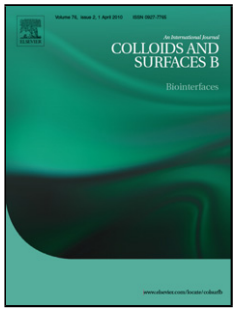
Accepted Manuscript



Title: Toxicity study in blood and tumor cells of laser produced medicines for application in fabrics

Author: M.Carmen Morán Tatiana Tozar Agota Simon Andra Dinache Adriana Smarandache Ionut Relu Andrei Mihai Boni Mihail Lucian Pascu Francesca Cirisano Michele Ferrari

PII: S0927-7765(15)30001-1
DOI: http://dx.doi.org/doi:10.1016/j.colsurfb.2015.06.041
Reference: COLSUB 7171

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received date: 18-4-2015
Revised date: 15-6-2015
Accepted date: 22-6-2015

Please cite this article as: M.Carmen Morán, Tatiana Tozar, Agota Simon, Andra Dinache, Adriana Smarandache, Ionut Relu Andrei, Mihai Boni, Mihail Lucian Pascu, Francesca Cirisano, Michele Ferrari, Toxicity study in blood and tumor cells of laser produced medicines for application in fabrics, Colloids and Surfaces B: Biointerfaces http://dx.doi.org/10.1016/j.colsurfb.2015.06.041

Toxicity study in blood and tumor cells of laser produced medicines for application in fabrics

M. Carmen Morán[a,b,*],Tatiana Tozar[c,d], Agota Simon[c,d], Andra Dinache[c,d], Adriana Smarandache[c,d], Ionut Relu Andrei[c], Mihai Boni[c,d], Mihail Lucian Pascu[c,d], Francesca Cirisano[e] and Michele Ferrari[e]

[a]*Departament de Fisiologia; Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII s/n, 08028-Barcelona-Spain*
[b]*Institut de Nanociència i Nanotecnologia-IN^{2}UB, Universitat de Barcelona, Avda. Joan XXIII s/n, 08028-Barcelona-Spain*
[c]*National Institute for Laser, Plasma and Radiation Physics, 077125 Magurele, Romania*
[d]*Faculty of Physics, University of Bucharest, 077125 Magurele, Romania*
[e]*CNR – Istituto per l' Energetica e le Interfasi, 16149 Genova, Italy*
*Corresponding author: **mcmoranb@ub.edu**

Highlights

► ► Phenothiazines water solutions exposed to UV laser beam are stable one month. ► Irradiation yields to stable photoproducts of CPZ and PZ, producing oxygen-independent photohaemolysis. ► Irradiation process of CPZ and PZ promotes the generation of substances with higher cytotoxic character that the parent ones. ► Irradiation results in an enhancement of the wetting and distribution of the CPZ and PMZ substances in the fabrics.

Abstract

Phenothiazine derivatives are non-antibiotics with antimicrobial, fungistatic and fungicidal effects. We exposed to a high energy UV laser beam phenothiazines solutions in water at 20 mg/mL concentration to increase antibacterial activity of resulting mixtures. Compared to previous results obtained on bacteria, more research is needed about UV laser irradiated phenothiazines applications on cancer cell cultures to evidence possible anticancerous properties. Evaluation of the safety of the newly obtained photoproducts in view of use on humans is also needed. Due to expensive animal testing in toxicology and pressure from general public and governments to develop alternatives to *in vivo* testing, *in vitro* cell-based models are attractive for

preliminary testing of new materials. Cytotoxicity screening reported here shows that laser irradiated (4 hours exposure time length) chlorpromazine and promazine are more efficient against some cell cultures. Interaction of laser irradiated phenothiazines with fabrics show that promethazine and chlorpromazine have improved wetting properties. Correlation of these two groups of properties shows that chlorpromazine appears to be more recommended for applications on tissues using fabrics as transport vectors. The reported results concern stability study of phenothiazines water solutions to know the time limits within which they are stable and may be used.

Keywords: laser, phenothiazines, hemolysis, culture cells, *in vitro* cytotoxicity, fabrics

1. Introduction

Recent reports have shown that phenothiazines solutions as parent compounds, when exposed to ultraviolet (UV) laser radiation generate photoproducts that have different molecular structures and properties with respect to them [1-4]. More recent studies show that solutions of phenothiazines in water once exposed to laser radiation have modified wetting properties with respect to fabrics [5]. These data suggest to extend the toxicity studies of phentothiazines exposed to laser radiation towards applications on cell cultures and to broaden the studies of their wetting properties on fabrics in view of further applications. The antimicrobial activity of irradiated irradiated Chlorpromazine (CPZ) against Mycobacterial strains [6] and Gram-positive and Gram-negative bacterial strains [7] was investigated. Cytotoxicity effects on human acute monocytic leukemia cell lines were determined [6]. CPZ irradiated at 20 mg/mL different time intervals with 266 nm laser beam presented enhanced activity with respect to parental compound for *Staphylococcus aureus* ATCC 25923, HPV 107 and *Escherichia coli* K-12 AG100, K-12 AG100A and $AG100_{TET8}$ and $AG100A_{TET8}$ [7]. For *Salmonella enterica serovar Enteritidis* NCTC 13349, 104, 5408, 104_{CIP} and 5408_{CIP}, irradiated CPZ exhibited mild antimicrobial activity but presented an inhibition of efflux pumps [7]. Cytotoxicity assay showed that half-maximal inhibitory concentration (IC_{50}) decreased with increasing of irradiation time. Irradiated CPZ has enhanced activity against Mycobacterial strains [6].

At a consensus conference of the European Society for Biomaterials in 1986, the word “biocompatibility” was defined as “the ability of a material to perform with an appropriate host response in a specific application”. Herein, we use biocompatibility to

include the deleterious effects caused by the unirradiated and irradiated phenothiazines, covering the *in vitro* haemolytic and cytotoxic assessments. An important feature in the development of delivery systems for parenteral administration is to determine their ability to cause hemolysis by interaction with cell membrane. The potential uses of colloidal self-assemblies as drug delivery systems make hemolysis evaluation very important. To this end, we examined this interaction by using erythrocytes as a model biological membrane system, since erythrocytes have been used as a suitable model for studying the interaction of amphiphiles or other species with biological membranes [8-10].

The safety evaluation of new products or ingredients made for human use is crucial prior to exposure. Due to the expense of animal testing in toxicology and pressure from general public and governments to develop alternatives to *in vivo* testing, *in vitro* cell-based models may be more attractive for preliminary testing of new materials [11]. The toxicity prediction is difficult, but cytotoxicity screening, which is routinely used in drug screening, is a good indicator of potential adverse effects in cells. Rapid, sensitive and reliable bioassays are required to examine toxicity. Established cell lines are useful alternative test systems for this kind of toxicological studies [12]. However, they must be chosen with care considering their origin [13].

Cytotoxicity assays are among the most common *in vitro* endpoints used to predict the potential toxicity of a substance in a cell culture [14]. Cell damage is manifested in several ways, including mitochondrion and plasma membrane dysfunction and fluctuating intracellular reduction capacity [15]. Current standard approaches to gauge the degree of cell damage include assays that measure various aspects of cell viability, such as metabolic activity and plasma membrane integrity. MTT reduction assay based on reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide by cellular dehydrogenases, is among the most commonly used endpoints. This method measures the reduction of MTT salt to a coloured insoluble formazan in active mitochondria in viable cells and also, in certain cases, outside the mitochondria [16-17]. The neutral red uptake (NRU) assay, also widely used in biomedical applications, measures the uptake of neutral red dye by viable cells with intact plasma membrane, and its concentration in lysosomes [18].

One of the most common non-epithelial cell line used in short- and long-term toxicological *in vitro* studies on cytotoxicity, biocompatibility, or mechanisms of cellular uptake of nanoparticles contains 3T3 fibroblasts. These are readily available, undergo contact inhibition, and are closely representative of a physiologic model cell line [19]. HeLa cell line is the oldest and most commonly used human cell line, derived from

cervical cancer cells [20]. Since they were put into mass production, HeLa cells have been used for research into cancer, AIDS, effects of radiation and toxic substances, gene mapping, and countless other scientific pursuits [21]. Differences in the sensitivity of endpoints, together with the type of cell model and the nature of tested chemicals, may affect the final outcome [22-23].

Biomedical fabrics with antibacterial or, more generally, therapeutic purposes are a challenging manufacturing task in textile industries. From one side, the fabric itself hosts the bacteria acting as a medium between the human organism and the microorganisms; on the other hand, it is regarded as a well assessed tool for drug delivery in contact of skin for external applications [24-26]. Therefore wide market sectors push textile industries to concentrate more on antibacterial fabrics manufacturing to improve fabrics quality and product sales. Natural antibiotics are preferred by industries not to degrade product quality. In [27] the authors propose benzalkonium chloride as an antibiotic in fabric finishing being more effective in skin protection against bacterial infection and less irritating in hand sanitizers. Also, wound treatment is a biomedical field where both natural and synthetic polymers are excellent candidates for applications [28-29]. Because of their versatility and biocompatibility even small changes in drugs molecular structure can bring large changes in their interactions with components of biological tissues [30].

In this paper the authors propose a cytotoxicity study of new molecules produced by laser irradiation as potential new therapeutic agents and their applicability to biomedical fabrics. The stability of the new products has been investigated in order to forecast a better protection or action with the target cells for a longer time, also in view of limiting the influence on shrinking and tearing properties of fabrics.

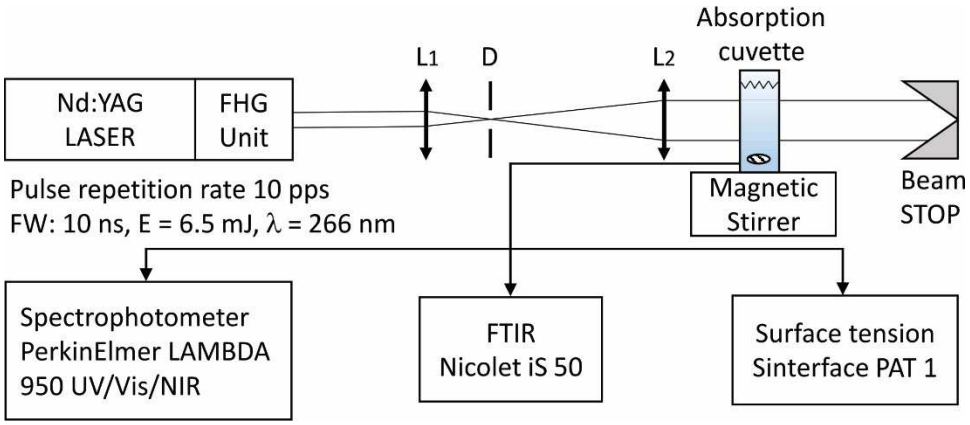
A detailed study is made on the stability of phenothiazines solutions in order to know the time limits within which exposed solutions are stable and may be used for applications. Secondly, a study of the cytotoxic properties of the irradiated solutions is presented and their interaction with fabrics is evaluated in view of biomedical use.

2. Materials and methods

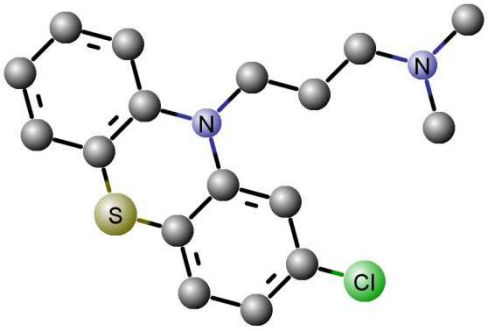
2.1 Set-up

The set-up utilized to study the stability of the irradiated phenothiazines solutions is shown in Fig.1a where a variant of a more general system described in [3, 4] is drawn. The pulsed laser beam sent on bulk sample (2mL) containing phenothiazines at 20 mg/mL in water, had 6.5 mJ energy and 266 nm wavelength. The irradiation time was

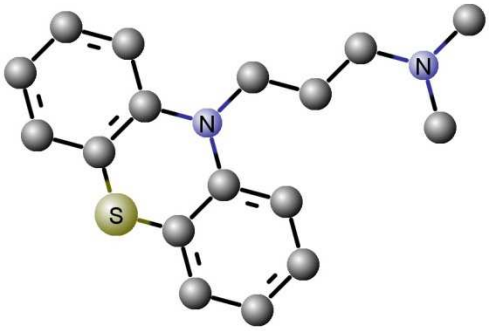
for all samples 4 hours and solutions were stirred during exposure to avoid precipitates formation.



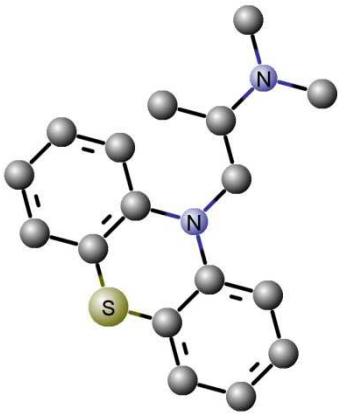
a)



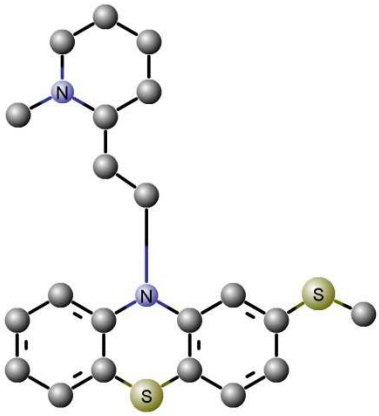
A.



B.



C.



D.

b)

Fig. 1

After irradiation, absorption and FTIR spectra of bulk samples were measured as well as surface tension of droplets of 15 µL volume.

2.2 Materials

2.2 Materials

The studied phenothiazines, described in [3] were chlorpromazine (CPZ; $C_{17}H_{19}ClN_2S$; IUPAC name: 3-(2-chloro-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethyl-propan-1-amine; molecular mass for the hydrochloride form 355.33 g/mol), promazine (PZ; $C_{17}H_{20}N_2S$; IUPAC name: N,N-dimethyl-3-(10H-phenothiazin-10-yl)-propan-1-amine; molecular mass 284.42 g/mol), promethazine (PMZ; $C_{17}H_{20}N_2S$; IUPAC name: (*RS*)-*N, N*-dimethyl-1-(10*H*-phenothiazin-10-yl)propan-2-amine; molecular mass 284.42 g/mol) and thioridazine (TZ; $C_{21}H_{26}N_2S_2$; IUPAC name: 10-{2-[(*RS*)-1-Methylpiperidin-2-yl] ethyl}-2-methylsulfanylphenothiazine; molecular mass 370.577 g/mol) prepared as solutions at 20mg/mL in ultrapure water as shown in [6-7]. The 3D not optimized structures of the utilized phenothiazines are shown in Fig.1.b. The identification of some of the photo-products and their relative concentrations regarding CPZ exposure to 266 nm laser beam was made by liquid chromatograph - time of flight – mass spectrometer (LC-TOF/MS). This is one of the most suitable methods to identify new photoproducts since it is based on their distribution with respect to the m/z values (where *m* is the molecular mass number of the compound and *z* to the charge number of the corresponding ion measured in the time of flight system) [4]. The LC-TOF/MS technique is presented in detail in [4] and offers an extremely accurate mass measurements (<5 ppm) due to the TOF high degree of certainty detection. The identified photo-products were: PZ, Promazine sulfoxide or Hydroxy-promazine, 2-Hydroxyy-promazine and other 3 compounds with molecular masses at 292, 300 and 308 amu, respectively [3]. In general, irradiation of CPZ and PZ generates subproducts with similar cytotoxic activity [3].

2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT) and neutral red dye (NR) were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco´s modified Eagle´s

medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin-EDTA solution (170,000 U/L trypsin and 0.2 g/L EDTA) and penicillin-streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin) were obtained from Lonza (Verviers, Belgium). The 75 cm2 flasks and 96-well plates were obtained from TPP (Trasadingen, Switzerland).

2.3. Interaction with Erythrocytes

2.3.1. Preparation of Red Blood Cell Suspensions

Red blood cells were obtained in accordance with the procedure described in [31]. Blood was obtained from human donors and drawn into tubes containing EDTA to prevent coagulation. The serum was removed from blood by centrifugation and by subsequent suction. The red blood cells were then washed three times by centrifugation at 3000 rpm with isotonic saline PBS solution (pH 7.4).). Prior to experimentation, an aliquot of the suspension is adjusted with PBS to give an extinction of about OD (optical density) = 2.0 at 575 nm wavelength after hypotonic lysis with distilled water in 1.0 ml final volume [32]. This OD corresponds to a cells density of 8 × 109 cell/mL.

2.3.2. Hemolytic Study

The membrane-lytic activity of the systems was examined by a hemolysis assay. A series of different volumes of a solution (1 mg/ mL) of the unirradiated compounds (20 mg/ mL) and irradiated phenothiazines samples (20 mg/ mL, 240 min, 6.5 mJ, and 266 nm) were placed in polystyrene tubes and an aliquot of erythrocyte suspension was added to each tube. The tubes were incubated at room temperature for 1 h under shaking conditions using an Atom 190 shaker (Atom). Following incubation, the tubes were centrifuged (5 min at 10000 rpm). The degree of hemolysis was determined by comparing the absorbance (540 nm; Shimadzu UV-160A) of supernatant with that of the control samples totally hemolysed with distilled water. Positive and negative controls were obtained by adding an aliquot of erythrocyte suspension to distilled water and isotonic PBS solution, respectively.

The degree of hemolysis was determined by the following equation:

$$\text{Hemolysis (\%)} = 100\times (\text{Abs}–\text{Ab}_0)/(\text{Abs}_{100}–\text{Abs}_0)$$

where Abs, Abs_0, and Abs_{100} are the absorbance of test samples, the suspension treated with isotonic physiological buffer saline (PBS), and the suspension of complete hemolysis treated with distilled water water, respectively.

2.4. Cell cultures

Murine Swiss albino fibroblast, 3T3 and human epithelial carcinoma, HeLa cell lines (purchased from the Celltec UB, Barcelona (Spain) were grown in DMEM medium (4.5 g/ L glucose) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/ mL streptomycin at 37ºC, 5% CO_2. The 3T3 and HeLa and HaCaT cells were routinely cultured in 75 cm^2 culture flasks and were trypsinised using trypsin-EDTA when cells reached approximately 80% confluence.

2.5. Cytocompatibility assays

The 3T3 (1 x 10^5 cell/ mL) and HeLa (5 x 10^4 cells/ mL) were seeded into the central 60 wells of a 96-well plate After incubation for 24 h under 5% CO_2 at 37ºC, spent medium was replaced in the wells with 100 µL of fresh medium supplemented with 5% FBS containing unirradiated compounds (20 mg/mL) and irradiated phenothiazines samples (20 mg/ mL, 240 min, 6.5 mJ, 266 nm) at the required concentration range (2.5-150 µg/ mL).

2.5.1. MTT assay

The MTT assay is based on the protocol first described by Mosmann [33]. In this assay, living cells reduce the yellow tetrazolium salt MTT to insoluble purple formazan crystals. After 3T3 and HeLa cell incubation for 24h, phenothiazines-containing medium was removed and 100 µL of MTT in PBS (5 mg/mL) diluted 1:10 in medium without FBS and phenol red was then added to the cells. The plates were incubated for a further 3 h, after which the medium was removed. Thereafter, 100 µL of DMSO was added to each well to dissolve the purple formazan product. Plates were then placed in a microtitre-plate shaker for 10 min at room temperature and absorbance of the resulting solutions was measured at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of tetrazolium salt reduction by viable cells against the untreated cell control (cells with medium only).

2.5.2. NRU assay

Based on the protocol described by Borenfreund and Puerner [34], NRU assay determines the accumulation of NR dye in the lysosomes of viable, undamaged cells. Following exposure to phenothiazines-containing medium, 3T3 and HeLa cells were incubated for 3h with NR dye solution (50 µg/mL^{1}) dissolved in medium without FBS and phenol red. Cells were then washed with PBS, following by the addition of 100 µL of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water to extract the dye. Plates were gently shaken for 10 min to ensure complete dissolution. We then measured absorbance of the extracted solution at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of uptake of NR dye by lysosomes against untreated cell control (cells with medium only).

2.6. Statistical analyses

Experiments were performed at least three times on independent occasions unless otherwise stated. Results are expresses as means standard error of the mean (SEM). Data were analyzed by PASW Statistics 18 software using one-way analysis of variance (ANOVA) with Scheffé post-hoc tests for multiple comparisons. Each experiment was performed at least three times on independent. Differences were considered statistically significant at $p<0.05$ or $p<0.005$. In the figures significant differences were illustrated with asterisk.

2.7. Interaction with fabrics

Medicines solutions were tested on different materials such as support.:Cotton (CO) and polyester (PES) purchased from WfkTestgewebe Test Materials and Concepts (Germany). The surfaces preparation consisted in washing CO and PES materials (10 and 5 times, respectively) in a recipient (1L) using warm tap water. Afterwards, the surfaces were cut into small pieces and placed on an adjustable sample holder for stretching

Scanning Electron Microscopy (SEM) coupled with EDS probe has been used to characterize the fabrics with and without interaction with irradiated medicines (LEO model WP1450- EDS model INCA, Oxford Instruments). A given amount of both irradiated and unirradiated sample has been dispensed on fabrics and let it dry. The samples have been then gold coated by sputtering.

3. Results and discussion

3.1 Phenothiazines stability studies

Prior to use irradiated phenothiazines on cell cultures and on fabrics, a stability study was performed in order to identify the time interval within which, after irradiation, the solutions remain stable and may be used in applications. The stability studies were made first by measuring the absorption spectra of solutions of CPZ, PMZ, PZ and TZ at 20mg/mL irradiated 4 hours with a pulsed UV laser beam of 6.5 mJ energy. The spectra were plotted at this relatively high concentration since along the measured spectral range (visible – near infrared, i.e. 450 nm – 1100 nm) the absorption is not high enough to saturate the absorption signal but is significant enough to evidence small modifications of the spectra which might suggest molecular modifications in the samples. FTIR spectra were also plotted for the phenothiazines solutions and surface tension measurements were made on droplets of solutions of 15 µL volume. In general, phenothiazines solutions are quite stable in time after irradiation, although changes are observable in the spectra or surface tension curves. As an example, in Fig.2, absorption spectra are shown for CPZ (Fig.2a) and PZ (Fig.2b); these two compounds were chosen since their action was the most efficient on the studied cells cultures. Each spectrum has a broad peak in the visible around 510 nm which is most probably constituted out of contributions belonging to some generated photoproducts; this peak does not vanish at long time intervals of the order of months. Besides this peak, both solutions exhibit after irradiation near infrared absorption maxima which vanish quite rapidly and belong most probably to transient, short lifetime compounds. The modifications in the NIR spectra of irradiated CPZ and PZ show that the mixtures of photo-products are not stable in the first 24 h from the end of the irradiation, but they are afterwards remarkably stable. So, only the concentrations of the short lifetime compounds change in time after irradiation. These compounds do not interact with culture cells in the experiments because the experiments were made at more than one month after medicine solutions irradiation. On the other hand, time evolution of the peak in the visible shows a reasonably stable set of compounds in solution at long time intervals, although the variations of absorption peak intensity is larger than the experimental errors in measuring the absorption spectra (drawn in Fig.2 on the curves at 7 days from exposure, according to [2]).

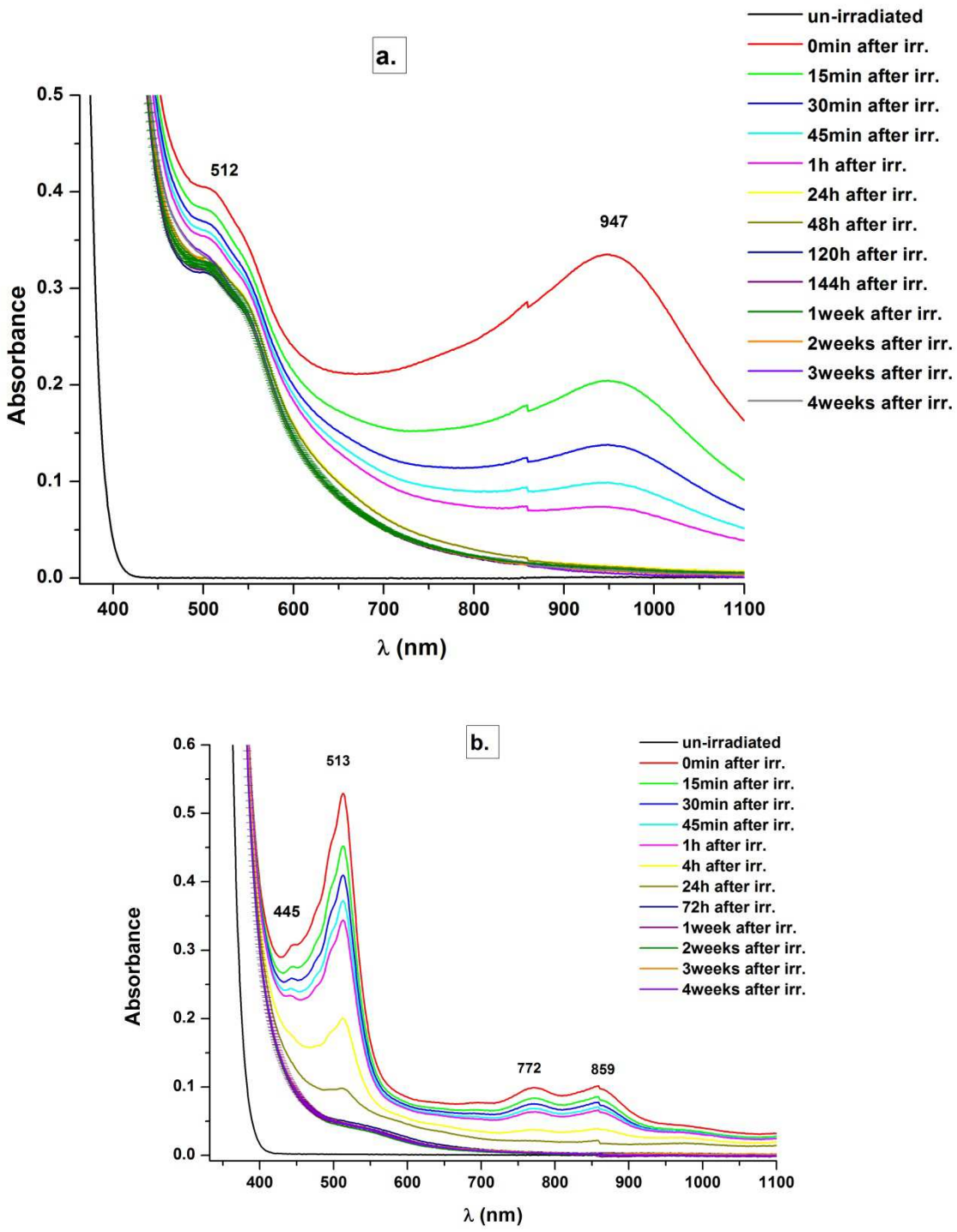


Fig. **2**

The same conclusions may be drawn from the FTIR spectra made on bulk samples and from the surface tension measurements on droplets of solutions having 15 µL volume (data not shown).

3.2. Hemolytic Assessments of Unirradiated and Irradiated Phenothiazines

Safety evaluation of new products or ingredients destined for human use is crucial prior to exposure. One important factor in development of novel systems for parenteral administration is to determine their ability to cause hemolysis by interaction with the cell membrane. To this end, we examined this interaction by using erythrocytes as a model biological membrane system, since erythrocytes have been used as a suitable model for studying the interaction of amphiphiles and other molecules with biological membranes [35-36].

The hemolytic activity of unirradiated and irradiated phenothiazines compounds was assessed at different concentrations. In these experiments, hemolysis was determined at a fixed time (after 1 h incubation) in the presence unirradiated and irradiated phenothiazines in the range of 50 to 150 µg/ mL. The hemolytic potential of a material is defined as a measure of the extent of hemolysis that may be caused by the system when it comes into contact with blood. Data analysis shows two different types of behavior. Thus, one can distinguish between (i) CPZ and PZ compounds, for which unirradiated and irradiated samples lead to differences in hemolytic response and (ii) TZ and PMZ derivatives, for which no apparent effect of the UV irradiation on the hemolytic response is observed.

When the hemolytic responses of unirradiated/irradiated CPZ and PZ compounds are compared, a strong increase of hemolytic response of irradiated samples could be observed. In CPZ case, 80 % of hemolysis was already observed at the lowest concentration, in comparison with the 7 % of hemolysis observed at 150 µg/ mL of the unirradiated CPZ sample (Fig.3a). In the case of PZ derivative, unirradiated sample showed hemolysis values lower than the permissible level of 5% [35] during the entire concentration interval studied. In the case of irradiated PZ sample, hemolysis progressively increases until achieves 80 % of hemolysis (Fig. 3b).

Quite different profiles were obtained for TZ and PMZ derivatives. A general trend is that the effect of the UV irradiation can be considered negligible. However, TZ and PMZ derivatives showed a very different behavior. In the TZ case, both unirradiated and irradiated samples showed dose-response curves that progressively increase until achieve 100 % of hemolysis (Fig. 3c). For PMZ derivative (Fig. 3d), for all the concentrations assayed, the extent of hemolysis was lower than the permissible level of 5%. PMZ-UV slightly exceeds this value at the highest concentration studied.

From the fitting of the obtained curves, HC_{50} values for different unirradiated and irradiated phenothiazines have been determined. Fig.3e summarizes the obtained results.

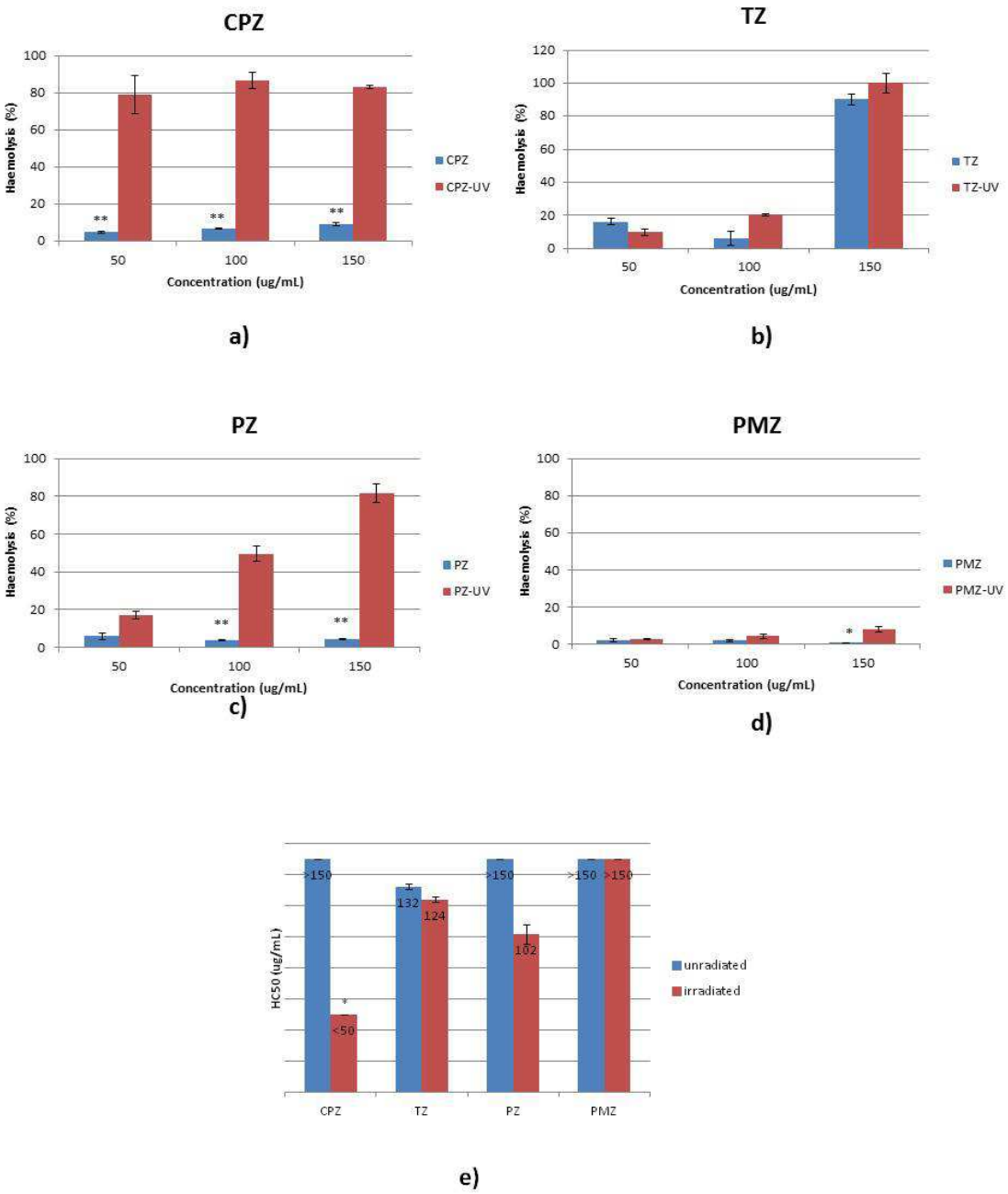


Fig 3.

The hemolysis assay showed that the phenothiazine derivatives as well as the irradiation process are controlling parameters of the hemolytic properties of these compounds. These results can be compared with those obtained by the determination of the photohemolysis of several phenothizines [37]. Drug-induced photosensitivity refers to adverse cutaneous responses which follow the combined or successive

exposure to certain chemicals (photosensitizers) and to light. Several phenothiazines have been reported to cause clinical phototoxicity [38].

Phototoxic reactions are broadly divided into those which are oxygen dependent and a lower number which do not require oxygen. The mechanism of damage is one of colloid osmotic hemolysis mediated by peroxide formation following porphyrin excitation [39] and due to stable photoproducts since the drug irradiated alone failed to induce subsequent cell lysis. Although under different experimental conditions (drugs at 50 µg/mL were exposed to UVA (350 nm, 2.5 mW/cm) for 30 min), the results described by Kim and Baek are in the light of our results. These authors showed that stable photoproducts were formed by CPZ, producing oxygen-independent photohemolysis. On the other hand, TZ was not forming photoproducts. The damage of TZ is one of colloid osmotic hemolysis mediated by peroxide formation following porphyrin excitation. As for the PMZ derivative, its irradiation had no effect on hemolysis.

3.3. Cytotoxic Assessments of Unirradiated and Irradiated Phenothiazines

It was of great interest in this study to characterize unirradiated and irradiated phenothiazines considering the cytotoxic response they induced. Dose–response curves were determined by the MTT and NRU assays using 3T3 and HeLa cells lines. 3T3 fibroblast cell line gave an appropriate *in vitro* model for skin irritation. HeLa, is the oldest and most commonly used human cell line. The cytotoxicity assays were performed in the concentration range 2.5-100 µg/mL.

For comparative purposes, an initial experimental series of cytotoxicity studies were performed at the similar range of concentration with that carried out on hemolytic studies (25-100 µg/mL). Fig. 4 shows the cytotoxic response of HeLa cell line treated with unirradiated and irradiated phenothiazines, determined by MTT and NRU assays. Cell viabilities of down to 5 % were observed in all compositions when MTT method was used. By NRU method, cell viabilities of up to 10 % were observed in almost all compositions. It is well established that MTT assay is a measurement of cell metabolic activity within the mitochondrial compartment, while NRU assay measures membrane integrity. NR dye diffuses through intact cell membranes to accumulate within lysosomes [16-18]. Based on the mechanisms of cell damage detected by each cytotoxicity assay, our results suggest that the toxicity mechanism of unirradiated and irradiated phenothiazines involve an earlier interaction with the mitochondrial compartment while plasma membrane and/or lysosomal compartments could be

affected at a later stage. Under these conditions, in all cases there are significant differences between the obtained values by MTT and NRU methods.

Concerning potential differences between unirradiated and irradiated samples, in the CPZ and PZ derivatives cases, for some conditions, irradiated phenothiazines seems to be more compatible with HeLa cells that the irradiated one, by NRU method. (Fig.4a). However, for PMZ derivatives, at the lowest assayed concentration, HeLa cells seem to be more sensitive to the deleterious effect of the irradiated sample than the unirradiated one, determined by MTT (Fig. 4d). At that point, due to the lack of cell viability observed under these conditions only is possible to confirm that the corresponding IC_{50} values would be lower than 25 µg/mL.

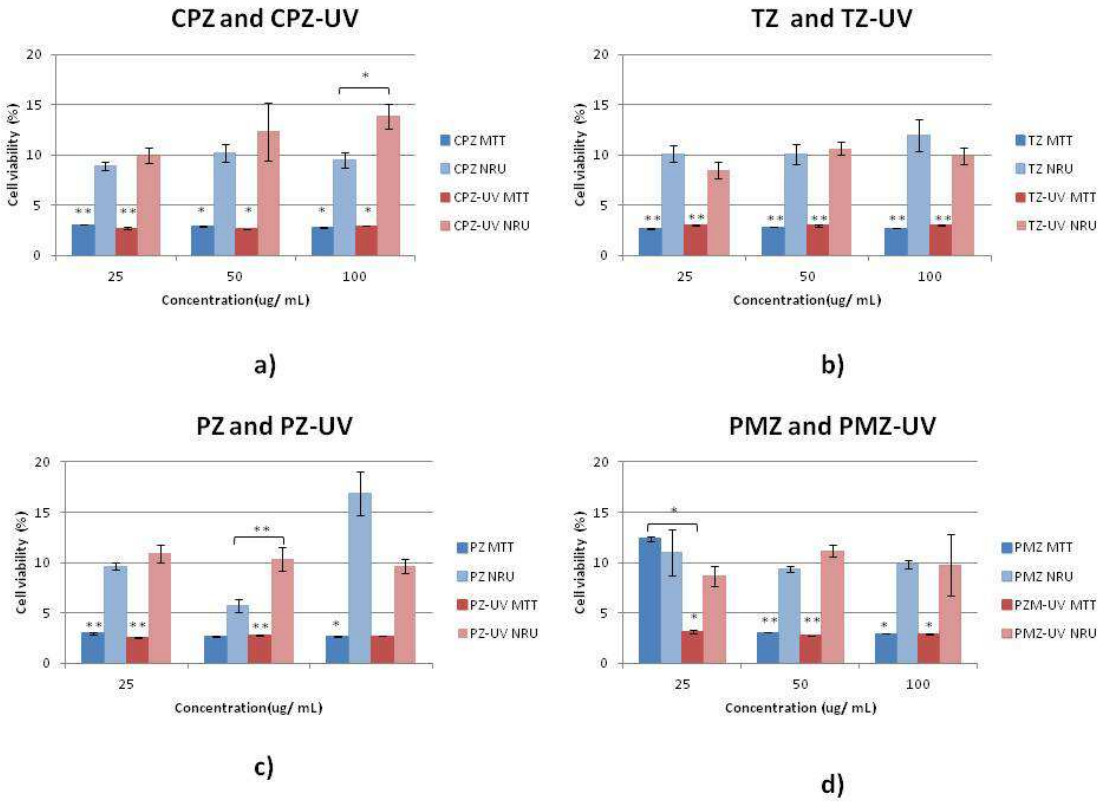


Fig. **4**.

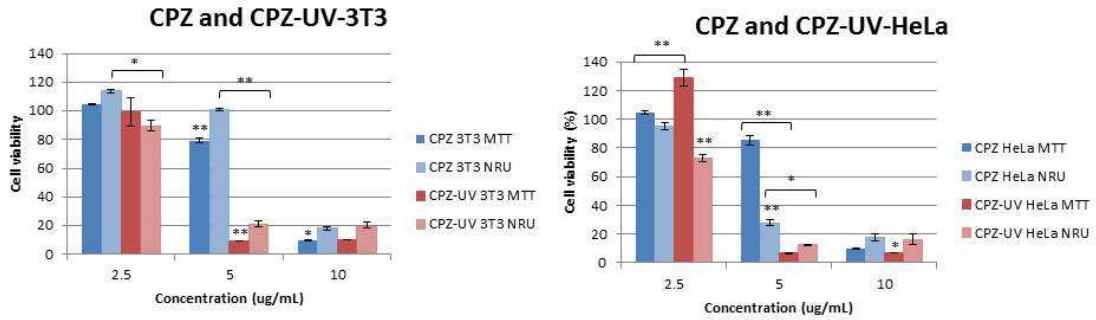
Taken into account these results, subsequent cytotoxicity studies were carried out at lower concentration in order to establish the cytotoxic potency of unirradiated and irradiated phenothiazines. Fig. 5 shows the cytotoxic response of 3T3 and HeLa cell lines treated with unirradiated and irradiated phenothiazines in the concentration range 2.5-10 µg/mL, determined by MTT and NRU assays. As can be observed, under this concentration range, it is possible to discriminate the conditions for which viable and

dead cells can be observed. Together with some significant differences on cell viabilities observed as a function of the endpoint method and cell line type, the most remarkable differences have been observed as a function of the irradiation process on the phenothiazine derivative. In a way similar with that observed in hemolytic studies, the data analysis shows two different types of behavior. Thus, we can distinguish between (i) CPZ and PZ compounds, for which unirradiated and irradiated samples lead to differences in the cytotoxic response and (ii) TZ and PMZ derivatives, for which the effect of the UV irradiation on the cytotoxic response seems to be less remarkable.

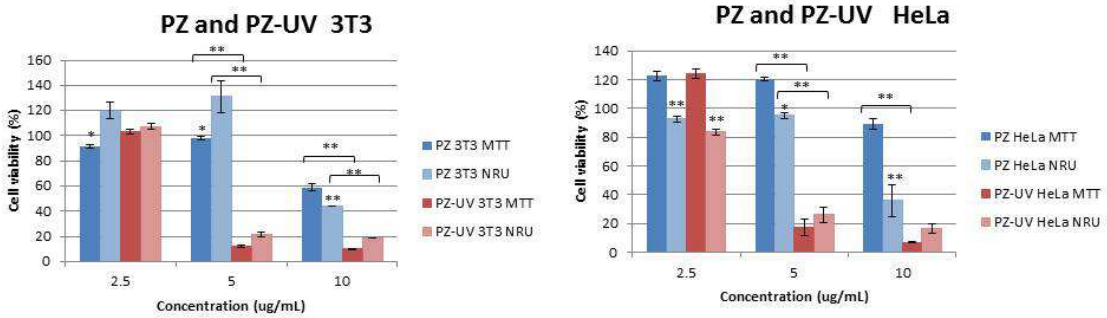
A general trend in cytotoxic responses of CPZ and PZ derivatives is that the irradiation process might promote the generation of substances with higher cytotoxic character than those present in the unirradiated samples. Fig. 5a and 5b show the cytotoxic responses of 3T3 and HeLa cell lines in the presence of CPZ and PZ derivatives, respectively. When 3T3 cells were incubated in the presence of CPZ derivatives at the lowest and intermediate concentrations, the cytotoxic response between unirradiated and irradiated samples appeared to be significantly different, with the NRU assay. In a similar way, HeLa cells showed significant differences between the cytotoxic response between unirradiated and irradiated samples, especially with the MTT assay. At the highest assayed concentration, the lack of cell viability would explain the observed similarities between unirradiated and irradiated samples. Concerning cytotoxic responses of PZ derivatives (Fig. 5b), the most notable differences between unirradiated and irradiated samples have been observed at intermediate and highest concentrations. When 3T3 cells where incubated in the presence of unirradiated and irradiated samples, significant differences have been observed, with both MTT and NRU methods. For HeLa cell line, significant differences between the cytotoxic response of unirradiated and irradiated samples were observed, especially with the MTT assay.

Quite different cytotoxic responses were obtained in the case of TZ and PMZ derivatives. A general trend is that the effect of UV irradiation can be considered negligible or promotes the formation of species that result to be less cytotoxic than the unirradiated samples. For TZ derivatives, at the lowest assayed concentration, together with some significant differences as a function of the endpoint method, 3T3 and HeLa cells resulted to be more sensitive to the deleterious effect of unirradiated samples determined by MTT and NRU, respectively (Fig. 5c).The lack of cell viabilities observed at such high concentrations (25-100 µg/mL) would explain the observed similarities between unirradiated and irradiated samples. Concerning the cytotoxic effects of PMZ

derivatives, significant differences between unirradiated and irradiated samples can be found at the intermediate assayed concentration, by NRU. Only at the highest assayed concentration, irraditated PMZ seems to be more cytotoxic to HeLa cells, measured by MTT method (Fig. 5d).



a)



b)

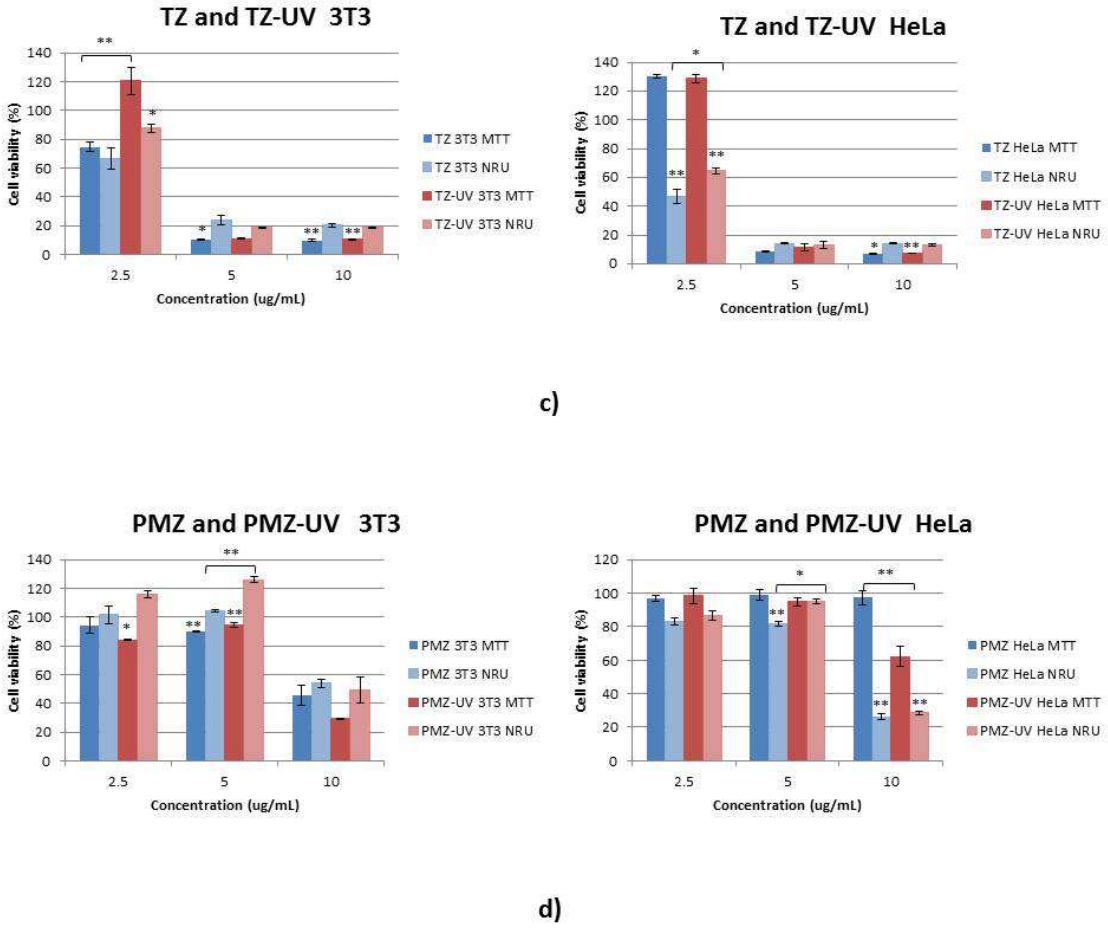


Fig. **5**

From the fitting of concentration-dependent viabilities curves, the corresponding IC_{50} values have been determined. The obtained results have been summarized in Fig. 6. In general, the IC50 values of unirradiated and irradiated phenothiazines were similar to the 3T3 cells, and only in the case of CPZ derivatives showed a significant decrease ($p<0.005$) in the toxic response after irradiation, as determined by MTT and NRU methods (Fig. 6a). In contrast, more significant differences between unirradiated and irradiated phenothiazines were observed with HeLa cells. Together with some significant differences on the cell viabilities observed as a function of the endpoint method, the four types of phenothiazines showed significant differences between the cytotoxic responses promote by unirradiated and irradiated compounds (Fig. 6b). For CPZ, PZ and PMZ derivatives, lower IC_{50} values were obtained for the formulations containing irradiated compounds in comparison of those obtained with unirradiated ones, especially with the MTT method. In contrast, lower IC_{50} values were obtained in

the case of unirradiated TZ derivative in comparison with irradiated one, determined by the NRU method.

When the results obtained between the two different endpoints were compared, it can be deduced that the NRU assay gave lower IC_{50} values than the MTT assay did (Figure 6c and 6d). However, the latter assay was more sensitive in detecting the irradiation effects within the two studied cell lines (Fig.4c). These results suggest that irradiated compounds have a greater effect on the metabolic activity than on plasma membrane on the cells, especially in the case of the HeLa cell line. In contrast, by NRU methods, 3T3 cells seem to be more sensitive to the deleterious effect of CPZ derivatives. The significant effect of CPZ derivatives on plasma membrane on 3T3 cells resemble that observed in the haemolytic studies, where significant differences were only observed in the case of the CPZ derivatives, in comparison with other phenothiazines.

The effect of irradiation on CPZ derivative is in agreement with previous obtained results [6]. When *in vitro* toxicity against the human THP-1 cell lines was determined by MTS method, the unirradiated CPZ showed an IC_{50} value of 5.51 µg/mL. The level of toxicity increased with the 4h exposure of CPZ to the 266nm laser beam (2.59 mg/L).

Selectivity index is an important factor in the development of anticancer agent, because this index is the factor that ensures the safety of the tested compound [40, 41]. The selectivity indexes toward cancer cells (Table 1) were calculated as a ratio of the average IC_{50} values for 3T3 fibroblast cells and the IC_{50} values for the corresponding cancer cell line (Hela cell line).

From values on Table 1 can be deduced that the selectivity index seems to be dependent on the endpoint method. In the case of MTT method, SI-MTT values are lower than 1, indicating no selectivity on the action of the unirradiated and irradiated phenothiazines for 3T3 and HeLa cell lines. In all cases, however, SI-MTT values for irradiated compounds are higher than those obtained for unirradiated ones. For NRU method, the obtained SI values are always higher than 1, demonstrated that the tested compounds exhibit high degree of selectivity.

In order to evaluate the effect of the UV-irradiation on the selectivity action of the studied phehothiazines, a pseudoselectivity index has been calculated. This index would to emphasize differences between the unirradiated and irradiated compounds, for both cell lines and both endpoint methods. As can be observed from results

summarizes in Table 1, the UV-irradiation of TZ derivative didn´t indorse any selectivity. However, the irradiation process can induce selectivity that is dependent of the phehothiazine derivative. Thus, in the case of PMZ, SI values ranged between 1 and 1.6 have been obtained. When CPZ compound is irradiated, the SI values increased up to 2.0, in several cases. For PZ compound, an interesting selectivity is observed. Although SI values are always higher than 2.0, SI values corresponding to the HeLa cell line, determined by the MTT method, increased up to 4.5 demonstrating a great selectivity.

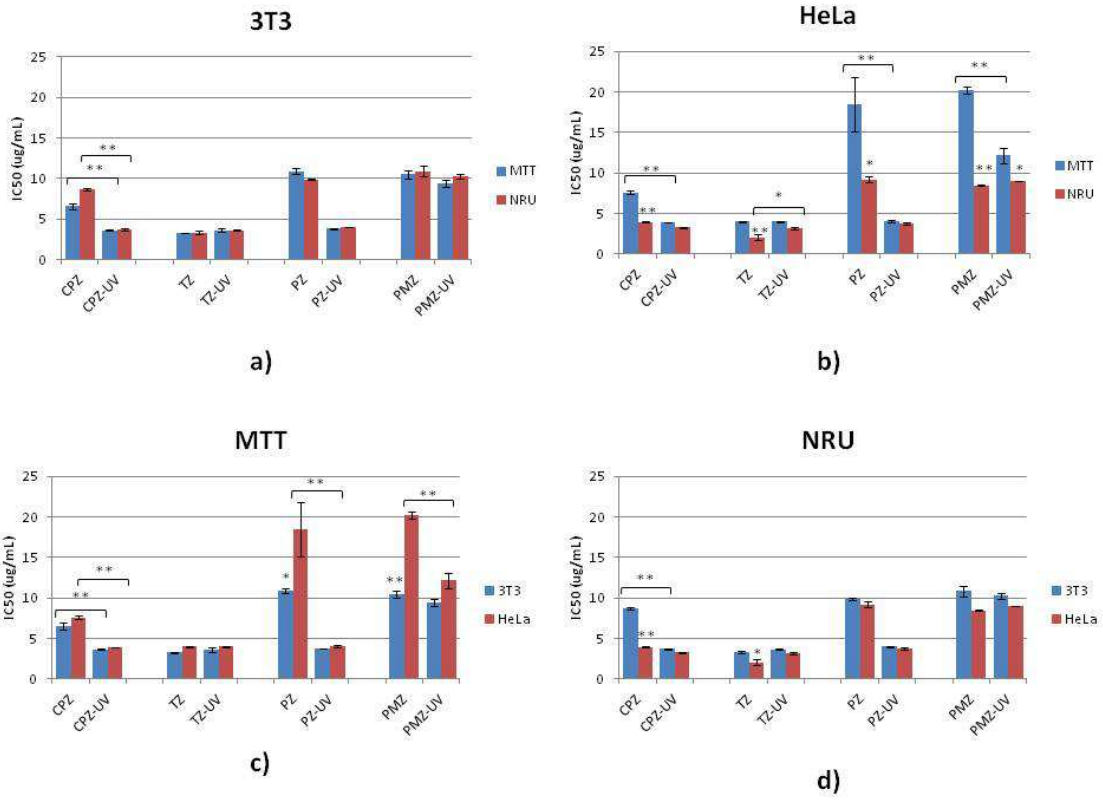


Fig 6.

Table 1. Comparison of cytotoxic activities of the unirradiated and irradiated phenothiazines. From the fitting of concentration-dependent viabilities curves, the corresponding IC_{50} values have been determined.

	3T3-MTT	3T3-NRU	HeLa-MTT	HeLa-NRU	SI-MTT	SI-NRU	SI(UV) 3T3 MTT	SI(UV) 3T3 NRU	SI(UV) HeLa MTT	H
	6..55 ±0.42	8.68 ±0.19	7.58 ±0.22	3.94 ± 0.07	0.864	2.205	1.806	2.352	1.934	
V	3.63 ±0.09	3.69 ±0.10	3.92 ±0.04	3.26 ±0.07	0.926	1.131				
	3.27 ±0.05	3.33 ±0.18	3.97 ±0.01	2.07 ±0.32	0.825	1.607	0.903	0.918	1.000	
	3.63 ±0.25	3.62 ±0.05	3.97 ±0.05	3.16 ±0.10	0.914	1.147				
	10.90 ±0.34	9.86 ±0.10	18.52 ±3.36	9.19 ±0.40	0.588	1.074	2.885	2.464	4.595	
	3.78 ±0.01	4.00 ±0.06	4.03 ±0.10	3.70 ±0.15	0.937	1.081				
	10.48 ±0.45	10.86 ±0.67	20.21 ±0.37	8.47 ±0.05	0.519	1.282	1.114	1.060	1.663	
V	9.41 ± 0.45	10.25 ±0.32	12.15 ±0.98	8.99 ± 0.02	0.775	1.140				

3.4. Interaction of Unirradiated and Irradiated Phenothiazines with Fabrics

The surfaces preparation consisted in washing procedure of cotton (CO) and polyester (PES) materials as shown in [5], (10 and 5 times, respectively) in a recipient (≈ 1 L) utilizing warm tap water to eliminate contaminations. Basically, in [5], at the end of every washing/cleaning stage/cycle, the water's surface tension used for washing was measured, to compare it with the value of the clean tap water. In the case of the CO surfaces, after 10 cycles the used water's surface tension reached the clean water's value. For PES surfaces 5 cycles were sufficient to obtain the same effect, due to the specific properties of PES.

The EDS maps reveal the distribution of the characteristic element present in the medicines acting as tracing agents coupled with morphology observations. In this case S and Cl maps have been extracted from the whole spectra.

Morphology investigations performed by Scanning electron microscopy (SEM) have given a microscale insight in the wetting properties of irradiated and not irradiated samples observed in [5, 7]. In Fig.7 the unirradiated PMZ sample seems to accumulate in zones of the fabric without a more homogeneous distribution (see maps of S and Cl) where the central part is more densely populated. The presence of more surface active compounds as produced by irradiation appears to be effective in the wetting properties of the irradiated solutions with respect to a more homogeneous distribution and uniformity of the wet fibers. The foam-like structure present in PMZ before the irradiation disappears after the laser treatment producing a more homogeneous film bridge between the fibers in comparison to the unirradiated sample.

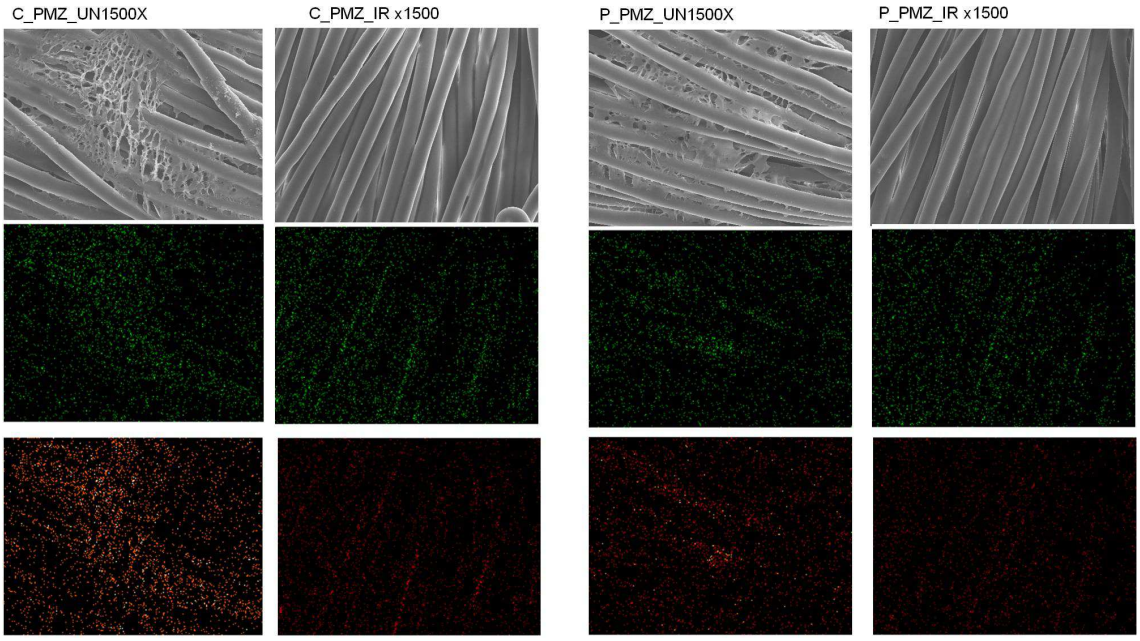


Fig. 7

CPZ also shows improved wetting properties on the fibers with prevalent hydrophilic nature like cotton (Fig. 8). On the other hand, the behavior toward the more hydrophobic polyester results quite independent from the irradiation stage.

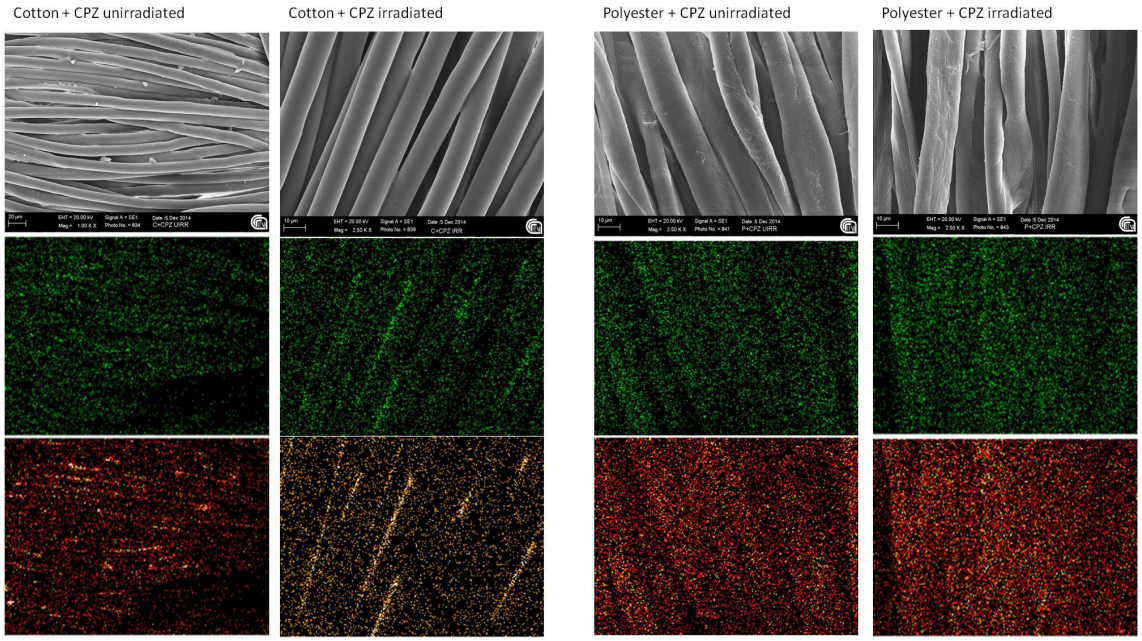


Fig. **8**

In general, irradiation produces more wetting agents able to let the substance better distribute along the fibers with some differences between cotton and polyester according to the more hydrophobic or hydrophilic percentage of surface active agents in the solution after irradiation [42]. For PZ the irradiation didn't appear to change appreciably wetting properties and distribution among the fibers. Plain cotton and polyester have been taken as a reference with the signal coming from the samples, but featuring a lower intensity throughout. On the other hand, the data reported here about the wettability of fabrics confirm the results reported in [5] where optical/optofluidic methods were used to measure wetting properties. SEM and EDS methods used in this paper confirmed the results shown previously in [5]." tracking at nano – micro scale the distribution of the new molecules along the fibers.

4. Conclusions

An important number of drugs employed currently in medicine have their origins in the chemical manipulation of phenothiazines. In general, chemical manipulation of a compound for generation of new derivatives is limited by existing organic chemistry, is time consuming and the percent yield of an active product is quite small. Exposure of phenothiazines solutions to UV laser radiation generates photoproducts that have different molecular structures and properties with respect to them. These data suggest to extend the toxicity studies of phentothiazines exposed to laser radiation towards

applications on cell cultures and to broaden the studies of their wetting properties on fabrics in view of further applications.

Together with some significant differences on the cell viabilities observed as a function of the endpoint method (MTT and NRU) and cell line type (3T3 and Hela cell lines), the most remarkable differences have been observed as a function of the irradiation process on the phenothiazine derivative. Of significant importance is that the irradiation photoproducts of CPZ and PZ derivatives have higher *in vitro* cytotoxicity against the studied cell cultures, suggesting that this approach may be useful for the development of compounds more bioactive than the parental species. Not all the phenothiazines have this kind of effect, such as it is the case of PMZ and TZ; this may be due to the different photoproducts obtained from their modification by exposure to UV laser radiation. The pseudoselectivity index values demonstrated that the irradiation process can induce selectivity in their mode of action. Whereas the UV-irradiation of TZ derivative didn´t indorses any selectivity, PMZ and CPZ increased selectivity as a result of the irradiation process. For PZ derivative, the irradiated compound demonstrated to be 4.5 times more selectivity against HeLa cell line than the unirradiated compound, determined by the MTT method.

On the other hand the interaction of unirradiated and irradiated phenothiazines with fabrics show that the most important modifications of the wetting properties may reported for CPZ and PMZ whereas these properties are not significantly improved for TZ and PZ. In view of possible biomedical applications, the irradiation procedure results in the production of more surface active agents in CPZ and PMZ holding to an enhancement of the wetting and distribution of the substances in cotton or polyester fabrics fibers with some differences according to their hydrophilic/hydrophobic features.

If the effects of the irradiated phenothiazines on cell cultures are correlate with the improvement of the wetting properties the irradiated phenothiazines it results that the most recommended phenothiazines for applications if CPZ because its irradiated water solutions are more effective on bacteria cultures and at the same time the same solutions better wet the studied fabrics. So, knowing the conditions in which cotton and polyester textiles can be wetted more easily, it would be possible to use them as drug delivery systems. This kind of properties may be useful in designing fabrics with controlled release of medicines to targets.

Though, PZ and PMZ may be considered as candidates for specific applications due to their respective advantages which may be used, namely a higher treatment efficiency showed by PZ and a better overall wetting characteristic of PMZ.

Acknowledgements

M.C. Morán acknowledges the support of the MICINN (Ramon y Cajal contract RyC 2009-04683). This study was financially supported by Project MAT2012-38047-C02-01 from the Spanish Ministry of Science and Innovation. The authors from NILPRP acknowledge the financial support of CNCS – UEFISCDI project number PN-II-ID-PCE-2011-3-0922 and by the NUCLEU program project PN 0939/2009. A. Smarandache was supported by the project POSDRU/159/1.5/S/137750. This work was conducted under the umbrella of COST MP1106 Action.

FIGURE CAPTIONS

Fig. 1. Set-up for exposure of the phenothiazines solution to UV laser light a) and the 3D not optimized structures of the utilized phenothiazines b).

Fig. **2.** Absorption spectra of CPZ and PZ measured immediately after exposure to UV laser radiation and kept in dark at 4ºC for time intervals of one month (CPZ) and 3 weeks (PZ). a) CPZ irradiated 4h; laser beam energy 6,5mJ; laser beam wavelength 266nm; b) PZ irradiated 4h; laser beam energy 6,5mJ; laser beam wavelength 266nm;

Fig 3. Dependence of human erythrocyte hemolysis on unirradiated (blue symbols) and irradiated (red symbols) CPZ (a), TZ (b), PZ (c) and PMZ (d) concentration. Erythrocytes were incubated for 1 h at room temperature at different concentrations, and the amount of released hemoglobin was determined. HC_{50} values of the corresponding unirradiated and irradiated phenothiazines (e). The data correspond to the average of three independent experiments ± standard deviation. Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test; *$p < 0.05$ denotes significant differences.

Fig. **4**. Concentration-dependent relative viabilities of HeLa cells treated with unirradiated (blue) and irradiated (red) CPZ (a), TZ (b), PZ (c) and PMZ (d) derivatives for 24 h determined by MTT (solid lines) and NRU (dotted lines) assays. The data correspond to the average of three independent experiments ± standard deviation. Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test; *$p < 0.05$ and *$p < 0.005$ denotes significant differences.

Fig. **5**. Concentration-dependent relative viabilities of 3T3 (left) and HeLa (right) cells treated with unirradiated (blue) and irradiated (red) CPZ (a), PZ (b), TZ (c) and PMZ (d) compounds for 24 h determined by MTT (solid color) and NRU (soft color) assays. The data correspond to the average of three independent experiments ± standard deviation.

Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test; *p < 0.05 and *p < 0.005 denotes significant differences.

Fig. **6**. IC_{50} values of the corresponding unirradiated and irradiated phenothiazines on 3T3 (a) and HeLa (b) cell lines on varying the endpoint method, and determined by MTT(c) and NRU (d) methods on varying the cell line type. The data correspond to the average of three independent experiments ± standard deviation. Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test; *p < 0.05 and *p < 0.005 denotes significant differences.

Fig. 7 SEM morphology(1500X) and EDS maps for S (green) and Cl (red) for irradiated and unirradiated PMZ (20mg/mL) on different fabrics (C for Cotton, P for Polyester)

Fig. **8** SEM morphology (1500X) and EDS maps for S (green) and Cl (red) for irradiated and unirradiated CPZ (20mg/mL) on different fabrics (C for Cotton, P for Polyester)

References

[1] M. L. Pascu, V. Nastasa, A. Smarandache, A. Militaru, A. Martins, M. Viveiros, M. Boni, I. R. Andrei,l A. Pascu, A. Staicu, J. Molnar, S. Fanning and L. Amaral, Recent Patents on Anti-Infective Drug Discovery 6 (2011) 147.

[2] A. Smarandache, J. Kristiansen and M.L. Pascu, LDDD 9 (2012) 352.

[3] M. L. Pascu, B. Danko, A. Martins, N. Jedlinszki, T. Alexandru, V. Nastasa, M. Boni, A. Militaru, I., R. Andrei, A. Staicu, A. Hunyadi, S. Fanning and L. Amaral, PLoS ONE 8 (2013) .

[4] T. Alexandru, A. Staicu, A. Pascu, E. Radu, A. Stoicu, V. Nastasa, A. Dinache, M. Boni, L. Amaral and M, L. Pascu, Journal of Biomedical Optics 20 (2015) 051002.

[5] A. Simon, T. Alexandru, M. Boni, V. Damian, A. Stoicu, V. Dutschk and M. L. Pascu. International Journal of Pharmaceutics 475 (2014) 270.

[6] A. M. Armada, T. Alexandru, D. Machado, B. Danko, A. Hunyadi, A. Dinache, V. Nastasa, M. Boni, J.Ramos, M. Viveiros, J. Molnar, M. L. Pascu and L. Amaral, In vivo 27 (2013) 605.

[7] T. Alexandru, A. Armada, B. Danzo, A. Hunyadi, A. Militaru, M. Boni, V. Nastasa, A. Martins, M. Viveiros. M. L. Pascu, J. Molnar and L. Amaral**,** Biochem. Pharmacol. 2 (2013) 1.

[8] M. P. Sheetz and S.J. Singer, Proc Natl Acad Sci USA1 71 (1974) 4457.

[9] B. Isomaa, H. Hägerstrand and G. Paatero, Biochim Biophys Acta 899 (1987) 93.

[10] M. P. Vinardell and M. R. Infante, Comp Biochem Physiol Part C 124 (1999) 117.

[11] J. M. Hillegass, A. Shukla, S. A. Lathrop, M. B. MacPherson, N. K. Fukagawa and B. T. Mossman, WIREs Nanomed Nanobiotechnol 2 (2009) 219.

[12] C. L. Crespi, Adv Drug Res, 26 (1995) 179.

[13] A. Jondeau, L. Dahbi, M. H. Bani-Estival and M. C. Chagnon, Toxicology 226 (2006) 218.

[14] V. Martinez, E. Corsini, M. Mitjans, A. Pinazo and M. P. Vinardell, Toxicol Lett 164 (2006) 259.

[15] H. Kim, S. C. Yoon, T. Y. Lee and D. Jeong, Toxicol Lett 184 (2009) 13.

[16] M. V. Berridge, P. M. Herst and A.S. Tan, Biotechnol. Annu. Rev. 11 (2005) 127.

[17] Y. Liu, D. A. Peterson, H. Kimura and D. Schubert, J Neurochem 69 (1997) 581.

[18] G. Repetto, A. Peso and J. L. Zurita,Nat. Protoc. 3 (2008) 1125.

[19] D. Napierska, L. C. Thomassen, V. Rabolli, D. Lison, L. Gonzalez, M. Kirsch-Volders, J. A. Martens and P. H. Hoet, Small 5 (2009) 846.

[20]. Rahbari, T. Sheahan, V. Modes, P. Collier, C. Macfarlane, R. M. Badge, M. Sheahan, Mc Collier, BioTechniques 46 (2009) 277.

[21] V. Smith,Wonder Woman: The Life, Death, and Life After Death of Henrietta Lacks, Unwitting Heroine of Modern Medical Science". *Baltimore City Paper*. Retrieved 2012-08-19.

[22] E. Fröhlich, C. Meindi, E. Roblegg, A. Griesbacher and T. R. Pieber, Nanotoxiciology 6 (2012) 424.

[23] N. A. Monteiro-Riviere, A. O. Inman and L. W. Zhang, Toxicol. Appl. Pharmacol. 234 (2009) 222.

[24] M. Das, S. Mardyani, W. C. W. Chan, E. Kumacheva, Advanced Materials 18 (2006) 80

[25] Y.C. Hseu,H.T.Tsou, K. J. Senthil Kumar,. K.Y. Lin,H.W. Chang,and H.L. Yang, Evidence-Based Complementary and Alternative Medicine, Article ID 197309, (2012)

[26] M. Ghoneum, A. Ghoneum, L. Tolentino and J. Gimzewski, In Vivo, 24 (2010) 141.

[27] D. Mathiyalagan, SSRG International Journal of Pharmacy and Biomedical Engineering (SSRG-IJPBE) 1 (2014) 16.

[28] J. Kumirska, M. X. Weinhold, J. Thöming and P. Stepnowski, Polymers 3 (2011)1875.

[29] V. Leung, R. Hartwell, H. Yang, A. Ghahary, F. Ko, Journal of Fiber Bioengineering & Informatics 4 (2011) 1.

[30] R.Rathinamoorthy and L. Sasikala, Int J Pharm Pharm Sci 3 (2011) 38.

[31] M.P. Vinardell and M. R. Infante, Comp Biochem Physiol Part C 124(2) (1999)117.

[32] Red Blood Cell (RBC) Lysis and Protein Denaturation DB-ALM Protocol n° 99 (EURL ECVAM DB-ALM: Protocol).

[33] T. Mosmann, J. Immunol. Methods 65 (1983) 55.

[34] E. Borenfreund and J.A. Puerner, Toxicol. Lett. 24 (1985) 119.

[35] B. Isomaa, H. Hägerstrand and G. Paatero, Biochim. Biophys. Acta 899 (1987) 93.

[36] S. B. Rao and C. P. Sharma, J. Biomed. Mater. Res. 34 (1997), 21.

[37] B. H. Kim and K. H. Baek, Kor. J. Environ. Toxicol., 5 (1990) 45).

[38] H. Ippen, Dtsch. Med. Wochenschr, 87 (1962) 480; M. A. Pathak and J. H. Epstein, Dermatology in General Medicine, McGraw-Hill, New York (1971) 412).

[39] C. L. Harber and R. C. Baer, J. Invest. Derm. 58 (1972) 327; E. A. Emmett, Toxic responses of the skin, Macmillan, New York, (1986) 412)

[40] R. B. Badisa, S. F. Darling-Rees, P. Joseph, J. S. Cooperwood, L. M. Latinwo and C. B. Goodman, Anticancer Res. 29 (2009) 2993.

[41] R. B. Badisa, D. A. Mina, L. M. Latinwo and K. F.A.Soliman, Anticancer Res. 34 (2014) 5447.

[42] M.L. Pascu, I.R. Andrei, M. Ferrari, A. Staicu, A. Smarandache, A. Mahamoud, V. Nastasa, L. Liggieri Colloids and Surfaces A: Physicochem. Eng. Aspects 365 (2010) 83)