Porphyrin – Cis-Platin drug system for HeLa cells photodynamic treatment

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Abstract

Photodynamic therapy (PDT) is a clinical approach that use light-activated drugs for the treatment of different kind of tumor tissues. HeLa tumor cells, as an experimental model for study the new biomedical concept of associated PDT with 5,10,15,20-sulphonato-phenyl-porphyrin (TSPP) and an antitumor agent – cisplatin (CisPt), yielded to an enhanced effect on the suppression of tumor cells in vitro. The ultrastructure changes of cells caused by the action of the new concept and laser irradiation were analyzed, putting into evidence a linear survival curves, by the linear quadratic model. The ultrastructural morphologic aspect of the HeLa cells phototreated with TSPP reinforces the results that a great number of cells with plasma membrane damage, characteristics of irreversible cellular lesions were observed.

1. Introduction

Photodynamic therapy (PDT) is a novel treatment for cancer and certain non-cancerous diseases that are generally characterized by overgrowth of unwanted or abnormal cells [1]. By using a combination of a photosensitizer and a light source, the procedure require exposure of cells or tissues to a photosensitizing drug followed by irradiation with light of the appropriate wavelength, compatible with the absorption spectrum of the drug [1,2]. In PDT, photosensitizers are used to absorb energy from a light source after its administration to tumour cells, producing reactive oxygen species that will cause cell death.

Intense research has been devoted to understanding the molecular processes involved in apoptotic cell death and deciding about the strategies that can restore the apoptotic potential to tumor cells. PDT with most of the sensitizers tested, acts via singlet oxygen production. Singlet oxygen has been indicated as the active PDT agent causing injury to cells and tissues. Because of the short half live of this excited species in cells (<0.1 ms) and short radius of action (<0.02 mm), damage will occur mainly next to the region the sensitizer is concentrated. Incubation time of cells with photosensitizer, is another parameter that will affect the mode of cell death during exposure to light. For short period of incubation, the plasma membrane is an important site of damage [3]. Prolonged incubation with Photofrin®, the first PDT photosensitizer to win approval by regulatory agencies in several countries, tends to localize in the mitochondria membrane.

Many of the sensitizers used in the experimental or clinical PDT, localize in the plasma membrane, mitochondria, endoplasm reticulum and lysosomes [4]. In photodynamic therapy, the cellular photo-modification consists of: photosensitizer transport to the active site; possible binding or aggregation; light absorption by the site; production of energetic intermediate states; reaction with cellular bio-molecules; modifications of cellular function.

Proteins, lipids and nucleic acids are the most vulnerable cellular targets which can support their photo-oxidation.

As documented in the literature, many efforts have been made to discover new sensitizers with high singlet oxygen yield and high photodynamic activity. Among different molecules, porphyrins can be considered ideal sensitizers because of their optimal photodynamic activity [5]. Cisplatin (*cis*-diamminedichloroplatinum; *cis*Pt) is a potent inducer of growth arrest and/or apoptosis in most cell types and is among the most effective and widely used chemotherapeutic agents

employed for treatment of human cancers. Cisplatin as one of the most widely used metal-containing anticancer drug, is one of the most effective agents used to treat various types of human cancer (bladder, testicular, ovarian, and head and neck tumors) and, but its clinical effectiveness has been limited by significant undesiderable side effects, such as dose-dependent nephrotoxicity and neurotoxicity. Moreover, the use of cisplatin is limited by lack of activity against tumors with neutral or acquired resistance to this drug. Pt(II) compounds have also an extensive history exhibiting virucidal activity, including a recent report of anti HIV-1 activity [6]. In this context there is a clear evidence that the carrier ligand influences the antiviral activity and modifications of the carrier ligand in cisplatin may broaden the range of antitumor and antiviral activities, therefore, there is still a need to synthesize platinum(II) complexes with novel ligands and to test them for antitumor activity, in the hope of overcoming the above mentioned limitations. There is an urgent need for the development of new anticancer drug candidates to replace CisPt causes apoptosis by DNA fragmentation. The toxicity of platinum anticancer drugs presents a major obstacle in the effective treatment of tumours. Much of the toxicity stems from a lack of specificity of the drugs for the sites at which they are able to exert maximum anticancer activity. An improved understanding of the behaviour of the drugs in the tumour environment may assist in the rational design of future platinum anticancer agents with enhanced specificity and reduced toxicity.

Platinum complexes currently make up one of the three most widely used groups of anticancer drugs in the world. The anticancer activity of cisplatin (cis-[PtCl2(NH3)2], see Figure 1) was discovered serendipitously in the 1960s. Since 1978 it has been used in the clinic against a variety of cancers, including testicular, ovarian, head and neck, bladder, cervical, lymphoma and melanoma. Treatment with cisplatin often causes severe side effects such as nausea, vomiting, nephrotoxicity, neurotoxicity, myelotoxicity, and emetogenesis. These side effects arise mainly as a result of the limited selectivity of cisplatin for tumour cells as compared to healthy cells, and may also be due to reactions with thiol-containing species in blood plasma, such as cysteine and human serum albumin. In spite of its widespread clinical use, many tumours are unresponsive to cisplatin treatment due to intrinsic (eg. colon cancer, non-small-cell lung cancer) or acquired resistance (eg. Ovarian cancer, small-cell lung cancer). The cellular mechanisms of cisplatin resistance have been identified and recently reviewed. The main factors that modulate resistance include decreased drug accumulation, increased levels of intracellular thiols that can deactivate cisplatin, and an increased capability of cells to repair or tolerate DNA damage caused by cisplatin. Other processes have also been implicated.

Porphyrins, however, have limited photo stability, so their association with other drugs, in order to increase both light stability and photodynamic efficiency, is strongly recommended [7-10].

Photosensitizer as TSPP (TSPP = 5,10,15,20–tetra-sulphonated-phenylporphyrin) are able to decrease the fraction of single stranded circular genomic DNA by converting it to linear form [11]. Porphyrin and/or metalloporphyrin mediated cleavage of nucleic acids occurs via oxidative attack on the sugar moiety with consequent nucleobase modifications leading to strand scission; or by a photo induced mechanism involving either the porphyrin excited state or singlet oxygen.

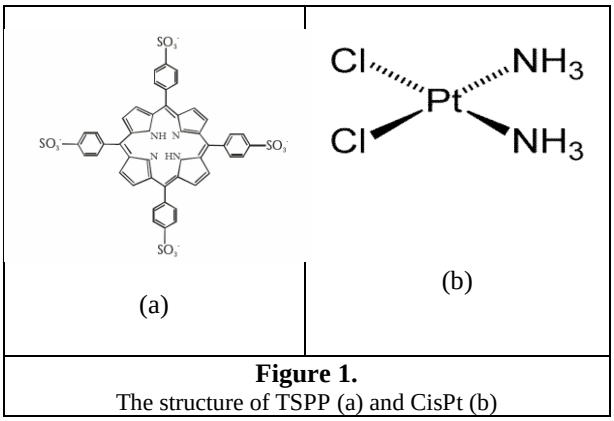
The photosensitized tumor therapy, carried on in the presence of certain chemical agents, can lead to enhanced tumor and tumor cell response. In this context, the combination between a porphyrin, TSPP, and a drug, cisplatin (CisPt), appears to be a useful and promising system, which has been studied, and it will be described hereafter.

In this work, HeLa tumor cells were used as an experimental model for studying the action of different new sensitizers in photodynamic therapy (PDT). Tumor monolayer cultures were incubated for 18 h at 37° C with TSPP and CisPt, and observed before and after photodynamic treatment with He-Ne laser. The association of a sensitizer with an antitumor agent, such as cisplatin, has an enhanced effect on the suppression of tumor cells in vitro which is more pronounced than that caused by individual components. Also, the ultrastructure changes caused by the action of two photosensitizers and laser irradiation on HeLa (neoplastic) cells were analyzed by transmission electron microscopy. The results showed induction of apoptosis.

2. Experimental part

2.1. Photosensitizers

TSPP (Figure 1a) has been prepared and purrified according to a published procedure [12].



(a) (b)

Figure 1.
The structure of TSPP (a) and CisPt (b)

Cisplatin (CisPt) (Figure 1b) has been used as pharmaceutical product without any further purification; the optimal concentrations were between 20-50 ng (105 cells) incubated for 18 h at 37° C.

2.2. Cell culture

Human HeLa cervical adenocarcinoma cells (ATCC CCL-2) were cultured at 37 °C in a humidified sterile atmosphere of 95% air and 5% CO2 at 37 °C, using DMEM supplemented with fetal calf serum (10%, v/v), glucose (4.5 g/l), L-glutamine

(292 mg/l), streptomycin sulfate (10 mg/l) and potassium penicillin (10000 U/l). HeLa cells were maintained frozen in DMEM with 10% DMSO. 1.8 ml CryoTubes™ (Nunc, Nalge Nunc International, IL, USA) were filled with the cellular suspension and then were placed in a cell Cryo 1 °C Freezing Container (Nalgene, Nalge Nunc International, IL, USA) to be slowly frozen up to -80 °C at a cooling rate of -1 °C/min for successful cell cryopreservation. Frozen cells were rapidly transferred to a liquid nitrogen container (-196 °C) and stored. HeLa cells are adherent cells which grow up to form cellular monolayers toward confluence after inoculation. Cell viability was evaluated with 0.2 % trypan blue solution. HeLa tumor cell cultures were treated with TSPP, CisPt and TSPP/CisPt.

2.3. Methods and apparatus

All spectroscopic measurements were carried out in 1-cm quartz cuvettes (Hellma, Germany) at room temperature and, in the case of cell suspensions, the samples were continuously stirred.

2.4. Cellular uptake

The cellular uptake of the photosensitizers was estimated by flow cytometry, using Foreskin cells cultured in 75 cm^2 tissue culture flasks as indicated above. Our compounds, dissolved in DMSO:water (0.05%-99.95%), were added to the flasks at a concentration of 1 μM when a confluence of 80–85% was reached and the cells were incubated with the sensitizer for different periods of time in the dark. Immediately after each time, the cells were washed with PBS to remove the non-entrapped sensitizer. Trypsinization was carried out using PBS containing 0.2% trypsin and 0.5 mM EDTA. The suspension of cells was centrifuged at 1500 rpm for 5 min. The pellet was suspended in PBS and prior to flow cytometric analysis, the new suspension was filtered through nylon filters (Nytal, 70 μm mesh, Sefar Maissa S.A., Barcelona, Spain) to exclude cellular aggregates.

2.5. Irradiation protocol

Cell suspensions were subjected to photodynamic therapy (PDT), namely pre-incubated for 1h-18h with the derivatives, and subjected to irradiation after washing them 3x in culture medium and resuspended at 3×10^5 cells/mL. The cell suspension irradiation was performed with a polychromatic lamp in a quartz cuvette, exposure time 30 minutes. Cellular photodegradation kinetics were recorded during irradiation with an UV-VIS double-beam spectrophotometer (Carl Zeiss Jena), connected to an external computer for data processing. Cell numbers were evaluated by means of maximum absorption at the band located at 275 nm, and fitted with a mathematical model. The absorption of UV in this region in a fixed volume of solubilized cells is proportional to the cell number, and therefore can be used as a simple means of obtaining a cell count. Cell counts obtained in this way can be combined with measurements of the inhibition of DNA synthesis ([3H]-thymidine incorporation) by test compounds, to produce an index of cytotoxicity [17]. A time dependency of log (No/N) was plotted, representing the decrease in the actual number of intact cells during the irradiation period, where No = initial number of cells and N = number of cells at a given time point. As cellular controls, we have used unloaded cells suspensions subjected to irradiation.

After irradiation, cell suspensions were washed twice for removal of cell debris generated during irradiation. After PDT, cell suspension was recorded in the

Counting Chamber (Roth) with 0.4% Trypan Blue Stain and the actual decrease in the number of cells subjected to PDT was presented.

2.5. Singlet oxygen test

Measurements were carried out in a quartz cell (1cm x 1cm) at 20°C. A DMSO solution (2.3 ml) containing sensitizer (4.8×10^{-5} M) and DPBF (2.7×10^{-5} M) was irradiated with light beam from a UV-Vis spectrophotometer. Solutions of sensitizers were freshly prepared and kept in the dark before measurements. The decreasing of the DPBF concentration was followed by a special programm ruled on a computer at the absorbance from 415 nm (the molar coefficient of absorption for DPBF is 23300 $M^{-1}cm^{-1}$) as function of the irradiation time (irradiation cycles 50 x 25 s). The reaction showed a zero order-kinetics in the first 100 s. The incident photon flow was 4.65×10^{-9} $M.s^{-1}$. Using the absorption spectra of the photosensitizer, the absorbed photon flow (I_{abs}) was evaluated. The quantum yield of the photooxidation of DPBF was calculated from the eq.l

$$\Phi_{DPBF} = ([DPBF]/I\ abs.V);\ V= 3\ cm^3 \quad (1)$$

The quantum yield for singlet oxygen generation was calculated from eq.2.

$$1/\Phi_{DPBF}=1/\Phi_{1O2} + (1/\Phi_{1O2}\ K_d/K_a)(1/[DPBF]) \quad (2)$$

From the intercept of the Stern-Volmer plots,we obtained the quantum yield for singlet oxygen generation.

2.6. Cell phototoxicity after irradiation

The cells were plated at a number of 106 cells/ml in each well of a 24 wells plate (Nunc, Denmark) as follows: six wells for light and photosensitizer and, six wells for light only (control). After 24 h of culture, cells were incubated with 10 μM of TSPP or Cis Pt in culture medium without serum for 60 min. Cells were washed twice with phosphate buffered saline (PBS) and 200 μl of fresh PBS was added for irradiation. Dark barriers were placed between wells to avoid scattered light during irradiation. Another dark barrier with an orifice of the diameter of the wall was placed on the top of the 24 wells plate for the same purpose. The irradiation was done in the dark with a He-Ne laser light exposure (Jena, 632.8 ms, power 50 mW) was performed for different times at 37° C in bottles with quartz windows, the cell suspensions being gently stirred during irradiation.

After He- Ne laser treatment, cell suspension were 1:10 diluted with growth medium ($1x10^5$ cells/ ml final concentration). The cellular suspensions were incubated for 24 h at 37° C in 5% CO_2 atmosphere. After irradiation, PBS was removed and culture medium with 10% FBS was added to the cells for 24, 48 and 72 h of culture in a humidified 5% CO_2 at 37ºC. After each period of incubation, the number of living cells was counted by the Trypan blue exclusion test.

2.7. Proliferation assay

The growth inhibitory effect of the studied compounds (TSPP, CisPt, or a combination of the two) towards HeLa cells was evaluated by using the spectrophotometric MTT assay [13]. The cells, grown in the culture flasks, were trypsinized and seeded at a density of 10^6 cells/well in well plates. DMEM growth medium (with 10% FBS) was used, and the cells were incubated overnight at 37°C in humidified environment containing 5% CO_2 to allow adherence. The tested compounds were diluted in FBS-free growth medium and then administered in growing doses (1, 10, 100 and 200 μM); their cytotoxic effect was evaluated using different periods of incubation (24, 48 and 72 hours). The MTT solution (5 mg/ml) was added to each well (10 μl) at the end of the incubation time with inhibitors and further incubated for 4 h at 37°C. After this period, the medium was aspirated and the purple crystals of formazan, which had been formed, were dissolved by addition of isopropanol/HCl 0.04 N (100 μl/well). Absorbance at 550 nm was measured on a spectrophotometer. The absorbance of the treated cells was then given as percentage of that of control cells and the resulting data are based on the mean value of 8 wells ± S. D.

2.8. Kinetic model

Irradiation of cells with light radiation produces linear survival curves [14]. The relationship between the surviving fraction S and the light dose D is then:

$$S = \exp(-\alpha D) \quad (3)$$

where:

S is the number of surviving cells;

$-\alpha$ is the slope

D is the radiation dose delivered.

The relationship is more commonly represented as:

$$S= \log N/No = \exp(-D/Do) \quad (4)$$

by defining Do as $1/\alpha$. When D=Do, $S = e^{-1} = 0.37$.

2.9. Electron microscopy

After irradiation, PBS was replaced by medium with serum. After 24 h of culture, cells were washed twice with PBS and fixed with 2.5% glutaraldehyde and 4% freshly prepared formaldehyde in phosphate buffer 0.1M (pH 7.2) for at least 2 h at 4ºC. Cells were detached from the dish with a cell scraper, centrifuged three times (1500g, 10 min) with 0.1M fresh phosphate buffer and post fixed in 1% osmium tetroxide in phosphate buffer for 30 min. Finally, cells were wahsed again and dehydrated in acetone and embedded in Epon. After sectioning, cells were contrasted with uranyl acetate for 30 min and lead citrate for five min. Transmission electron microscopy was performed using a Zeiss 900 and a Zeiss EM10 microscope.

2.10. Fluorescence microscopy

It was performed in order to investigate the cellular loading efficiency. Thus, in an Nikon E300 inverted fluorescence microscope with image capture, HeLa cells loaded 24h with non-toxic concentration of the investigated compounds were investigated with the V2A filter, excitation 380-420nm, emission < 450nm. After loading, cells were washed and in RPMI1640 medium without phenol red resuspended at $5x10^6$ cells/mL concentration. Live cell suspensions were laid on fluorescence slides (Marienfeld). Image was captured both in fluorescence and in phase contrast.

2.11. Confocal fluorescence microscopy

HeLa cells were viewed using a BIO RAD Radiance Plus Confocal Microscope. Images were obtained using a 100 x oil immersion objective (Nikon). The compounds CisPt, TSPP and CisPt-TSPP were excited at 543 nm, and detected at λ_{em} = 555 – 626 nm. Stains were excited using λ_{ex} = 488 nm, and detected at λem = 500-560 nm. HeLa cells were viewed using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Welwyn Garden City, UK). Images were obtained using a 63x objective oil immersion objective. The compounds were excited at 543 nm, 76% laser strength, and detected at λ_{em} >560 nm. Stains were excited using λ_{ex} = 488 nm, 10-15% laser strength, and detected at λ_{em} = 505-530 nm.

2.11. Caspase-3 activity

It was measured from cell lysates by a colorimetric method (CaspACE™ Assay System, Promega Corporation), using its high sensitive substrate coupled to a cromophore (p-nitroaniline, pNA). The free pNA released from substrate upon enzyme action, is spectrophotometrically detected at 405 nm. Using a calibration curve and the actual proteic concentration in cell lysates (Bradford method) the specific caspase activity was calculated to the total proteic content of each lysate and normalized to $1x10^5$ cells. Results are presented as microMpNA/$1x10^5$ cells. Cell cultures were tested in different stages of the cell cycle, different batches of the same cell lines or primary cell cultures in order to have the statistical significant basal caspase 3 activity related to the cell type.

2.12. Apoptosis

It was evaluated by flow cytometry, using the annexin V / propidium iodide method (BD Biosciences kit). Annexin V (Ann) highlights apoptosis-associated loss of plasma membrane asymmetry, namely phosphatidylserine (PS) translocation from the inner to the outer leaflet of the plasma membrane, which is recognized by annexin V. Propidium iodide (PI) is used as vital stain. Annexin V staining identifies early apoptosis events, while cells double positive (Ann+PI+) are in late apoptosis or already dead. Positive controls were camptothecin 1microM (apoptosis inducer) treated cells. Samples were analyzed by flow cytometry (FACScalibur cytometer, Becton Dickinson) within one hour, using CellQuest software.

3. Results and discussion

Exposition of HeLa tumor cells to a combination TSPP with cisplatin showed a significant synergistic effect, which improves the efficacy of the antitumor drug, Figure 2.

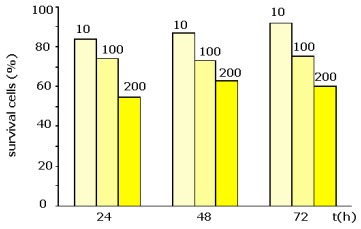


Figure 2.
Survival rate of HeLa in the presence of TSPP/CisPt

In the treatment with TSPP, at 10mM concentration of the drug, the surviving cell population was reduced to 60% after 24 h, and to less than 20% after 48 h of incubation respectively (the effect being more important for higher cisplatin concentration), Figure 3.

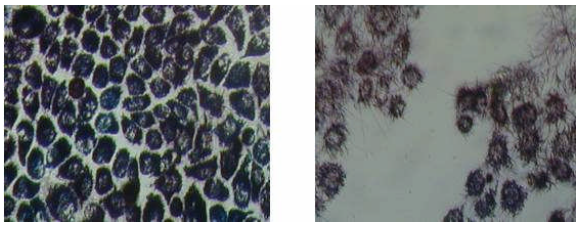


Figure 3.
MTT test for HeLa cells (control)-left and incubated with TSPP-CisPt 4 mM (right)

HeLa tumor cells were exposed to a photosensitizer (TSPP) alone or in association with an antitumor agent (cisplatin). When HeLa cells, in a stationary development stage, were treated with TSPP in concentrations ranging between 1 and 200 μM (or 5 and 500 ng/10^5 cells), the surviving rates were almost 100% after 24 h, and not less than 90% after 48 h of incubation (Figure. 4).

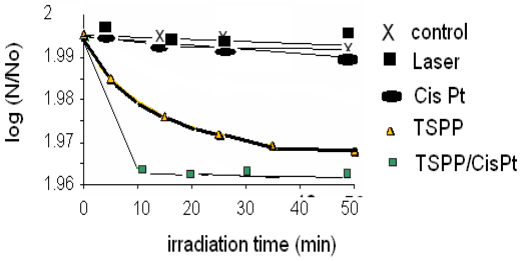


Figure 4.
Number of living cells (HeLa) after photodynamic treatment with TSPP, CisPt, TSPP/CisPt at different times.

On irradiation of HeLa tumor cells only with He-Ne laser, or Cis Pt not significant cytotoxic effect had been observed.

HeLa tumor cells were then irradiated wit He-Ne laser before and after treatment with TSPP, CisPt, or a combination of the two, and SEM examination of treated and untreated cells was also performed.

It is known that laser radiation can stimulate cell proliferation, a mechanism dependent on the fluency applied. In this work, the fluency used (0.5 J/cm^2) has an inhibitory effect of He-Ne laser (632.8 nm) in the region of 300 to 600 mJ/cm^2 in HeLa cells. It is widely accepted that non-ionized species can cross the plasma membrane more easily than charged compounds [15]. TSPP that is soluble in water is not capable of penetrating into the cell by means of passive diffusion. It is endocytosed by the cell and therefore localized in endosomes and lysosomes. Many authors consider that the cell uptake of the sensitizer is more efficient for lipophilic photosensitizers due to the better penetration through the cell membranes.

The ultrastructural morphologic aspect of the HeLa cells phototreated with TSPP reinforces the results that a great number of cells with plasma membrane damage, characteristics of irreversible cellular lesions were observed. These are characteristics of the cell necrosis process. Despite the knowledge that TSPP localizes preferentially in the mitochondrial membrane and that after laser irradiation activation of proteins leads to the apoptotic process, the initial localization of the photosensitizer in the plasma membrane and the interaction time in our experiments suggest a process of cell death with necrosis like characteristics.

PDT of HeLa cells with TSPP, shows ultrastructure features that suggest apoptotic cell death. A characteristic of this kind of death is the conservation of membrane integrity. According to Zhang [16], the relation between the mode of cell death (apoptosis or necrosis) and the dose of PDT, may be dependent mainly on the cell line and the photosensitizer used. Our results demonstrate cell apoptosis occurring in the neoplastic cell line. This may be related to the fact that lesion to the lysosomes membrane are followed by enzyme leakage to the cytoplasm. The activation of these enzymes causes enzymatic digestion of cellular components evidenced by nuclear alterations like picnotic nuclei and the characteristic ladder pattern of DNA fragmentation. TPPS as hydrophilic sulfonated porphyrin is taken up into lysosomes by endocytosis. PDT with either of the membrane- localizing photosensitizers resulted in increasing numbers of cells becoming apoptotic (TUNEL positive) during the first 12 h, but apoptotic bodies were not observed. In contrast, after photoactivation of the lysosome localized photosensitizers, apoptotic cells were not detected until after 12 h but extensive fragmentation of the cells into apoptotic bodies was found. These data provide evidence for at least two distinct pathways by which PDT can induce apoptosis. HeLa cells present a specificed morphological aspect, where only mitochondrial alteration is observed. However, maintaining the cell membrane integrity as a whole. The oxygen free radicals partially reduced are highly toxic molecules that cause lesion to cell membranes and other cell constituents [17]. Mitochondria and lysosomes have been identified as key components in the induction of apoptosis [18, 19].

SEM scan of untreated HeLa tumor cells, cultured 72 h at 37° C, showed a continuous, uniformly distributed monolayer without any cellular alteration (Figure 5). After laser irradiation the following features could be observed: (i) cyto plasmatic extensions branching out towards the periphery and contacting the neighboring preelongations and membranes, (ii) swellings on the cells surface, and finally (iii) some breaks (ruptures) at the cells periphery. Some cytoplasmatic prelongations appear, some of them having the tendency of branching with others cells.

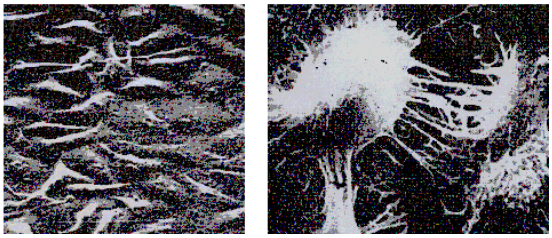


Figure 5.
SEM scan of untreated HeLa tumor cells, cultured 72 h at 37° C (left) and laser irradiated (right)

a b c

Figure 6.
HeLa tumoral cells after PDT treatment for control cells (a), TSPP treated (b) and TSPP/ CisPt (c)

The main explanation resides in the fact that cisplatin induces apoptosis in HeLa tumor cells implying attack on mitochondria (Fig. 6).

With TSPP, under He-Ne laser irradiation, presence of microvilli with or without microswellings, flattening tumoral cells, smooth surface without microvilli but with swellings on their surface, small craters in the central zone resulted from cytoplasmatic swellings, cytoplasmatic prelongations some of them having the tendency of branching with others cells.

The interpretation of the shape of the cell survival curve is still debated, as is the best way to fit the types of data mathematically.

For a linear kinetic curve (single hit, single target) the parameter Do can then be used to characterize the sensitivity in the linear region of the curve. Extrapolation of the terminal straight line portion of the curve back to the abscissa defines a value, n, the extrapolation number.

In the shoulder region of the curve the proportion of the cells killed, two interpretations are possible:

• Cell death results from the accumulation of events that are individually incapable of killing the cell, but which become lethal when added together (target models).

• Lesions are individually repairable but become irreparable and kill the cell if the efficiency of the enzymatic repair mechanisms diminishes with number of lesions and therefore the dose (repair models).

In this case, linear-quadratic model is the most used model for HeLa cells, from the following point of view:

• Do, the initial slope, due to single event killing, the dose to reduce survival to 37%, valuable for all the case except TSPP/CisPt;

• D, the final slope, interpreted as multiple-event killing, the dose to reduce survival by 67% from any point on the linear portion of the curve, which could apply for TSPP/CisPt.

The linear quadratic model assumes that a cell can be killed in two ways.

• Single lethal event

• Accumulation of sub lethal events.

From kinetic point of view, the survival fraction is evaluated as a plot of log ($N(t)/N_0$) versus irradiation time. In this case, could be applied the Theory of Dual Radiation Action, where the lesions responsible for cell photo destruction result from the interaction of sub lesions, resulted from unrepaired DNA double-strand breaks.

In the presence of the combined drugs TSPP-CisPt and under He-Ne laser irradiation could be observed flattened and covered cells with microswellings, micro craters at the cell periphery, lysis of the swellings and cells with smooth aspects.

Photodynamic therapy (PDT) is a standard treatment for various cancers (lung, esophagus, stomach, cervix, bladder, etc.) as well as for non-malignant conditions such as age-related macular degeneration, actinic keratoses and psoriasis. It is based on the selective retention of a previously administered nontoxic photosensitizer in the target cells, and irradiation of these cells with visible light at the appropriate wavelength (1). Upon illumination, the photosensitizer generates reactive oxygen species (singlet oxygen and free radicals, such as OH^-, HO_2^- and $.O_2^-$; Figure **7**).

These reactive species ultimately eliminate highly proliferating cells by damaging membranes, DNA and other cell structures, and also by affecting extracellular matrix (ECM) components.

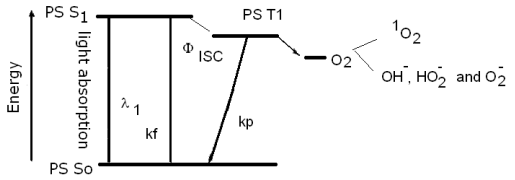


Figure 7.
Photosensitization process represented by a modified Jablonski diagram. PS S_0 = singlet ground state photosensitizer; PS S_1 = short-lived singlet excited state photosensitizer; PS T_1 = long-lived triplet state photosensitizer; $h\nu_f$ = fluorescence; $h\nu_p$ = phosphorescence; Φ_{isc} = intersystem crossing; $^1O_2$ = singlet oxygen.

Cell membranes have been identified as an important intracellular target, and many of their natural constituent macromolecules are readily susceptible to the organism, reacting with the singlet oxygen produced during the photochemical pathway, typically present in the PDT process. Such membranes include the plasma membrane surrounding the cell, the membranes of the endoplasmic reticulum distributed throughout the cytoplasm and the membranes of mitochondria and Golgi apparatus.

Cytoplasmatic residues resulted from cells desintegration as a consequence of the combined treatment. Scanning examination of He La tumor cells, exposed to photodynamic treatment (TSPP) associated with CisPt put into evidence various morphological lesions such as: microswellings, microcraters at the cell periphery, lysis of the swelling.

Apoptosis, also known as 'programmed cell death' or 'cellular suicide', is an active form of death with particular changes in cell morphology and protein activity. It is characterized by cell shrinking, surface membrane blebbing, chromatin condensation and DNA fragmentation. Apoptosis can be initiated in various manners, including PDT, and the common effector mechanism is to induce caspase-mediated cleavage of substrates. Initiator caspases are responsible for the first proteolytic events, e.g. cleavage of the cytoskeleton and related proteins including actin, and fodrin (a membrane-associated cytoskeletal protein). Amongst others, these early apoptotic events are thought to be responsible for the characteristic cell surface blebbing.

Three principal mechanisms are suggested for PDT action: cellular damage of targeting (photodamage by involving the process of apoptosis), vascular damage and immunological response.

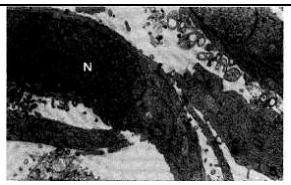


Figure 8.
Control HeLa cells. (N= nuclear chromatin; arrows = mitochondria).

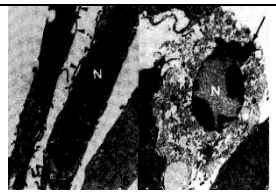


Figure 9.
HeLa cells 24 h after photodynamic treatment with TSPP-CisPt.

By transmission electron microscope structural alteration it was a found that characterize the apoptotic death mechanism for example, in the organization of the citoplasmatic membrane (figures 8,9), the condensed chromatin and aggregated chromatin in the nuclear peripheral, the condensation and presence of apoptotic bodies Our results indicate that the morphological criteria (apoptotic cell rounding and shrinkage) allow distinguishing the apoptosis as cell death mechanisms of photodynamic treatment. Thus, the morphological analysis under light microscopy and transmission electron microscope constitutes a very important and even decisive tool to identify the specific type of cell death unambiguously.

After photodynamic treatment with TSPP-CisPt, HeLa cells presented condensed chromatin, cell elongation, and cytoplasm condensation. All these are proves for apoptosis. Aspects of apoptotic bodies generated into cells are visualized in Figures 10,11.

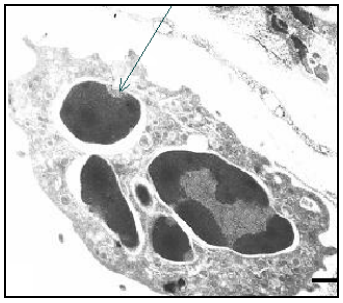


Figure 10. Apoptotic bodies inside HeLa cells (arrowhead).

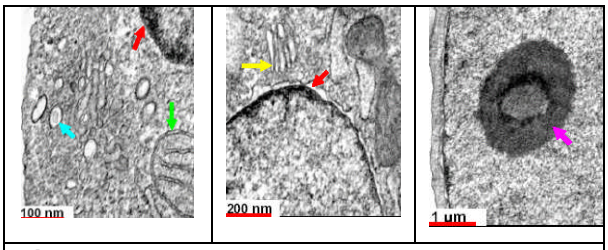


Figure 11. TEM micrographs of control HeLa cells

Figure 12 show that the CisPt-TSPP probe was confined to the nuclear compartment. It should be noted that shorter (<1 hour) and longer incubation times (>24 hours) revealed similar distribution patterns. Hence the above compound do not localise in the nuclei of the cells.

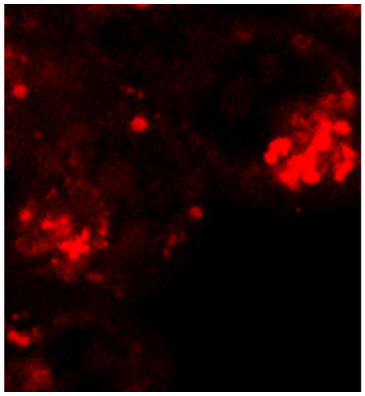


Figure 12.
Confocal image of CisPt-TSPP in HeLa cells

4. Conclusion

Photodynamic therapy (PDT) is a clinical approach that uses light-activated drugs for the treatment of different kind of tumor tissues. HeLa tumor cells, as an experimental model for study the new biomedical concept of associated PDT with 5,10,15,20-sulphonato-phenyl-porphyrin (TSPP) and an antitumor agent – cisplatin (CisPt), yielded to an enhanced effect on the suppression of tumor cells in vitro. The ultrastructure changes of cells caused by the action of the new concept and laser irradiation were analyzed, putting into evidence a linear survival curves, by the linear quadratic model.

In the presence of the combined drugs TSPP-CisPt and under He-Ne laser irradiation could be observed flattened and covered cells with microswellings, microcraters at the cell periphery, lysis of the swellings and cells with smooth aspects. Cytoplasmatic residues resulted from cells desintegration as a consequence of the combined treatment. Scanning examination of He La tumor cells, exposed to photodynamic treatment (TSPP) associated with CisPt put into evidence various morphological lesions such as: microswellings, microcraters at the cell periphery, lysis of the swelling.

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