A375, we can see that porphyrin Br$_4$THPP, has a higher photodynamic effect than the non halogenated porphyrin an clearly higher than Photofrin®. Meanwhile some of the other porphyrins, though having halogens in the structure and even an higher singlet oxygen quantum yield such as I$_4$THPP, Φ_Δ=0,58, are actually much less efficient.

The *"in vivo"* activity of sensitizer Br$_4$THPP was also studied using xenotransplants of colon adenocarcinoma WiDr and of melanoma A375 implanted in immunodeficient Balb/c nude rats, Fig. 17. Experiments were made in rats 6-8 weeks of age and the xenotransplants developed after subcutaneous injection of 2x10^6 cells of human cell lines. Rats were injected intraperitoneally with the sensitizer (2mg/kg) when the size of tumors was of 300-500 mm^3, and after an interval to spread the compound the tumor irradiated using a laser red light using a fluency of 100 mW/cm^2 up to a total energy of 150 J. These experiments proved high inhibitory activity of tumor growth as shown in Fig. 17. The efficiency depends of the interval between the injection and irradiation.



Toxicity Br$_4$THPP>THPP>Photofrin>Br$_{12}$THPP>I$_4$THPP

FIGURE 16
Phototoxicity over cells of melanoma A375 of some halogenated photosensitizers comparatively to that of Photofrin®.



- Br$_4$THPP shows photodynamic activity in xenotransplants
- Dependent of absorption interval and cell line

FIGURE 17
Study of photodynamic activity of Br$_4$THPP «*in vivo*».

Preceding results highlighted the need to guide the modeling of sensitizers not only on the basis of simple theoretical grounds based on physical-chemical principles, but through the screening of the therapeutic efficiency observed in the *"in vitro"* and *"in vivo"* tests, and also data of internalization meanwhile obtained and its correlation with the efficiency of cell destruction.

The photodynamic effect over the cells is achieved through the action of singlet oxygen promoting cell degradation via peroxidation of lipids or oxidation of membrane proteins. This action is impaired by the extremely short lifetime of that species, less than 40 ns, originating a range of action lower than 0.02 µm. Therefore, the action triggering the cell degradation is strictly confined to the near proximity of the local of $^1O_2$ generation. The point of action of singlet oxygen is, consequently, conditioned by the morphology of the cell structure affecting the internal localization into the cell.

It is appropriate now to present here a schematic view of the cell structure and of the accepted mechanisms of degradation. The potential sensitizer targets in the cell, and consequently of the reactive oxygen species generated by the photodynamic effect are essentially the cell membrane and the membranous organelles: mitochondria, lysosomes, endoplasmic reticulum, … It is on these that the mechanisms of cell death act. The proposed mechanisms will be: attack to the cell membrane degrading its structure and necrosis death, attack to the endoplasmic reticulum, ER, rising the intracellular Ca^{2+} concentration promotes apoptosis due to activation of calcineurin originating dephosphorylation of the pro-apoptotic protein *Bad* which migrates to the mitochondria inducing cytochrom C release. Cytisolic cytochrom C release and the high concentration of intracellular Ca^{2+} activate the caspase cascade originating DNA cleavage and the destruction of intracellular proteins. A schematic representation of this mechanism is presented in Fig. 18. The lack of interest to go on originating from the change of internal policy by the pharmaceutical company owner of the first patent, after closure of the above mentioned project Prime, led us to become alone in the objective of saving the results and to the need of finding a new partnership. In those circumstances we tried to capitalize the experience and expertise obtained during the project Prime in the partnership with Filomena Botelho from Faculdade de Medicina in our University conducting in Portugal the studies with cells and xenotransplants in small animals looking for new partnerships intended to lead the study of or sensitizers to advanced phases of clinical trials.

In the studies of photodynamic activity performed with the diversity of sensitizer structures previously referred we observed significant differences from sensitizers of high activity to others of very low efficiency, irrespectively of all of them having a common basic structure incorporating functionalities selected on the basis of expected benefit to optimize some physical-chemical parameter, such as, halogens to increase the singlet oxygen quantum yield.

Attempting a rationale for the collected data, we came to believe to be worth following our endeavor of optimizing an answer looking for similar sensitizers, which while including the functionalities considered as advantageous were even simplified. By

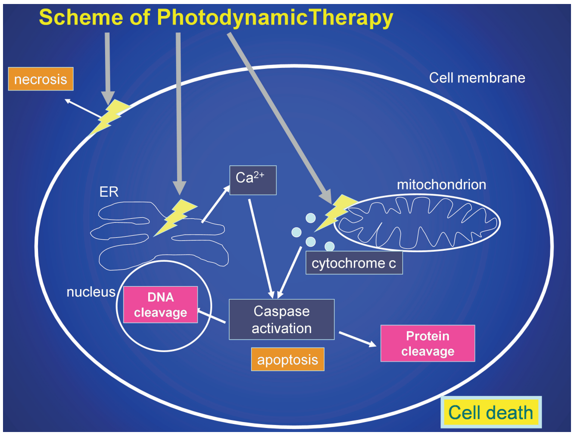


FIGURE 18
Schematic representation of the mechanisms of cell death promoted by photodynamic therapy.

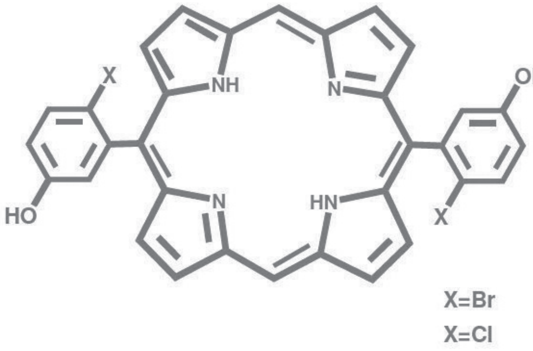


FIGURE 19
Structure of porphyrins 5,15-diaryl substituted.

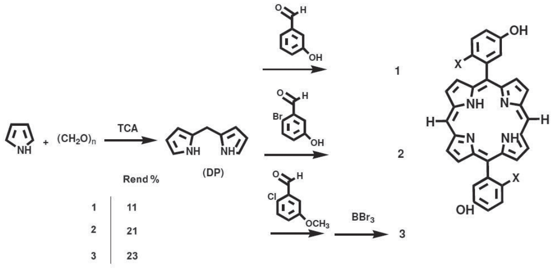


FIGURE 20
Scheme for the synthesis of 5,15-diaryl substituted porphyrins.

affinity, we considered appropriate exploiting an analogue of *meso*-tetrakis(2-bromo-5-hydroxyphenyl) porphyrin Br_4THPP, the best sensitizer found in previous studies, eliminating two of the *meso*-phenyls, and the corresponding chlorinated porphyrin, Fig.19. Such molecules keep the physical-chemical characteristics of the model sensitizer, hydroxyl and halogen groups, but have two free *meso*-positions being smaller. The absence of phenyl groups in these *meso*-positions make them more similar to natural porphyrins without substituents in those positions, making elimination probably easier since natural porphyrins are eliminated through an oxidative process consisting in an attack to those *meso*-positions leading to formation of bile pigments. A desired characteristic of PDT sensitizers is the capacity to be eliminated by the body as quickly as possible after the required photodynamic effect.

The most convenient synthetic approach to synthesize porphyrins having only two *meso*-phenyls and the other two free, corresponds to compounds having binary symmetry such as *meso*-bis(2-bromo--5-hydroxyphenyl)porphyrin, Br_2DHPP. Such molecule can be built following two different routes, Fig. 20.

We developed efficient experimental conditions to build the required dipyrrylmethanes and to join the two halves forming the desired *porphyrin*[18].

Using the two new porphyrins Br_2DHPP, e Cl_2DHPP, and Photofrin® in a comparative study of photodynamic activity using cells of colon adenocarcinoma WiDr and melanoma A375 we got the results illustrated in Fig.s 21, and 22 showing the brominated compound an activity superior to Photofrin® and to Br_4THPP, IC_{50} significantly lower than the other two[19].

In Fig.s 23, and 24, the activities of sensitizers Br_2DHPP, and Cl_2DHPP, are matched against others of our range of sensitizers relatively to their activity on human bladder cancer cells CRL 1472 for incubations of 24 and 48 hours respectively demonstrating the high efficiency of Br_2DHPP.

The preceding comparative study shows that the photodynamic activity of sensitizer Br_2DHPP is practically equivalent to that of the non-brominated sensitizer. However, in a study of cell internalization performed with incubation of these two sensitizers after a period of 6 H and concentration of incubation

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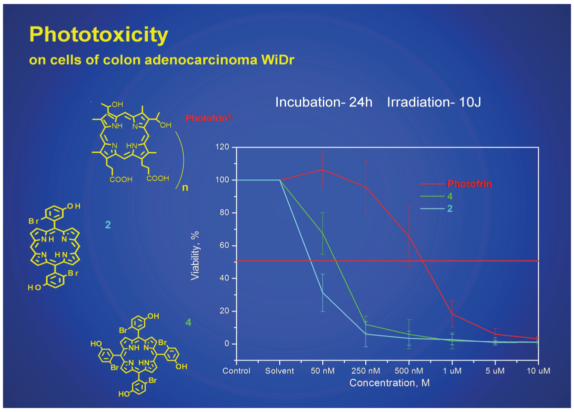
[18] Serra, A., Pineiro, M., Santos C. I., Rocha Gonsalves, A. M. d'A., Abrantes, M., Laranjo, M., Botelho, M. F., «In vitro photodynamic activity of 5,15-bis(3-hydroxyphenyl)porphyrin and its halogenated derivatives against cancer cells», Photochem. Photobiol. 86, 206-212 (2010)

[19] a) «Approaches to a broad range of high performance PDT sensitizers», António M. d'A. Rocha Gonsalves, Arménio C. Serra, Marta Pineiro, and M. Filomena Botelho, Proc. of SPIE (2009) Vol. 7190, 719005, 1-10; b) «In Vitro Photodynamic Activity of 5,15-bis(3-Hydroxyphenyl)porphyrin and its Halogenated Derivatives Against Cancer Cells», A. Serra, M. Pineiro, C. I. Santos, A. M. d'A. Rocha Gonsalves, M. Abrantes, M. Laranjo, M. F. Botelho,Photochemistry and Photobiology, 2010, 86(1), 206-212; c) «Halogenated Derivatives of 5,15-bis(3-Hydroxyphenyl)porphyrin as photosensitizers for photodynamic therapy», Inventors: Gonsalves AMDR, Serra, AC, Pineiro M., Botelho, F., PT 104511, 11/04/2009

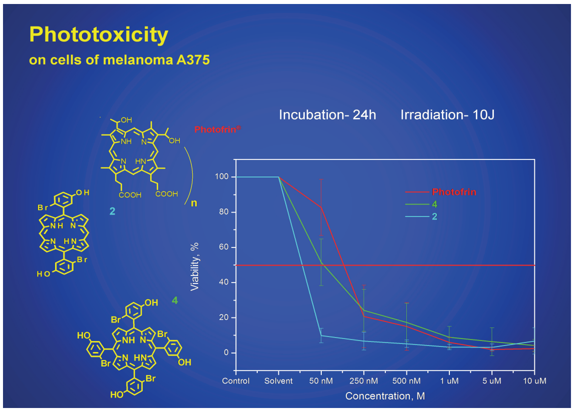


Phototoxicity

on cells of colon adenocarcinoma WiDr

Incubation- 24h Irradiation- 10J

FIGURE 21
Phototoxicity of sensitizers Br_2DHPP, Br_4THPP and Photofrin® on cells of colon adenocarcinoma WiDr.

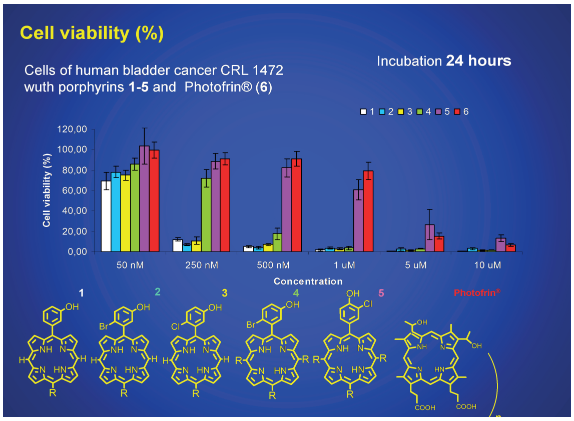


Phototoxicity

on cells of melanoma A375

Incubation- 24h Irradiation- 10J

FIGURE 22
Phototoxicity of sensitizers Br2DHPP, Br4THPP and Photofrin® on cells of melanoma A375.

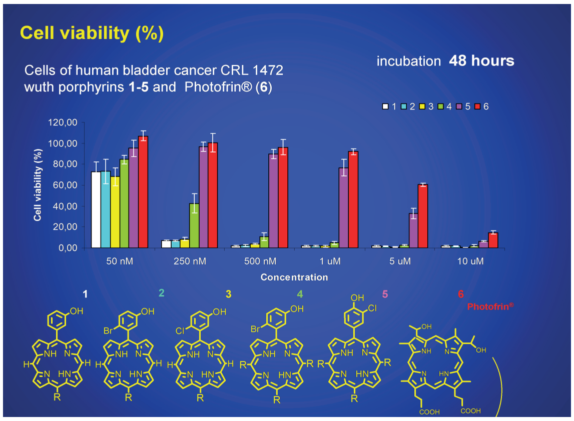


Cell viability (%)

Cells of human bladder cancer CRL 1472
wuth porphyrins **1-5** and Photofrin® **(6)**

Incubation **24 hours**

FIGURE 23
Study of cell viability for cells of human bladder cancer CRL 1472 with porphyrins Br_2DHPP, Cl_2DHPP, DHPP and others taken as reference after 24 H incubation.



Cell viability (%)

Cells of human bladder cancer CRL 1472
wuth porphyrins **1-5** and Photofrin® **(6)**

incubation **48 hours**

FIGURE 24
Study of cell viability for cells of human bladder cancer CRL 1472 with porphyrins Br2DHPP, Cl2DHPP, DHPP and others taken as reference after 48 H incubation.

of 5 μM with cells of human bladder cells CR 1472 we saw that the internalization of the two sensitizers is considerably different as shown in Fig. 25. The intracellular concentration of the brominated sensitizer is approximately five times lower than that of the non-brominated compound. Having the two sensitizers a photodynamic activity practically equivalent when applied in the same concentration we can say that the brominated compound is much more efficient. We can quantify this situation using the concept of intrinsic photoactivity, defined as the ratio between IC_{50} and the cell capture, $ISP=IC_{50}$/Cell Capt. Being the halogenated porphyrin IC_{50}

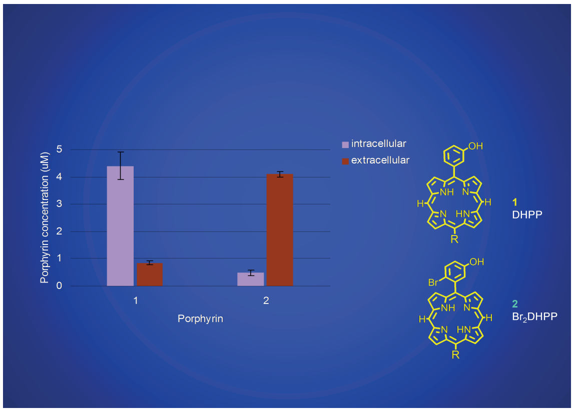


FIGURE 25
Internalization of porphyrins DHPP e Br2DHPP after 6 H incubation and 5 μM incubation concentration with human Bladder cancer cells CR 1472.

practically the same as that of the non halogenated its ISP is five times higher. We interpret this result assuming an intracellular localization more favorable to the photodynamic effect and also that a lower cellular internalization can also be advantageous concerning potential secondary effects.

In an attempt to gather information about the intracellular localization of our sensitizers, we managed some preliminary results illustrated through the images of observations using fluorescence microscopy presented in Fig. 26. There is evidence that sensitizer Br_2DHPP is located in the same position as the green mitochondrial probe, Mitrotracker GreenFM, and not inside the nuclei which is identified using the blue probe, Hoechst 33252.

The internal localization identified by fluorescence microscopy justifies the high efficiency demonstrated by our sensitizer on the accepted interpretation for the mechanism of cell death previously presented. Localizing in mitochondria, suppliers of energy to cells, these will be preferentially affected with catastrophic consequences. Nuclei will not be affected with likely advantage relatively to potential secondary effects promoted by the sensitizer.

Relatively to activity *"in vivo"* of porphyrin Br_2DHPP, the experiments performed on xenotransplants of colon adenocarcinoma WiDr and of melanoma A375, performed under the conditions of the preceding ones, led to results shown in Fig.s 27, and 28. In the experiments with tumor of colon adenocarcinoma WiDr, with irradiation after 72 H after injection of the sensitizer, it is observed a practical stoppage of growth. The same in the case of melanoma A375, irradiation performed in this case only 48 H after injection.

Having intense absorption bands in the red zone of the spectrum, and probably more easily eliminated after treatment, chlorins, and bacteriochlorins Fig. 29, have potential advantage as photodynamic sensitizers relatively to porphyrins. The study conducted by Bonnet leading to the approved and marketed sensitizer Foscan®

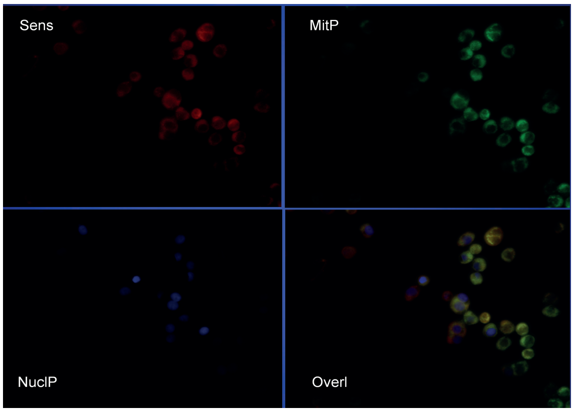
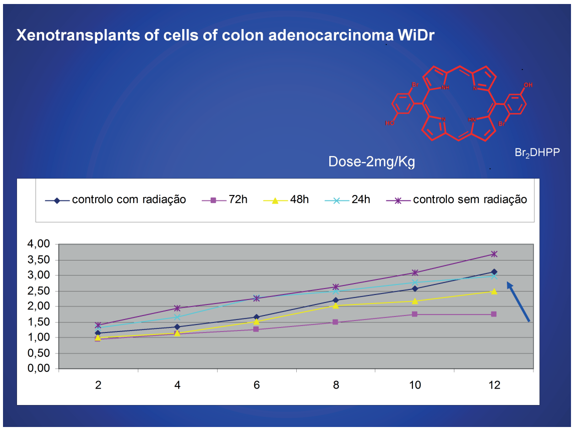
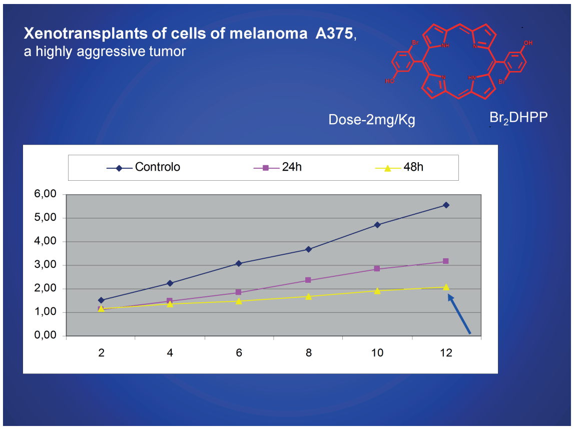


FIGURE 26
Images obtained by fluorescence microscopy submitted to sensitizer Br2DHPP, Sens, mitochondrial probe, MitP nuclear probe, NuclP, and images overlapping, Overl.


Xenotransplants of cells of colon adenocarcinoma WiDr

Dose-2mg/Kg

FIGURE 27
Photodynamic activity experiments "in vivo" from sensitizer Br2DHPP on xenotransplanted tumors of colon adenocarcinoma WiDr.


Xenotransplants of cells of melanoma A375,
a highly aggressive tumor

Dose-2mg/Kg

FIGURA 28
Photodynamic activity experiments "in vivo" from sensitizer Br2DHPP on xenotransplant tumors of melanoma A375.

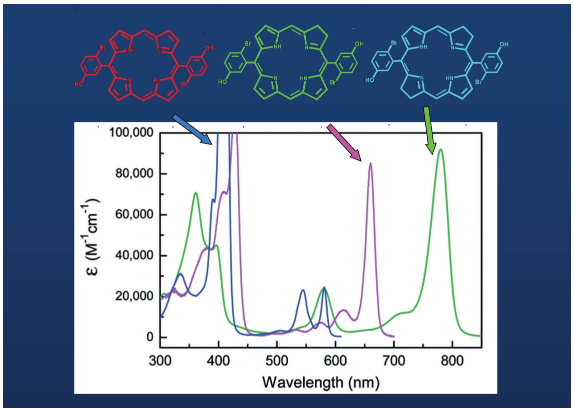
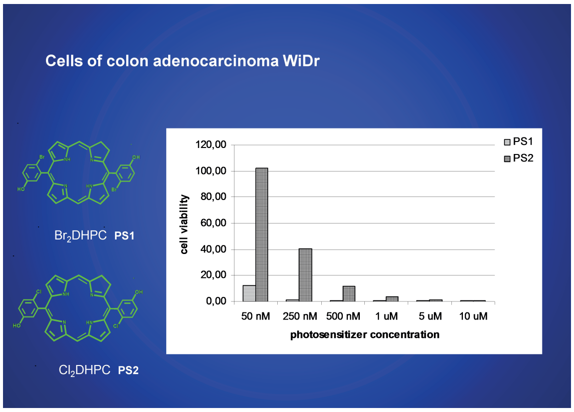


FIGURE 29
Absorption spectra typical of porphyrin, chlorin and bacteriochlorin.


Cells of colon adenocarcinoma WiDr

Br_2DHPC **PS1**

Cl_2DHPC **PS2**

FIGURE 30
Activity of chlorins Br2DHPC e Cl2DHPC on cells of colon adenocarcinoma WiDr.

evolved from the porphyrin firstly prepared to the corresponding chlorin. Our synthetic developments include, as said before, synthesis of chlorins either by direct synthesis or through reduction of the corresponding chlorin.[20] It was therefore justified to exploit the potential of the chlorin correspondent to porphyrin Br_2DHPP as photodynamic therapy sensitizer.

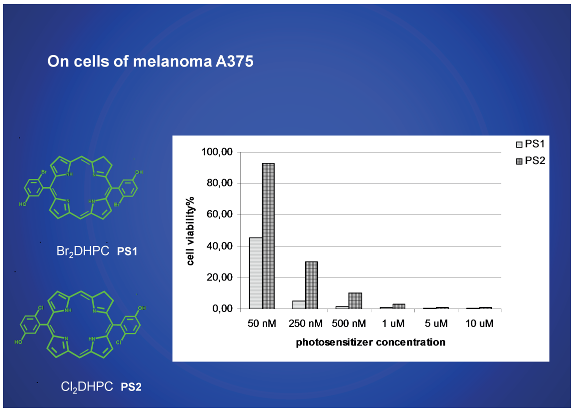
Interestingly, chlorin Br_2DHPC, has an oxygen quantum yield, Φ_Δ=0.98, superior to all porphyrins we studied before. Although our observations lead to conclude that this is not the main factor to define the photodynamic efficiency, it is nevertheless a significant property and potentially convenient.

The photodynamic activity of chlorin Br_2DHPC, and that of the corresponding chlorinated compound, Cl_2DHPC, Cl_2DHPC, in experiments with colon adenocarcinoma WiDr, and melanoma A375 cells under conditions as previously described is shown in Fig.s 30, and 31. The brominated compound proved to be the more active sensitizer.

The comparative study of photodynamic activity at cell level between clorin Br_2DHPC, and the corresponding porphyrin Br_2DHPP with colon adenocarcinoma WiDr, melanoma A375, and also human bladder cancer CRL1472 cells is illustrated in Fig.s 32, and 33 showing a much higher activity of the chlorin.

In Fig. 34 we see the result of experiments performed with esophagus cancer cells OE19 with chlorin Br_2DHPC. This study gave showed an IC_{50} of 25-50 nM for this sensitizer, comparing favorably with the lowest values found in the literature.

In an exploratory study of the activity "*in vivo*" performed with chlorin Br_2DHPC, following experimental conditions as previously described with


On cells of melanoma A375

Br_2DHPC **PS1**

Cl_2DHPC **PS2**

FIGURE 31
Activity of chlorins Br_2DHPC e Cl_2DHPC on cells of melanoma A375.

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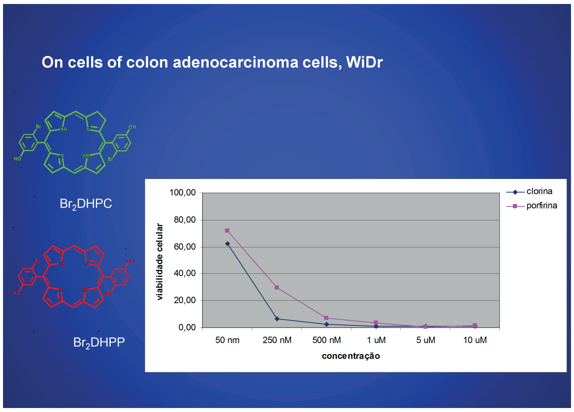
[20] e) «Controlled porphyrinogen oxidation for the selective synthesis of meso-tetraarylchlorins», Arménio C. Serra, António M. d'A. Rocha Gonsalves, Tetrahedron Letters 51 (2010) 4192–4194;137; f) «MnO_2 instead of quinones as selective oxidant of tetrapyrrolic macrocycles», Bruno F.O. Nascimento, António M.d'A. Rocha Gonsalves, Marta Pineiro, Inorganic Chemistry Communications 13, 2010, 395–398

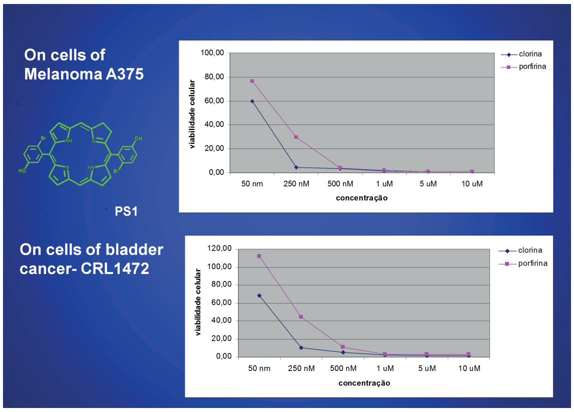


On cells of colon adenocarcinoma cells, WiDr

Br$_2$DHPC

Br$_2$DHPP

FIGURE 32
Comparative activity of sensitizers Br$_2$DHPC e Br$_2$DHPP on colon adenocarcinoma WiDr cells.



On cells of Melanoma A375

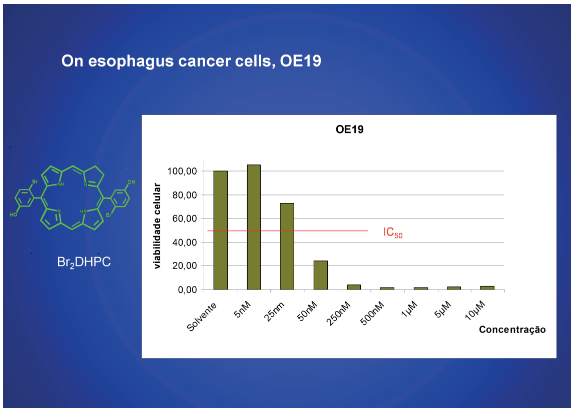
PS1

On cells of bladder cancer- CRL1472

FIGURE 33
Comparative activity of sensitizers Br$_2$DHPC e Br$_2$DHPP on melanoma A375, and human bladder cancer CRL1472 cells.

tumors of colon adenocarcinoma WiDr implanted in Balb/c nude rats, we obtained evidence of an efficient reduction in the rate of growth of tumors after one single PDT treatment after 72 H of the sensitizer injection, Fig. 35.[21]

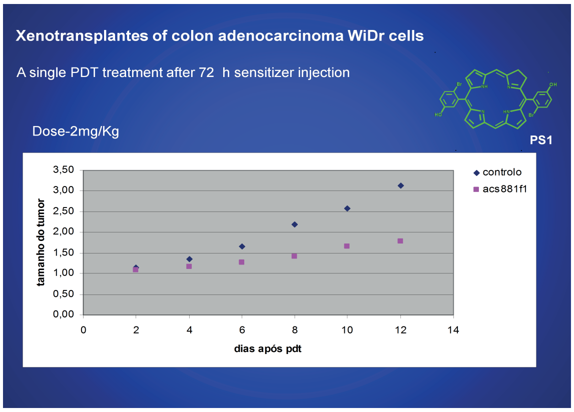
The efficiency demonstrated by chlorine Br$_2$DHPC, justified the expectation that it could become a sensitizer for practical use in photodynamic therapy. Looking forward to the preparation of an application for approval as a therapeutic agent we started the process of study of animal toxicity with encouraging results as shown in Fig. 36. Using rats Wistar injected with a dose 5x higher (10 mg/Kg) than the one used for the "*in vivo*" animal PDT studies, parameters correspondent to kidney, liver and cardiac functions were measured. Parameters relative to kidney and liver functions were normal either after 3 or 12 days. Only the creatine kinase and LDH levels related to cardiac function showed higher levels after 3 days injection but were back to normal after 12 days.



On esophagus cancer cells, OE19

Br$_2$DHPC

FIGURE 34
Activity of sensitizer Br$_2$DHPC in esophagus cancer cells OE19.



Xenotransplantes of colon adenocarcinoma WiDr cells

A single PDT treatment after 72 h sensitizer injection

Dose-2mg/Kg

PS1

FIGURE 35
Photodynamic activity of sensitizer Br2DHPC "in vivo" on xenotransplanted tumors of colon adenocarcinoma WiDr.

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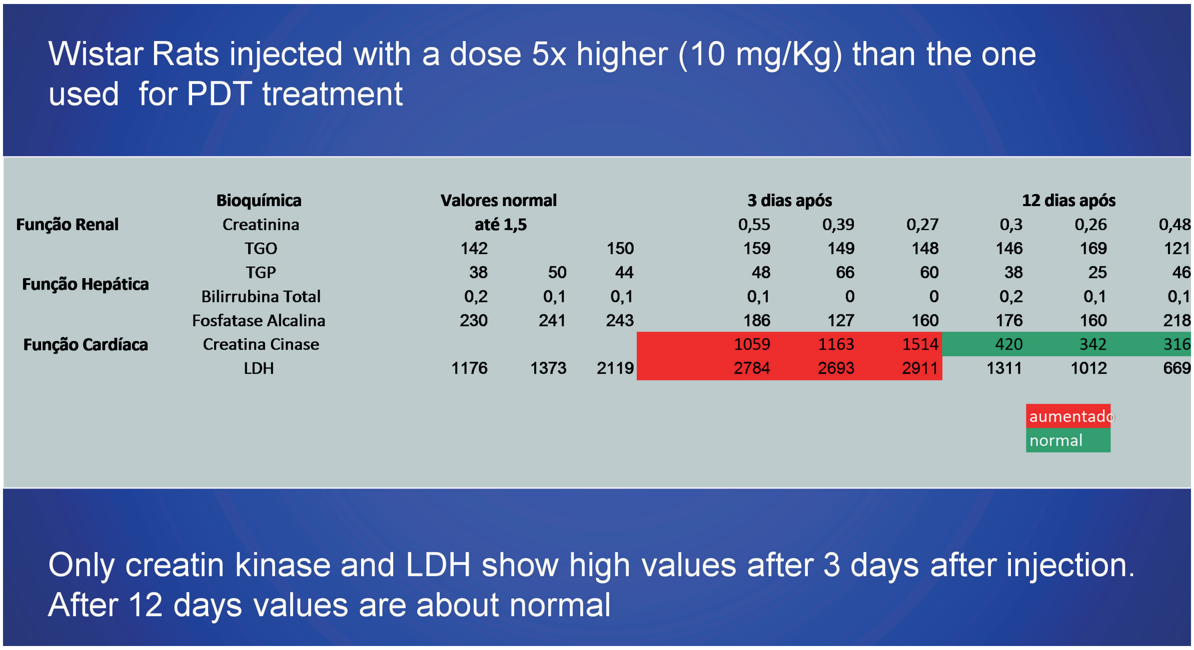
[21] «Halogenated Derivatives of 5,15-bis(3-hydroxyphenyl)chlorins as photosensitizers for photodynamic therapy», Inventors: Gonsalves AMDR, Serra, AC, Pineiro M., Botelho, F., Proprietor: Fundação Padre António Vieira, WO 2011/161065 A1, 29 Dec 2011, PCT/EP2011/060256, 20/06/2011, Priority 25.06.2010 PT105173B



Wistar Rats injected with a dose 5x higher (10 mg/Kg) than the one used for PDT treatment

	Bioquímica	**Valores normal**			**3 dias após**			**12 dias após**		
Função Renal	Creatinina	**até 1,5**			0,55	0,39	0,27	0,3	0,26	0,48
Função Hepática	TGO	142		150	159	149	148	146	169	121
	TGP	38	50	44	48	66	60	38	25	46
	Bilirrubina Total	0,2	0,1	0,1	0,1	0	0	0,2	0,1	0,1
	Fosfatase Alcalina	230	241	243	186	127	160	176	160	218
Função Cardíaca	Creatina Cinase				🟥 1059	🟥 1163	🟥 1514	🟩 420	🟩 342	🟩 316
	LDH	1176	1373	2119	🟥 2784	🟥 2693	🟥 2911	1311	1012	669

🟥 aumentado
🟩 normal

Only creatin kinase and LDH show high values after 3 days after injection. After 12 days values are about normal

FIGURE 36
Photodynamic activity of sensitizer Br2DHPC "in vivo" on xenotransplanted tumors of colon adenocarcinoma WiDr.

This study would not have reached the present advanced stage without the dedication of collaborators who, through the years where they built the work to obtain their degrees. I can not ignore that in the development here presented the fundamental contributions of two of them, Arménio Serra and Marta Piñeiro.

(COMMUNICATION TO ACADEMIA DAS CIÊNCIAS DE LISBOA
THE 1ST OF MARCH 2012)