# CU/ZN SUPEROXIDE DISMUTASE -POLYAZAAROMATIC RUTHENIUM COMPLEX-DNA ELECTRON TRANSFER. INFLUENCE OF THE REDOX POTENTIAL

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In previous work we have reported that Cu/Zn superoxide dismutase (SOD) induced an unexpected enhancement of the photonuclease activity of both $Ru(bipy)_3^{2+}$ and $Ru(bpz)_3^{2+}$. It was clearly shown, in the case of $Ru(bpz)_3^{2+}$, that this augmentative effect was not due to the involvement of superoxide anion and OH°. Moreover, in the presence of SOD a dramatic increase of the yield of photoadducts and oxidative damage at 5' guanine of GG sites generated by $Ru(bpz)_3^{2+}$ was observed. The participation of an electron transfer was proposed. This assumption was supported by the luminescence quenching of $Ru(bpz)_3^{2+}$ by the Cu/Zn SOD, a quenching constant of 4.69 $10^9$ $M^{-1}$ $s^{-1}$ was evaluated. Such behaviour was not observed with $Ru(bipy)_3^{2+}$. Moreover, low temperature EPR spectroscopy analysis of $Ru(bpz)_3^{2+}$, performed in the presence of SOD and under irradiation, revealed the formation of $Ru(bpz)_3^{1+}$. In contrast, with $Ru(bipy)_3^{2+}$ the formation of the reduced species $Ru^{1+}$ was not observed. This result points out the participation an electron transfer between $Ru(bpz)_3^{2+}$at the excited state and an amino acid of the metalloprotein. The redox potential of $Ru(bipy)_3^{2+}$ is apparently not high enough to induce such process.

In a second step, spin trapping experiments with N-tert-butyl-α-phenylnitrone and poly(dG-dC) have substantiated the ability of $Ru(bpz)_3^{2+}$ to photosensitize DNA *via* an electron transfer, giving rise to the formation of guanine radical. In the presence of SOD, the signal intensity corresponding to the guanine radical increased dramatically. Moreover EPR experiments using poly(dA-dT) irradiated in the presence of $Ru(bpz)_3^{2+}$ and SOD, revealed the formation of a signal corresponding to the adenine radical. These original results strongly suggest the formation of $Ru^{3+}$ species *via* an oxidation of $Ru(bpz)_3^{2+}$ by the SOD. Once formed, $Ru^{3+}$ is sufficiently potent thermodynamically to oxidize adenine within DNA. A survey of measured reduction potentials for $Ru^{3+}/Ru^{2+}$ of 1.86 V in acetonitrile versus 1.4V for DNA suggests that oxidation should be possible. In accordance with these different data and the redox potentials of both Cu/Zn SOD and $Ru(bpz)_3^{2+}$, a reaction mechanism was proposed, in which $Ru(bpz)_3^{2+}$ at the excited state is reduced by an amino acid of the SOD to generate $Ru^{1+}$. This step, not observed with $Ru(bipy)_3^{2+}$, is apparently necessary for the second redox reaction. In a second pathway $Ru(bpz)_3^{2+}$ at the excited state is oxidized by SOD to generate $Ru^{3+}$. Subsequently, $Ru^{3+}$ oxidizes the guanine. Tyrosine residues and the catalytic copper site of the SOD could be involved in these different electron transfer processes between the protein and $Ru(bpz)_3^{2+}$ at the excited state. In this study EPR spectroscopy was a powerful tool to examine radical reactions within DNA.

# UPREGULATION OF RHOB BY UVB IRRADIATIONS IN HUMAN KERATINOCYTES: A ROLE IN THE APOPTOTIC RESPONSE.

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Ultraviolet B irradiation generates many deleterious effects on skin tissue leading to fast adaptive cell response that contributes to maintaining their functions and survival. Dysregulation of this response promotes skin cancers and photo-aging. *rhoB* is an early responsive gene to DNA damaging agents and growth factors. RhoB is involved in vesicle trafficking, cytoskeletal organization, cell growth control and appears to play a crucial role in the cell response to genotoxics such as chimiotherapeutic agents and ionizing radiations.

In this study we have first investigated the regulation of RhoB expression by UVB in human keratinocytes. RhoB protein level was strongly induced by UVB irradiation (8-fold, 6 hours after exposure). Real-time PCR analysis showed a 5-fold increase in *rhoB* mRNA expression in HaCaT keratinocytes as well as in primary cultured keratinocytes. In order to understand the mechanism of UVB induction of RhoB, the expression of a *rhoB* promoter-*Luc* fusion construct was analyzed after transient transfection in HaCaT keratinocytes. These experiments showed a 3-fold increase of *rhoB* promoter transcriptional activity after irradiation. Moreover, actinomycin D treatment showed a stabilization of *rhoB* mRNA after UVB exposure. These results showed that RhoB upregulation by UVB is triggered by both transcriptional and post-transcriptional mechanisms.

One of the major response of keratinocytes to UVB irradiation is apoptosis. In order to understand the role of RhoB induction in UVB response, we then studied the effect of RhoB inhibition on UVB-induced apoptotis. The specific inhibition of RhoB induction by RNAi treatment induced a 2-fold increase in the apoptotic response of HaCaT keratinocytes to UVB irradiation as mesured by 3 types of experiments (DAPI staining of nucleus, detection of PARP clivage by western blotting, and quantitation of cytoplasmic histone-associated-DNA-fragments by ELISA).

Our data suggest that RhoB induction might act as a control element in the protective response to UVB radiation and should limitate apoptosis rate in response to UVB irradiation. It will be interesting to further characterize its role, notably in photocarcinogenesis.

# DNA REPAIR AND BIOLOGICAL RESPONSES IN UVB-IRRADIATED CULTURED HUMAN SKIN CELLS

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UVB light is known to induce the formation of dimeric photoproducts between adjacent DNA pyrimidine bases including cyclobutane dimers and (6-4) photoproducts. Using a highly sensitive and specific liquid chromatography-mass spectrometry (HPLC-MS/MS) assay, we assessed the repair of the latter lesions within the DNA of cultured normal human fibroblasts and keratinocytes upon UVB irradiation. Then, we investigated the relationship between repair of the main DNA lesions and cellular responses after UVB exposure. These included cell cycle and apoptosis studied by flow cytometry technique while proliferation was quantified by 5-bromo-2'-deoxyuridine incorporation. A major observation dealt with the fact that the rate of repair of TT and TC photoproducts significantly decreased with increasing UVB doses. Another significant result was that (6-4) photoproducts were efficiently repaired since they were completely removed within 24 h after irradiation. In contrast, cyclobutane dimers, the major lesions, persisted after a relative high dose of UVB (corresponding to 2 MED) in the two selected cutaneous models. Indeed, about 60 % of the initial amounts of cyclobutane dimers were still present 72 h after irradiation. In particular, UVB-induced apoptosis, which was observed 24 h after irradiation of keratinocytes did not lead to a significant decrease in the level of DNA damage. In addition, fibroblasts were found to proliferate normally 48 h after irradiation, after a transient cell cycle arrest, in spite of the fact that a high yield of DNA lesions was still present in their genome. In the other hand, no apoptosis was observed after irradiating these cells at 500 $J.m^{-2}$. Altogether, these results show that, in the two skin cell types, repair of the global genome is not required for the proliferation of cells after exposure to UVB radiation. This observation is strongly suggestive first of a major role played by transcription coupled repair in the cellular response to UV irradiation, and second of the importance of cyclobutane dimers in UV mutagenesis.

**"HOT SPOTS" ASSOCIATED WITH THE PHOTOINDUCED BINDING OF RUTHENIUM TRISBIPYRAZINE TO C-RAS DNA AND INHIBITION OF THE REPLICATIVE T4 POLYMERASE**

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Trisbipyrazine ruthenium(II) complex is known to form covalent photoinduced linkages with DNA *via* an electron transfer process. These photodamage result from the attachment of a bipyrazine ligand to the guanine which is the nucleobase with the lowest redox potential. In order to determine the sequence selectivity of this chemistry "foot printing experiments", using the DNAse I, were performed. In this study a 60 mers oligonucleotide corresponding to the sequence of codons 9 to 14 of the proto-oncogene H-ras was taken as target. Polyacrylamide sequencing gel revealed "hot spots" which occurred at adjacent, guanines (GG, GGG), with a particularly strong preference for GGG sites. These guanines were also selectively oxidized by the dye at the exited state. These data are consistent with our previously postulated mechanism for the covalent binding chemistry which involves an electron transfer from the deoxyguanine to $Ru(bpz)_3^{2+}$ at the exited state. To get further insight on the biological relevance of DNA photo-adducts of $Ru(bpz)_3^{2+}$, we have studied their influence on the function of T4 DNA polymerase. In vitro replication studies were performed using a 17 mers DNA primer radiolabeled with $\gamma^{32}P$ and hybridized to its complementary sequence located on the 3'-side of the 60-mer matrix. After illumination, polymerase action is inhibited at multiple sites in the vicinity of the dye lesions corresponding to GG and GGG sites. These observations support a model in which $Ru(bpz)_3^{2+}$ reacts with guanine responsible of potential gaps in DNA and can in this way impede primer elongation by T4 DNA polymerase. This assumption was supported by original data showing a covalent binding of $Ru(bpz)_3^{2+}$ on the 8-oxoguanine, which has a lower redox potential than the guanine, and a selective stop of the DNA polymerase T4 action at this site. For this study a single 8-oxoguanine was introduced in the sequence of the proto-oncogene H-ras. All these results open new perspectives for future application of $Ru(bpz)_3^{2+}$ as a new drug for the photodynamic therapy.

# AUTOFLUORESCENCE ANALYSIS OF CHANGES OF CEREBELLAR CORTEX DURING NORMAL AND ALTERED DEVELOPMENT

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Autofluorescence emission from biological substrates depends on nature, amount, distribution of endogenous fluorophores (proteins, NAD(P)H, flavins), and is influenced by tissue optical properties (absorption, scatter), providing a tool for the direct diagnosis of tissues morpho-functional conditions. Autofluorescence potential was investigated for the diagnosis of cerebellum molecular and morphological alterations, occurring during post-natal development to lead to definitive cytoarchitecture. Cerebellum rat development (in particular lobules VI and VII-neocerebellum), is delayed by cisplatin treatment. Drug injection to 10-days old rats induces a high incidence of apoptotic cells in the external granular layer (24 h), restoration of the proliferative activity (7 gg), and absence of the external granular layer (20gg); delay of Purkinje cell differentiation in the external germinative matrix (24 h), and alteration of Purkinje dentrites branching at longer times. Changes of molecular features of developmental morphogens were also found (Pisu et al., Guioli et al., FENS Forum 2002). The influence of layers alterations on tissue light penetration was investigated through high sensitivity imaging. Sagittal sections of fresh tissue were placed on a slide, 366nm excitation light was delivered on the surface of target lobules with a fiber-optic probe, and images were recorded from the orthogonal direction. The pattern profile was analysed by non-linear regression to estimate the depth of tissue involved (mm at which the signal is = 1/e of the incident light). In neocerebellum this was 0.75, 0.37, 0.23 mm for control rats of 10, 17 and 30 days, and 2.22, 1.63, 0.51 mm for the corresponding treated rats. Paleocerebellum showed neither structural alterations nor depth values changes. Microspectrofluorometric analysis showed small differences in the spectral shape among the layers of untreated rats cerebellum, and no appreciable differences among the layers of treated rats. Spectral fitting analysis, evaluating the contribution of each fluorophore to the whole emission, similarly to an *in-situ* biochemical analysis, evidenced an increase in oxidised flavins and lipopigments in treated rats, indicating an increase in oxidised state. Results obtained evidence the influence of structural organization in addition to biochemical composition on autofluorescence emission properties, and confirm its diagnostic potential. *Supported by MIUR Cofin grant 2002 to G. Bernocchi.*

# FEASIBILITY OF FIBEROPTIC CONFOCAL MICROSCOPY IN THE DIAGNOSIS OF ORTHOTOPIC RAT BLADDER CANCER MODEL.

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**Purpose:** We report the feasibility of fibered confocal fluorescence microscopy for the endoscopic imaging of rat bladder urothelium *in situ* by means of a minimally invasive imaging device (Cell-viZio, MKT France).

**Materials and methods**: 0.5 ml of a 100 µM Rhodamine 123 solution (a mitochondrial marker) was instilled intravesically in female Fischer rat bladders (healthy controls and AY27 (transitional cell carcinoma) bearing rats for 30 minutes. At the end of instillation bladders were washed with normal saline before imaging. The Cell-viZio probe was then inserted and placed in gentle contact at different spots on healthy controls or tumor bearing rat bladders. In parallel, frozen sections were prepared and examined with a standard fluorescence microscope and HE stained for pathological confirmation.

**Results**: In healthy bladders the confocal fluorescence imaging system allows a clear distinction between the three cellular layers of rat bladder epithelium: the upper layer of polygonal umbrella cells, the underlying intermediate cells and cuboidal basal cells. In tumor bearing bladders, tumor cells appear much smaller with a higher cell density and higher fluorescence intensity. The same cells features were retrieved with fluorescence microscopy in *ex vivo* experiments and were confirmed by HE stained sections.

**Conclusion**: We report the feasibility to perform endoscopic fiberoptic confocal microscopy *in vivo* in small animals. The use of Rhodamine 123 as fluorescent probe enables us to make a clear distinction between four different cells types: umbrella cells, intermediate cells, basal cells and tumor cells.

## DIODE LASER WELDING OF THE CORNEA: AN EXPERIMENTAL STUDY

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An *ex vivo* and *in vivo* experimental study, evaluating the application of laser welding of the cornea, is presented. The proposed technique is based on stromal collagen photothermal activation, induced by near-infrared (810 nm) low power (80 mW) diode laser radiation, strongly absorbed by the chromophore Indocyanine Green (ICG), applied in the corneal wound to be repaired. The result is a localized and controlled welding effect, with immediate sealing on the wound edges and good mechanical resistance. The laser especially developed for this application was a AlGaAs diode laser equipped with a fiber optic delivery system (200 and 300 micron fiber core diameter). Each fiber terminates in a hand piece which enables easy operations under surgical microscope. *Ex vivo* experiments on freshly enucleated porcine eyes were set up for studying ICG absorption curve in corneal tissue and evaluating local heating. As it is known, ICG optical absorption spectrum strongly depends on its concentration and on the solvent. From the literature and from some measurements performed on saturated and non saturated solutions of ICG in distilled water, the absorption peaks are far from laser wavelength (779 nm for non saturated and 699 nm for saturated solutions, as measured). We measured ICG absorption spectra at different concentrations when dissolved in corneal collagen by means of an integrating sphere connected to a spectrophotometer. All the recorded absorption curves showed the principal peak at 803+/- 1nm, almost coincident with the diode emission wavelength. An infrared camera was used to estimate temperature variations on the cornea surface in dependence of laser power and spatial distribution of the heated zone. At a laser power of 80 mW the temperature rise was found to be around 10-15°C. At higher power values (120 mW), the temperature rise was 30°C and the welded tissue showed unacceptable local damage of the corneal stroma. *In vivo* experiments to evaluate laser welding and the following healing process were performed on 30 rabbits. Corneal cuts of 5 mm in length were sutured by diode laser irradiation in association with ICG. Then the animals were subjected follow up on 7, 15, 30, 60 post-operative days. Morphological and histological observations revealed that the healing process in the laser group was more effective and faster than that of the control group subjected to traditional stay suturing.

In conclusion this experimental study shows the characterization and optimization of cornea laser welding procedure, that could be proposed as a valid alternative to the standard suturing procedure.

# AUTOFLUORESCENCE SPECTROSCOPY OF NORMAL AND MALIGNANT ESOPHAGEAL EPITHELIUM CELLS

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The intensity and spectral distribution of near UV induced autofluorescence are related to the tissue structure (through collagen and elastin emissions), the cellular metabolism (through NAD(P)H and flavins emissions) and are strongly affected by the optical properties of tissue (absorption and diffusion). The purpose of this study is to characterize and compare the autofluorescence spectra of normal and two types of tumoral esophageal epithelial cells, considering both spectral shape and intensity, to discriminate between the contribution of intrinsic cellular components and of the structural part of the tissue autofluorescence.

The autofluorescence spectra of subcellular volumes were recorded with a confocal microspectrofluorimeter, under excitation wavelength 351 nm. To ensure a good statistical analysis, at least 100 cells of each histological type were measured, resulting in the acquisition of more than 1000 spectra. The emission fluorescence spectra of cell suspensions were also recorded in the right angle geometry, using a fluorescence spectrophotometer, with excitation set at 351 nm, for cell concentrations in the range from $1.0x10^5$ to $1.5x10^6$ Cell/mL.

Spectral shapes of normal and tumoral cells are found to be similar in both configurations, consisting in an emission band extending to 600 nm, with a peak around 450 nm, that is attributed to protein-bound NAD(P)H. The mean autofluorescence intensity is significantly higher in tumoral cells than in normal ones (approximately by a factor two). A possible explanation for the larger amount of the NAD(P)H reduced form in tumoral cells is that aerobic energy metabolism is less important in neoplastic cells than in normal ones, which results in an increase in the reduced fraction of pyridine nucleotides.

The analysis of the contribution of the intracellular fluorescence to the global esophageal tissular autofluorescence allows a better understanding of the origins of the spectral modifications observed during the neoplastic transformation of the esophagus mucosa. It is a first step toward a quantitative interpretation of esophageal autofluorescence spectra.

# 4-HYDROXYMETHYL-4’,8 –DIMETHYLPSORALEN, A NEW FUROCOUMARIN FOR PEG-CONJUGATION STUDIES

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Furocoumarins are well-known photosensitizers capable of inducing severe effects in various biological substrates. Some of them are widely used in photomedicine (e.g., 8-methoxypsoralen) in PUVA therapy for the treatment of skin diseases, in photopheresis for prevention of organ transplantation and in sterilization of blood preparations. Furocoumarin photochemotherapy represents one of the usual treatment of the Cutaneous T-Cell Lymphoma (CTCL, group of diseases making up 5% of all cases of Non-Hodgkin’s lymphoma) which mainly affects the skin.

To improve the therapeutic indices of PUVA therapy and photopheresis, both usually used in all stages of CTCL, we designed a strategy for increasing drug concentration inside the tumor cells. This goal can be reach conjugating the drug to a suitable macromolecule by means of a weak linkage. In fact, it has been demonstrated that by means of the so called Enhanced Permeability and Retention effect (EPR), a conjugated drug can accumulate preferentially into tumor cells; then the conjugate can undergo to hydrolysis, thus releasing the active drug inside the target cells. This effect has been exploited for various antitumor drugs at present under clinical evaluation.

The aim of our study was to obtain a suitable conjugate of an active furocoumarin derivative. First of all, we synthesized a appropriate furocoumarin molecule for polymer conjugation. Considering the strong photosensitizing activity and the wide spectrum of mono- and bifunctional lesions induced by methylated psoralens, in particular by 4,4’,8-trimethylpsoralen, we synthesized an its derivative having a hydroxyl group, namely 4-hydroxymethyl-4’-8-dimethylpsoralen. Then, we planned different strategies for polymer conjugation, linking the hydroxyl group with different PEG polymers (mono- and bi-substituted). We also preliminarily studied the main photochemical and photobiological features of the new conjugates together with their stability at different pH values and in plasma.

# Further Studies on the Combined Action of a Furoquinolinone (FQ) with UVA 365 nm or NB-UVB 311 nm

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Photochemotherapy using 8-methoxypsoralen (8-MOP) has become a very powerful phototherapeutic tool in dermatology. The photocarcinogenic hazard associated with high cumulative doses of PUVA has increased interest in modifying existing protocols with a view to reducing long-term risks. For this purpose, we chose an alternative substance with a low carcinogenic risk as photosensitizer, a new ultraviolet light NB-UVB, and intraperitoneal pathway administration.

Previous studies have shown that FQ (1,4,6,8-tetramethylfuroquinolinone), one of the most interesting compounds synthesized in our Department, presents antiproliferative activity even in the dark, unlike the furocumarines 8-MOP and 4,6,4’-TMA. Coupled with narrowband-UVB radiation, it could enhance the own therapeutic activity.

In PUVA photochemotherapy, treatment by mouth is currently preferred since it produces fewer toxic side effects. However, results obtained with this type of administration are greatly influenced by uncontrollable factors which affect absorption velocity, particularly by the organs.

Conversely, a more homogeneus distribution and better reproducible results, are achieved by intraperitoneal administration.

The study was performed on the sera and organs of healthy and tumour-bearing mice, in the dark and irradiated with UVA (365 nm) and NB-UVB (311 nm). Outcomes are compared and discussed.

## FLUPHENAZINE PHOTOTOXICITY:
## A MECHANISM INVOLVING THE TRIFLUOROMETHYL GROUP

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Fluphenazine is a neuroleptic drug used for the long-term treatment of mental disorders, in particular in the therapy of various psychoses, including schizophrenia and mania.

Occurrence of skin phototoxic and photoallergic reactions is observed when patients expose themselves to sunlight during treatment with fluphenazine or other phenothiazine derivatives.

In order to identify the mechanism of fluphenazine phototoxicity, the drug was irradiated with UVA in different solvents and in presence of nucleophilic substrates of biological relevance, like serine, lysine and a serine-containing oligopeptide. The photoproducts were characterized by mass spectroscopy and, for three of them, by NMR spectroscopy.

A minor photoproduct in which a N-oxidation occurred was found in all conditions.

The major product which formed in water was a carboxylic acid derived from the hydrolytic defluorination of the $CF_3$ group. In methanol and ethanol the main product was the ester of the carboxylic acid. Serine also bound fluphenazine through the same mechanism. Although with low yield, a similar product was identified in a 14-peptide irradiated in presence of the drug. Lysine did not react in the same conditions.

These results suggest that, in vivo, photobinding of fluphenazine to serine residues of proteins may occur *in vivo*, thus initiating the processes leading to photoallergy.

# STUDY OF THE INTERACTIONS WITH UV LIGHT OF NON STEROIDAL ANTI-INFLAMMATORY DRUGS, THE SELECTIVE INHIBITORS OF THE CYCLOOXYGENASE-2: PHOTOSTABILITY AND PHOTOSENSITIZING PROPERTIES IN VITRO

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In the last years, skin photosensitization is appearing in the most sensitive subjects with a greater number of drugs (Antibiotics, Antifungals, Diuretics and Cardiovascular agents, NSAIDs and Antipsychotics) either applied directly on the skin or administered systemically, and results in the risk of tumors of the skin. It's likely that other drugs that induce moderate phototoxicity are not recognized as 'photosensitizers' since the induced cutaneous reactions are taken for mild sunburn or solar eczemas. The selective inhibitors of the cyclooxygenase-2 (CSIs), drugs entered recently the market, are here studied in terms of phototoxicity and photostability, since their chemical structure suggests their interactions with UV light. This reactivity could bring to changes of the molecule or to its activation, with induction of photosensitizing effects. Indeed, for the less recent Nimesulide, some phototoxic reactions have already been reported in humans. In fact, CSIs drugs are characterized by the presence of an aromatic structure with high degree of conjugation, of a sulphonamide moiety (similar to that of the Sulphonamides, the phototoxicity of which has been already demonstrated) and, in some of them, of a trifluoromethyl group highly unstable under irradiation (as in the case of Fluphenazine). The present study includes investigations on the photostability of PTPBS (4-[5-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide), and the evaluation of its phototoxic potential *in vitro* on cellular models and on isolated biomolecules.

## PHOTOPROTECTIVE EFFECTS OF CYANIDIN-3-O-β-GLUCOPYRANOSIDE AGAINST UVA-INDUCED OXIDATIVE DAMAGE IN HUMAN KERATINOCYTES

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Ultraviolet A (UVA) radiation (320-380 nm) - one component of the solar UV spectrum - penetrates through the dermis and beyond to the subcutaneous tissue and affects both the epidermal and dermal components of skin. Generation of high levels of ROS by UVA can overwhelm normal defenses to oxidative damage, leading to extensive cellular damage and eventual cell death, either by apoptosis or necrosis. We evaluated the protective effects of cyanidin-3-O-β-glucopyranoside (C-3-G) against UVA-induced apoptosis in a human keratinocyte cell line (HaCaT) in terms of DNA fragmentation and caspase-3 activity. Treatment of HaCaT cells with C-3-G before UVA irradiation inhibited DNA fragmentation (54%) and caspase-3 activity (33%). We also investigated antioxidant properties of C-3-G in HaCaT cells against ROS formation at apoptotic doses of UVA: C-3-G inhibited $H_2O_2$ release (an indicator of cellular ROS formation) after UVA irradiation. In addition, the inhibition of $H_2O_2$ release is inversely correlated (in a linear fashion) with UVA-induced apoptosis. Further confirmation of the potential of C-3-G to counteract UVA-induced ROS formation comes from our demonstration of its ability to enhance the resistance of HaCaT cells to the apoptotic effects of ROS such as $H_2O_2$ or the superoxide anion ($O_2^{\bullet-}$). Furthermore, in terms of Trolox Equivalent Antioxidant Activity, C-3-G treatment led to a greater increases in antioxidant activity in the membrane enriched fraction than in the cytosol (55% *vs.* 19%). The protective effects against UVA-induced ROS formation can be attribuited to the higher membrane levels of C-3-G incorporation. These encouraging in vitro results support further research into C-3-G (and other anthocyanins) as novel agents for skin photoprotection.

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# Chlorin conjugated to a VEGF receptor-specific peptide increases uptake by endothelial cells.

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Because of high VEGF receptor expression in endothelial cells in the tumor environment, its natural ligand VEGF could be an attractive candidate for the conception of targeting strategies for photosensitizers. An alternative can be the use of selected peptides able to interact with VEGF receptor.

An heptamer peptide has been reported in the literature as inhibiting the binding of VEGF to one of its receptor, VEGFR-2/KDR. Moreover, it inhibited endothelial cells proliferation, and suppressed angiogenesis *in vivo* (Binétruy-Tournaire R. *et al.* EMBO J; 19(7), 1525-33, 2000). Coupling a photosensitizer to this molecule could potentiate the vascular effect of photodynamic therapy.

We chemically synthetized this peptide by a solid phase method (Fmoc/piperidin strategy). We then attached to its amino-moiety an hexanoic spacer and 5-(p-monocarboxyl)phenyl, 10,15,20- triphenylchlorin (TPC1COOH). Crude product was purified by successive washing steps, preparative RP-HPLC using a water-0,1% TFA/ methanol gradient, followed by hexane precipitation. Product purity was checked by UV spectrometry, analytical RP-HPLC, 2D-RMN and mass spectrometry. Uptake kinetics were carried out in HUVEC endothelial cells. Cells were grown during 4 days, then incubated with either TPC1COOH or peptide coupled-TPC1COOH at 5 µM during 1h to 24h. Fluorescence measurements from the photosensitizers-loaded cells were performed, following sonication, in ethanol at 652 nm with an excitation at 415 nm.

Uptake kinetics revealed a significant increase in the uptake of the peptide conjugated-TPC1COOH compared to TPC1COOH, up to 15- to 20- fold after 15h contact.

# INTERACTION OF SULFONATED ANIONIC PORPHYRINS WITH HIV GLYCOPROTEIN GP120 AND ASSOCIATED PHOTODAMAGES

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The key role of gp120 in the cellular entry of HIV makes this glycoprotein an attractive target for new drugs. Various polyanions bind to the positive charged V3 loop of gp120. Here we consider a series of anionic porphyrins bearing two sulfonated groups and two carboxylic chains with various degree of esterification. These molecules cary an overall negative charge between 4 and 2. The interactions of these molecules with the V3 loop and another positively charged area, the non-exposed C5 region, are investigated in the dark by using specific antibodies and ELISA test. Competitive inhibition of the anti-V3 antibody is observed with an increased efficiency for the esterified compounds, but no evidence for binding to the C5 region is found. In contrast, when gp120 is irradiated with light in presence of porphyrin prior to the addition of the antibody, no effect is found on the V3 loop but we observe a strong inhibition of the anti-C5 antibody revealing irreversible photodamages in this area. Again, the esterified compounds are the most efficient. Despite the presence of large excess of dimers in the incubation solution, porphyrin monomers are identified as the photoactive forms. It is suggested that porphyrin bound to the V3 loop could produce photodamages at some distance, in particular within the C5 region that contains several photosensitive amino acids. This example of light-induced damages on non-exposed residues may offer new ways for drug design.

# RESPONSE TO UV-B IRRADIATION IN *EUPLOTES FOCARDII* INVESTIGATED WITH EXPRESSION ANALYSIS OF *HSP70* AND *RAD51* GENES

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*Euplotes focardii* (Valbonesi and Luporini, 1990) is an endemic hypotrich ciliate in Antarctic coastal seawater. This is a thermally very stable ambient, but subject to variable UV radiation, due especially to geographical position and seasonal ozone depletion in the stratosphere caused by various pollutants such as CFC. A simulation of this variable irradiance conditions has been set up in laboratory using UV-A and UV-B lamps and cut-off filters, in order to investigate the differential response of *Euplotes focardii* to UV irradiation by the analysis of the expression of *hsp70* and *rad51*, two genes related to environmental stresses.

## PARTIAL SEQUENCE OF A GENE OF A RHODOPSIN-LIKE PROTEIN IN *EUGLENA GRACILIS*

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Light-absorbing chromoproteins called rhodopsins are broadly distributed among different taxa throughout the three domains of extant life: Archea, Bacteria, Eucarya. This clearly indicates that these proteins have been highly conserved during evolution. Independent of their location, they share identical topologies characterized by an apoprotein, the opsin, consisting of seven transmembrane α-helices that form a pocket in which the aldehyde group of the retinal chromophore forms a protonated Schiff's base with a specific lysine residue in the seventh transmembrane helix. Upon light absorption, retinal isomerizes and does not fit the binding site, causing conformational changes of the opsin, which eventually trigger a signal reliably and reproducibily. Many experimental data showed that the photoactive protein in the unicellular eukaryotic flagellate *Euglena gracilis* is a rhodopsin-like protein. To further support these data, a fragment of the gene for this protein was sequenced. PCR primers were designed to amplify a 500 bp fragment. The amplification product was sequenced and named Euopsina. This amplificate shares primary and tertiary structure properties of the microbial rhodopsin-like proteins. Analysis of the potential gene and protein-coding regions for Euopsina was performed by using Blast software package. GenBank research assigned Euopsina to the same family of human rhodopsin, Family A, G-protein-coupled receptor-like proteins. Comparison between amino acid sequences of microbial rhodopsins and Euopsina revealed a similarity of 65-69 %. Identity between Euopsina and the sensory rhodopsin I (HsSRI) of *Halobacterium salinarum*, is higher (37%) than the identity between HsSRI and the other microbial rhodopsins. Moreover, 75% of the residues predicted to form the retinal binding pocket are fully conserved in the Euospina, while in the other microbial rhodopsins the conservation ranges from 55 to 75%. As in all the sensory rhodopsins, the primary sequence of Euopsina shows a neutral amino acid in the position of residue 96, which improves the efficiency of signal transmission. By using TMHMM software, Euopsina revealed the typical seven transmembrane α-helices structure of rhodopsin-like proteins.

# Structural changes of PsbS, a small subunit of Photosystem II during photoprotection

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PsbS subunit of Photosystem II in higher plants plays a crucial role in pH- and xanthophyll-dependent non-photochemical quenching (NPQ). This process (NPQ) is known to contribute to the defence mechanism against photoinhibition by dissipating the excess absorbed light energy in form of heat. We produced a specific antibody against the stroma-exposed loop between the second and third putative helices of *Zea mays* PsbS. Thylakoid membranes of various higher plant species contained a protein, recognized by this antiserum, with the expected molecular mass of 21-kDa, and a 42-kDa protein band, indicating the formation of a dimer of the 21-kDa PsbS protein. The PsbS monomer/dimer ratio in isolated thylakoid membranes was found to vary with lumenal pH in a reversible manner, the monomer being the prevalent form at acidic and the dimer at alkaline pH. In intact chloroplasts and whole plants, dimer-to-monomer conversion was reversibly induced by light. Indeed, lumenal acidification is known to occur during photosynthesis. Sucrose gradient centrifugation revealed a prevalent association of the PsbS monomer and dimer with LHCII and PSII core complexes, respectively. The finding of the existence of a light-induced change in the quaternary structure of the PsbS subunit may contribute to understanding of the mechanism of PsbS action during non-photochemical quenching. Further, availibility of highly pure protein and its characterization might also help the elucidation of the detailed molecular mechanism for PsbS action.

# EUMELANINS AND PHEOMELANINS AS AN INDEX OF PHOTOSENSITIVITY

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Aim of our study was to correlate the concentrations of eu- and pheo-melanins and the values of delayed pigmentation as an index of photosensitivity.

Melanins concentration in the hair correlates with skin content of melanins so that in our study hair concentration of melanins was evaluated in 60 healthy volunteers. BTCA and TTCA are biochemical markers of pheomelanins, PTCA of eumelanins. For each subject, six square areas (1x1 cm) on the volar surface of the right forearm were irradiated with increasing doses of UVB. Evaluation of MED was performed after 24 hours

Pigmentation of MED area was evaluated at 5th , 9th, and 16th day from irradiation, either from a clinical or from a chromatometric point of view, by means of a dermaspectrometer

Erythema and pigmentation were correlated with eu- and pheo-melanin concentration, and then with phenotypes and phototypes of the 60 volunteers.

Results show that the ability to tan is better correlated to eumelanin amount in the hair, that is in the skin. MED is not an useful marker of photosensitivity.

Results will be better discussed.

## EPIDEMIOLOGIC DATA ABOUT PLE IN ITALY

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**Background**

Polymorphous light eruption (PLE) is the most common idiopatic photodermatoses.

It describes a broad clinical spectrum with chronic recorrences. It is often characterized by non scarring pruritic erythematous papules, vesicles or plaques. UV exposure is the main etiologic factor.

**Objective**

The aim of this study was to evaluate i) the incidence of PLE in Italy, ii) the main clinical features and iii) the clinical course and recurrences.

**Methods**

The study was carried out in 8 Dermatological services in Italy (University of Genova, Spedali Civili Brescia, S. Gallicano Institute Rome, University of Perugia, University of Siena, University “Federico II” Napoli, University of Milano, University of Verona). Subjects were required to fill a simple questionaire (41 questions) exploring the following topics: phototype and phenotype, modalities of solar exposure, clinical features of PLE, number of recurrences, familiar, pathological and pharmacological anamnesis.

The study was carried in healthy volunteers, not affected by a known photodermatological disease.

**Results and conclusion**

12378 subjects entered the study. The mean PLE incidence obtained was 5,3% without differences among the different latitude of our Country.

The plaque type of PLE was the most common clinical type (36,4%), the body site most frequently affected was the trunk (61,1%).

Other data obtained, statistically analyzed, will be discussed.

**CYTOTOXIC EFFECTS INDUCED BY THE COMBINATION OF CHEMOTHERAPY AND PDT IN TUMOUR CELLS *IN VITRO***

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The aims of this work are: i) to study the cytotoxic effects induced by the combination of chemotherapy and photodynamic therapy (PDT), in *in vitro* human cancer cells and ii) to evaluate the possibility to establish therapeutic protocols of combined treatments for esophageal and lung cancers. Toward this aim, the esophageal squamous carcinoma cells (KYSE-510) and the non-small cell lung cancer cells (NCI-2347) were treated with chemotherapics only, cis-platin, gemcitabine or vinorelbine, or with PDT only, using Photofrin as the photodynamic agent. Viability of the cells was measured by trypan blue dye-exclusion assay 24 h after treatment, as a function of the dose of each therapeutic agent. The data were used for selecting appropriate experimental conditions for subsequent experiments in which the two treatments were combined. The experimental protocols for combined treatments varied depending on cell line and chemotherapeutic agent used. The combination of cis-platin and PDT did not increase the death of KYSE-510 or NCI-2347 cells, as compared to single treatments, at least in cells simultaneously incubated with cis-platin and Photofrin before irradiation. On the contrary an additive effect could be observed between PDT and gemcitabine but this effect was dependent on gemcitabine concentration. The cytotoxic effects induced with protocols in which exposure to Photofrin is delayed with respect to cis-platin or gemcitabine is being evaluated as well. In order to clarify the reasons of the observed effects, the mechanisms of cell death and the cell cycle alterations induced by the single and the combined treatments are being studied.

## First biodistribution study of a triglucoconjugated derivative of *meso*-tetraaryl-porphyrin

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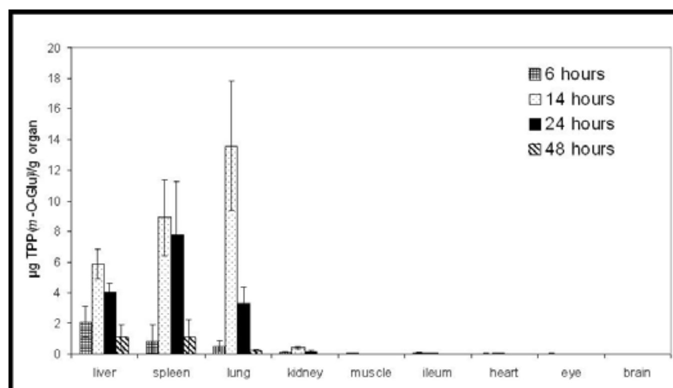
In the recent years, numerous new glycoconjugated derivatives of chlorins and porphyrins have been developed with modulated amphiphilicity and high photocytotoxicity. Among them the 5,10,15-*meso*-tri-(*meta*-O-β-D-glucosyloxyphenyl)-20-phenyl porphyrin [abbreviated TPP(*m*-O-Glu)$_3$)] seems to be a serious candidate for photodynamic therapy (PDT).

Biodistribution of the TPP(*m*-O-Glu)$_3$ was determined after intravenous administration of single dose (0.5 mg.kg$^{-1}$) to healthy rats. Animals were sacrificed at 6h, 14h, 24h and 48h after injection. Five rats were used in each case. A total of 9 organs were removed (liver, lung, kidney, ileum, heart, spleen, muscle, brain and eye). Quantitation of the TPP(*m*-O-Glu)$_3$ in tissue was performed using an analytical protocol combining L-L extraction and high performance liquid chromatography (HPLC) with NIR sensitive fluorescence detection.




Lung, liver and spleen constituted the TPP(*m*-O-Glu)$_3$ target tissues. In contrast small amounts of photosensitizer were found in muscle, kidney, ileum, heart. TPP(*m*-O-Glu)$_3$ was not detected in the brain or in the eye. The peak tissue was reached after fourteen hours. At 48 h the PS is almost completely eliminated.

This first *in vivo* study of a glucoconjugated porphyrin enables us to consider further experiments in rats inoculated with tumor models (corresponding to lung, liver and spleen cancers) with a laser irradiation performed 14 hours after photosensitizer systemic administration.

**PHARMACOKINETICS OF A TRIGLUCOCONJUGATED DERIVATIVE OF *MESO*-TETRAARYL-PORPHYRIN: A SINGLE DOSE STUDY IN THE RAT**

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The aim of our work is to establish, for the first time, the pharmacokinetic profile of a glucoconjugated porphyrin derivative, the 5,10,15-*meso*-tri-(*meta*-O-β-D-glucosyloxyphenyl)-20-phenyl porphyrin [abbreviated TPP(*m*-O-Glu)$_3$]. This new photosensitizer for photodynamic therapy (PDT) needed to be evaluated *in vivo*.

Pharmacokinetics of the TPP(*m*-O-Glu)$_3$ was determined after intravenous administration of single dose (0.25, 0.5 and 1 mg.kg$^{-1}$) to healthy rats and compared to Foscan® (0.3mg.kg$^{-1}$). Quantitation of both compounds in serum was performed using the same analytical protocol combining L-L extraction and high performance liquid chromatography (HPLC) with NIR sensitive fluorescence detection. The distribution of TPP(*m*-O-Glu)$_3$ towards blood elements was studied via the vacutainer CPT® tubes. Non-compartmental and compartmental pharmacokinetic parameters were estimated with the WinNonLin® software (version 1.1).

Pharmacokinetics parameters of TPP(*m*-O-Glu)$_3$ and Foscan® were derived from plasma concentration-time data using a two-compartment pharmacokinetic model. For the TPP(*m*-O-Glu)$_3$, its initial mean serum concentration ($C_0$) and the area under curve (AUC) were proportional to the dose (r = 0.996 and r = 0.999 respectively).

| Pharmacokinetic parameters | Foscan® (dose: 0.3 mg.kg$^{-1}$) | TPP(*m*-O-Glu)$_3$ (dose: 0.5 mg.kg$^{-1}$) |
|---|---|---|
| volume of distribution (ml) | 34.5 ± 27.90 | 27.8 ± 10.51 |
| terminal elimination half-life (h) | 17.4 ± 11.21 | 0.24 ± 0.06 |
| mean residence time (h) | 19.5. ± 12.39 | 6.1 ± 1.20 |
| total clearance (ml.h$^{-1}$) | 2.9 ± 1.70 | 4.6 ± 1.79 |

The TPP(*m*-O-Glu)$_3$ parameters are lower than those of Foscan® and not dose dependent. No metabolite of the glucoconjugated porphyrin was detected. Blood distribution experiments demonstrate that TPP(*m*-O-Glu)$_3$ did not bind to erythrocytes.

This first *in vivo* study of a glucoconjugated porphyrin shows a two-compartment pharmacokinetics with faster clearance from normal tissue and presumes less side effects than Foscan®.

# Singlet oxygen production and triplet state measurements of new metallo-porphyrins.

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Photodynamic therapy (PDT) is a treatment for several malignant and non-malignant diseases. The effectiveness of PDT depends on the photophysical properties of the dyes.

We report the results of singlet oxygen formation experiments by following its luminescence at 1.27 μm and transient triplet-state absorption experiments in different solvents for new metallo-porphyrins. The photosensitizers could be useful for photoinactivation of bacteria. The singlet oxygen measurements have been performed on a Spex 1680 with an IR detector. We used TPP (Tetra-phenyl porphyrin) as a reference photosensitizer ($^1O_2$ quantum yield of 0.55 in toluene). Porphyrins have been excited in the Soret Band (around 420 nm, according to the compound) at an optical density of approximately 0.2 in both chloroform and toluene in order to avoid aggregation and photo-bleaching. The transient triplet-state absorption experiments were performed in DMSO with an Infinity laser source and an OMA detector. The triplet state life times measurements were performed in air saturated solutions or in argon saturated solutions. The majority of the products showed singlet oxygen quantum yields around 0.5 in chloroform and toluene. For several compounds, we didn't observe any luminescence. The presence of palladium, ruthenium or tungsten didn't prevent the formation of satisfactory singlet oxygen quantum yields. Nevertheless, the presence of cadmium increases the singlet oxygen quantum yields. Aluminium and nickel prevented singlet oxygen formation. The triplet states lifetimes are reported to be in the order of microseconds and milliseconds in air saturated solutions and in argon saturated solutions, respectively.

The majority of the products showed singlet oxygen quantum yields at about 0.5 and the shorter triplet lifetimes obtained in air equilibrated solutions were, in general, consistent with a quenching by oxygen. These preliminary experiments represent a first simple step of selection for effective photoantimicrobial agents .

# PHOTOSENTITIZATION OF *ACANTHAMOEBA PALESTINENSIS* TROPHOZOITES AND CYSTS

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Phthalocyanines have been shown to act as efficient photoinactivating agents against different types of microbial pathogens (Soncin *et al*., *Photochem. Photobiol. Sc.* 1:815-819, 2002). Recent findings (Kassab *et al., Photochem. Photobiol. Sc.* 2:668-672, 2003) suggest that photoactivated phthalocyanines are also toxic against protozoa, thus opening new avenues for the application of photosensitized processes in the medical and environmental fields.

In order to explore this possibility more in detail, we undertook a thorough investigation on the photosensitivity of a specific protozoa, namely *Acanthamoeba palestinensis*, which is known to be responsible for important human diseases, including corneal ulcerations. In particular, we used a tetracationic phthalocyanine (RLP068) which was prepared by chemical synthesis in the laboratories of Molteni Farmaceutici (Florence, Italy). In a typical experiment, the *A. palestinensis* cells, in the vegetative and in the logarithmic phase of growth ($10^5$ cells/ml), were incubated at 30° C for 1 h with RLP068 concentrations in the 0.01- 2 μM range. After two washings with PBS, the cells were exposed to 600-700 nm light (50 mW/cm$^2$) for 10 min. and their survival was subsequently measured at 24 h after irradiation by the eosin exclusion test. The degree of photoinduced cell killing appeared to be dependent on the phthalocyanine concentration in the incubation medium: thus about 50% lethality was observed upon irradiation with 0.1 μM RLP068, while an essentially 100% drop in cell survival was induced by irradiation with 1.0 μM phthalocyanine. Under these experimental conditions, no effect on cell survival was observed after dark incubation with phthalocyanine, as well as by irradiation in the absence of the photosensitizer.

At present, we are extending our investigations to *A. palestinensis* cysts in order to assess both their affinity for RLP068 and their degree of sensitivity to red light irradiation, as determined by their extent of excystment as a function of phthalocyanine concentration, fluence-rate and total light fluence. The challenge of this segment of our studies is represented by the potential protective action carried out by the presence of two walls in the cysts.

# APPLICATION OF BNCT AND PDT IN TUMOUR THERAPY USING A BORON PHTHALOCYANINE AS A PHOTO- AND RADIO-THERAPEUTIC AGENT

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BNCT and PDT are different modalities to treat a tumour lesion and it would be interesting to combine these two therapies using a single compound. Boronated porphyrins and phthalocyanines can act as both a Boron carrier to malignant tissues and photo/radio-sensitizers. For this reason a tetrasubstituted Boron-phthalocyanine ($B_4Pc$) bearing 40 boron atoms per molecule has been synthesized and its photosensitizing activity has been initially studied on melanotic melanoma murine cells (B16F1). The cell uptake of $B_4Pc$ steadily increased from 5 min to 6 h incubation followed by a plateau corresponding with 0.6 nmoles of $B_4Pc$ per g of cell proteins. The cell-photosensitizing efficiency experiments were performed as a function of irradiation time (1-15 min) and concentration (7-28 μM) after 24 h incubation. The survival decreased by 95 % after 15 min irradiation (600-700 nm, 50 mW/cm$^2$). Fluorecence microscopy analysis of $B_4Pc$-loaded cells showed that the phthalocyanine was colocalised with an endosomal probe. The cell death mechanism was investigated by CPP32 caspase test under irradiation conditions causing extensive cell death (7 μM concentration, 24 h incubation, 15 min irradiation) and it resulted that the Pc-sensitized photoprocess causes apoptosis. *In vivo* studies were performed on C57BL\6 mice bearing melanotic B16F1 tumour. Recovery at 24 h after 3 mg/kg $B_4Pc$ i.v. injection gave maximal accumulation in the tumour (1.5 nmoles of $B_4Pc$ per g of tissue) and selectivity of Pc-photolocalization with a ratio 4:1 between tumour and skin. PDT experiments on mice were performed with a light source at 670 nm (250 J/cm$^2$) at different time (3-24-48 h) after 6 mg/kg $B_4Pc$ i.v. injection and, in mice irradiated after 3 h from administration, we observed a slower tumour growth rate than in control mice. We started also BNCT studies *in vitro* and *in vivo*. Mice bearing melanotic B16F1 tumour, irradiated 20-30 min with thermal neutrons at 24 h after 6 mg/kg i.v. injection, showed some degree of slowing down in tumour growth rate. B16F1 cells, incubated for 24 h with 7 μM $B_4Pc$ or not incubated, were irradiated with thermal neutrons for 20-30 min. Cell death was observed for cells irradiated both in the presence and in the absence of $B_4Pc$ and this suggests that one must improve irradiation conditions to be sure that the effect are only due to thermal neutrons.

# PHOTODYNAMIC THERAPY: A POSSIBLE ALTERNATIVE IN THE TREATMENT OF RETINOBLASTOMA

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**Introduction**: Retinoblastoma (RB) is the most common malignant intraocular tumour in children. Recently, synergistic tumour hyperthermia and carboplatin chemotherapy have successfully been used to cure retinoblastoma. But the short and long-term risks still remain for chemotherapy with carboplatin for those patients with constitutional anomalies of the RB1 gene. In this context, photodynamic therapy (PDT) with the administration of a sensitizer devoid of mutagenic properties could appear as a possible conservative approach. Glycoconjugated porphyrins bearing α-, β-O-galactosyl or α-O-mannosyl linked by a diethylene glycol spacer and their S-analogous have been synthesized with the aim to obtain efficient sensitizers with specific affinity for human retinoblastoma cell line Y79.

**Results** *In vitro*: Y79 drug uptake was determined by flow cytometry and extraction. Cells preincubation with the corresponding glycosylated albumin inhibits cellular uptake by 40% suggesting that mannosylated and galactosylated drug uptake is mediated by sugar moieties. A high phototoxicity (MTT assay after incubation and light exposure (514 nm light, 1J/cm$^2$)) was determined ($LD_{50}$ of 0.35 and 0.05 μM for the α-mannosyloxy and α-galactosyloxy respectively).

*In vivo*: One model of human retinoblastoma, derived from surgical specimens, xenografted into nude mice was used (Rb-102-FER). Injected mice received Foscan® as a reference{0.3 mg/kg (i.p.)} and α-mannosylated porphyrin (0.6 mg/Kg). The drug concentration at the tumour site, that has been previously shown to be related to the fluorescence drug intensity, was measured by optical fiber fluorimetry. It was found to increase up to 24 hours after injection remaining constant for up to three days in both cases. Light treatment (514 nm, 75 J/cm$^2$) was performed 24h after injection. In a first experiment PDT treatment efficiency was evaluated after Foscan® injection. The irradiation results in a significant regression suggesting that PDT may represent a new therapeutic approach for the conservative treatment of retinoblastoma.

# PHOTODYNAMIC THERAPY: OUR EXPERIENCE FROM 2001 TO 2003

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Photodynamic therapy (PDT) with topical δ-aminolaevulinic acid (ALA) and visible light has been successfully employed in the treatment of non-melanoma skin tumours, and pre-malignant conditions such as actinic keratoses. Recalcitrant hand and foot warts, i.e. not responsive to conventional therapies such as cryotherapy and keratolytic agents, have also been shown to respond to ALA-PDT.

In this study we report our experience of three years in the treatment of actinic keratoses (Aks), superficial and nodular basal cell carcinomas (BCCs), pigmented BCCs and recalcitrant plantar warts. 20% ALA emulsion was applied under occlusive dressing for 3 - 5 hours and the skin was then irradiated with 630 nm light ( Foto EC - PL 300 - Loto ). The total irradiative dose, per session, was 50 $J/cm^2$. Treatments were repeated weekly, until complete clinical disappearance of the lesion. ALA-PDT was interrupted in case of partial response if, after 3 additional treatments, no further improvement was observed. In order to enhance the ALA penetration, plantar warts have been treated with keratolytic ointment kept in place by a sticking-plaster for 7 consecutive days, followed by superficial curettage, before ALA-PDT. The final response rates are as follows: 100% Aks, 84% superficial BCCs, 60% nodular BCCs, 37.5% pigmented BCCs, plantar warts 75%.

**PHOTOFRIN MEDIATED PHOTODYNAMYC THERAPY IN LUNG CARCINOMA CELLS: MOLECULAR EFFECT ON BCL-2 ANTIAPOPTOTIC PROTEIN.**

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Photodynamic therapy is a novel procedure in cancer therapy, nowadays chiefly utilized for palliation in end-stage forms.

This treatment modality involves systemic administration of a photosensitizing agent, with selective localization in target tissues and successive lesion irradiation by suitable (wavelenght and intensity) source. Light exposure of the photosensitizer determines ROS formation that are responsible of the final cell damage. Several categories of photosensitizer have been used in PDT study, including both natural or synthetic dyes, drugs, and endogenous porphyrins. Photofrin is the most widely used drug for PDT in laboratories and clinics.

Experimental evidencies have shown that parameters such as drug dose, time and intensity of exposure and photosensitizer intracellular localization cause different biological effects. These events can therefore trigger several molecular pathways that may result in cell death including necrosis or apoptosis. The specific fate, indeed, seems to be related to the drug dose and/or light fluence.

Indeed, recent results recognizes, as important PDT target, proteins implied in cell death or surviving. In particular, it appears that $Bcl_2$ is a target of PDT since both degradation and phosphorylation have been observed following photosensitization of unrelated photo-sensitizing agents (phtalocyanines, ICG, hypericin). To date these observations have not found complete molecular explanation, although they may asume particular importance in view of the currente and future PDT use in humans.

The experimental model used in this study is represented by a) "non-small-cell lung carcinoma (H1299); b) H1299 transfectant clones overexpressing Bcl2 protein (1B8); c) six deletion mutants overexpressing a Bcl2 protein harboring a signal peptide that convey it to mithocondria (clones MAOB.15; MAOB.17), endoplasmic reticulum (clones Cb5.16; Cb5.21) and cytosol(clones ΔTM 5; ΔTM 24).

Protein expression was analyzed by Western blots of cell lysates obtained from the mentioned cells type 1 and 5 hours after irradiation with a high power lamp equipped wit a 630 nm filter at a fluence 0,54-1,08 J/cm$^2$). When parental cells were treated with PDT Bcl-2 appeared as a doublet implying possible posttranslational modification of the protein. It is widely known that photosensitizer intracellular localization depends on chemical structure of the drug, time of exposure and cell type. In this respect it was analyzed expression of targeted Bcl-2 protein to assess a possible preferential site of photodamage in the cell.

It is interesting to notice that using PDT, with a fluence of 0,54 J/cm$^2$, exogenous Bcl-2 protein, wherever targeted, i.e. mitochondrion, ER or cytosol remained definitely unmodified. The three conveyable Bcl-2 proteins share the same deletion in the transmembran domain suggesting some implication of this region in protein modification. To further assess whether the only the wild protein was available to modifications, we obtained another H1299 clone(1B8) overexpressing the wild type protein, Bcl2: Upon PDT, this protein resulted modified as the parental cell line. The nature of this posttraslational modification and its possible biological meaning is part of our current investigation.