MOLECULAR AND CELLULAR TARGETS IN PHOTOBIOLOGY

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With the development both of biochemistry, molecular biology, genomics, and of photochemistry and photophysics, the studies of how photobiological events take place, have been widely and deeply developed, and the role of molecular as well as cellular targets involved, is a strategic point of the research. In my lecture I shall deal with two different classes of photobiologically active compounds, chosen as example. The first class of compounds includes acridizinium derivatives, which show when irradiated with UVA light, an efficient ability to cleave DNA. These compounds, also called photonucleases exhibit a large potential for therapeutical applications or may also be used to mark, to probe or to characterize the nucleic-acid structure, because they are often inert until activated by light and permit to control the reaction both in a spatial and temporal sense. The second group of compounds concerns psoralens. These are naturally occurring or synthetic compounds, well known in popular medicine for the treatment of vitiligo and for inducing phytophotodermatitis, and in clinics for PUVA therapy. Cell membrane represents the first target for psoralens; these compounds for penetrating inside the cytoplasm physically interact with components of cell membrane, in which the most important targets are phospholipids. Psoralens intercalate inside the bilayer formed by the unsaturated fatty acids of phospholipids and when are photoactivated they give a cycloaddition reaction with olefinic part of the fatty acids forming a cyloadduct. This photoadduct is released from the modified phospholipid, by phospholipase A_2 hydrolysis., and plays the role of second messenger like DAG (diacylglycerol) stimulating PKC. This in turn activates tyrosinase leading to increased synthesis of melanin. The best-known target for psoralens is represented by nucleic acids and in particular by DNA, to which psoralens photoinduce mono and bifunctional lesions. It is commonly accepted that the DNA-psoralen photodamage is responsible of the cell death, and genotoxicity. Recently with the aims to verify whether other targets may be involved in cell death, we have studied the possible role of mitochondria in terms of psoralen-induced apoptotic cell death. Recent studies carried out by our research group have pointed out that apoptosis by far was prevailing over necrosis and involved mitochondrial dysfunction in different cellular lines (lymphocytes and keratinocytes) that lead to caspases activation. Moreover the photoinduced cell death is preceded by a cell cycle arrest in the G2/M phase. Herein, these new photobiological features of psoralens will be presented and discussed.

CELLULAR RESPONSE TO SOLAR UV-INDUCED DNA DAMAGE

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Exposure to solar ultraviolet radiation is a major risk factor in the development of skin cancer. Bipyrimidine photoproducts, and in particular cyclobutane pyrimidine dimers (CPDs), caused in DNA by UVB radiation are presumed to constitute a primary event in the initiation of skin tumours. Those DNA lesions are repaired by nucleotide excision repair. When unremoved from the genome, they lead to mutations that principally arise as a consequence of DNA synthesis past bipyrimidine photoproducts by specialized polymerases (involved in translesion synthesis). Indeed, in mutated p53 in skin tumours, two thirds of the mutations are C to T substitutions and about 10% are tandem CC to TT changes. The relative contribution of UVA radiation in skin cancer is still unknown. However, at UVA doses compatible with human exposure, CPDs, mainly at TT sites, are the major DNA damage produced, whereas 8-oxoguanine is three time less frequent in mammalian cells, including human keratinocytes. 8-oxoguanine may lead to GC to TA mutations, but is usually well removed by base excision repair. UVA radiation induce AT to CG and also GC to AT substitutions. In the meantime, mammalian cells respond to solar UV radiation by initiating a vast array of events in the nucleus, as well as at cell membrane and in the cytoplasm. Cellular response to DNA damage involves increased expression or activation of many early responsive genes, such as tumour-suppresor p53, Gadd45, c-fos, c-jun, NF-κB. Sensor proteins recognize DNA damage before or after processing, signal their presence to transducers, typically protein kinases, that amplify the signal by phosphorylating downstream target proteins. Typically, an activation within minutes of ATR by UVC/B –induced DNA damage is assumed, which phosphorylates p53 or Chk1 kinase, activating cell cycle checkpoints. Cell cycle arrest gives time to cell to repair their genome. If a cell is too heavily damaged, activated p53 will drive it to apoptosis. Recent studies have shown that apoptosis can also be initiated in UVC/B irradiated cells by non-nuclear events, such as Fas and TNF receptors clustering at the cell membrane, and activation by reactive oxygen species of MAP kinases (p38 and JNK) in the cytoplasm. The presence of multiple signaling pathways ensures either cell suvival, cell cycle arrest and DNA repair, or a timely elimination of heavy damaged cell (i.e. sunburn cells in skin which are apoptotic keratinocytes) to prevent proliferation of mutated clones and protect from cancer.

New insights into the photobiological effects of 8-methoxypsoralen (8-MOP) and UVA in eukaryotic cells using DNA microarray technology

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The response of eukaryotic cells to exposures to photosensitizing agents such as 8-MOP and UVA involves a cascade of events which determine the cytotoxic, mutagenic and carcinogenic responses. One of the crucial initial events is the induction of genes. To get a better understanding of these events we studied with genomic microarrays the transcriptional changes photoinduced in the 6300 genes of the yeast *Sacccharomyces cerevisiae* following exposures to 8-MOP plus UVA. This appeared to be of special interest since 8-MOP photoinduced oxidative damage as well as DNA monoadducts and interstrand cross-links are not only associated with genotoxic effects but also with greatly beneficial effects in phototherapeutic applications.

As shown before, the repair of 8-MOP photolesions implies the formation of DNA double-strands breaks (DSB) as intermediates, the activation of the homologous recombination pathway (involving the *RAD51* gene) and a signal transduction cascade (including the kinases Mec1/Rad53, equivalent to Atr in human cells).

DNA microarray analysis was performed after treatment with 8-MOP plus UVA in a haploid strain under well defined conditions controlling cell survival, growth delay, cell cycle stage and *RAD51* gene induction (as measured by Northern blot). Treatments leaving 25% survival induced 479 genes and repressed 475 genes during post-treatment incubation. Several genes of general cellular metabolism were affected. Many of them are known as genes responding to environmental stress such as thermal, oxidative, osmotic stress and amino acid starvation. Interestingly, also many DNA damage inducible genes like *RNR1*, *RNR2*, *RNR3*, *RNR4*, *DIN7*, *RAD16*, *RAD51*, *RAD54*, *SRS2*, *POL1*, *DUN1* were induced by 8-MOP plus UVA. Some of them were already recognized in the literature as specifically DNA damage responsive genes (see Gasch A.P. et al. *Mol.Biol.Cell* 2001;12: 2987-3003).

Our results suggest that 8-MOP plus UVA treatments induce specific transcriptional profiles showing a general signature of DNA damage but also reflecting the specific types of photodamage induced. Thus, responses of eukaryotic cells to 8-MOP plus UVA involve a specific network of genes conditioning its photobiological effects.

DISTRIBUTION OF UVB- AND UVA-INDUCED DNA DAMAGE IN HUMAN AND RODENT CULTURED CELLS

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The mutagenic and carcinogenic properties of solar ultraviolet radiation (UV) are associated with the induction of DNA damage. In the UVB range, the photoproducts are mostly cyclobutane dimers and (6-4) adducts between adjacent pyrimidine bases. Using a HPLC-tandem mass spectrometry assay, we have determined the distribution of UVB-induced dimeric photoproducts in CHO cells and in cultured normal skin fibroblasts and keratinocytes. In all cell types, the nature of the two pyrimidine bases involved in the dimerization has a drastic effect on the photochemical reactions. Indeed, CT and CC sites were found to be 10 times less photoreactive than TT and TC doublets. A drastic effect on the type of photoproducts was also observed. For instance, the ratio between the yield of cyclobutane dimer and that of (6-4) photoproduct was around 10 for TT while it was close to 1 for TC. Interestingly, exposure of cells to biologically relevant doses of UVB did not generate Dewar valence isomers of the (6-4) photoproducts in detectable amounts. The distribution of photoproducts is actually similar to that observed upon irradiation of isolated DNA, suggesting that the photochemistry of DNA does not depend on the cellular context, at least qualitatively. UVA radiation also exhibits mutagenic properties, although to a much lower extent than UVB. The underlying DNA damaging processes are not fully elucidated; although photosensitized oxidation is widely accepted as the major pathway. However, we have shown that TT cyclobutane dimers were produced in much larger yield than strand breaks and oxidized bases in UVA-irradiated CHO cells. Accordingly, a much higher frequency of TT cyclobutane dimers with respect to 8-oxo-7,8-dihydroguanine was observed in normal human skin cells exposed to UVA. Interestingly, the formation of (6-4) photoproducts was not detected using the sensitive HPLC-MS/MS method. This observation rules out a direct excitation of DNA bases and suggests a triplet energy transfer mechanism from a yet unidentified endogenous chromophore. In addition, UVA radiation was found to convert the (6-4) photoproducts into their Dewar valence isomers upon either combined or successive exposure of rodent and human cells to UVB and UVA radiations.

TRIPLET STATES AND IONIZATION OF DNA OLIGOMERS

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In our laboratory we have undertaken a systematic study of the photophysical properties of nucleic acids using optical spectroscopy from the femtosecond to the millisecond timescale as well as modelling of the excited states [1-4].

In the present communication we address the question of the formation of triplet states and ionization of the DNA oligomers $(dA)_{20}$, $(dT)_{20}$, $(dAdT)_{10}.(dAdT)_{10}$ and $(dA)_{20}.(dT)_{20}$. They are studied at room temperature by transient absorption spectroscopy, using 266 nm nanosecond laser pulses. The transient spectra obtained for the single and double helices are compared with those of the nucleotides, TMP and dAMP. We have found that the absorption band corresponding to the thymine triplet state is found for $(dT)_{20}$ but it is absent from the transient spectra of both double helices. The thymine triplet concentration in the single stranded oligomer is lower by an order of magnitude than that found for TMP under identical experimental conditions. The adenine triplet is not detected for $(dA)_{20}$. Neither thymine nor adenine triplet absorption is detected for $(dAdT)_{10}.(dAdT)_{10}$ and $(dA)_{20}.(dT)_{20}$. All the examined oligomers, both single and double stranded, undergo ionization at photon densities lower than that of the monomers. The reaction of the hydrated electrons with the adenine becomes less efficient when going from the nucleotides to the double strands. For $(dAdT)_{10}.(dAdT)_{10}$ it is shown that ionization takes place via a biphotonic process; the positive charge is located on the adenine moieties. In the case of $(dA)_{20}.(dT)_{20}$, the transient spectra are dominated by the absorption of the <6-4> photoproduct.

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Oxidative and reductive quenching of trisbipyrazyl ruthenium complex by the Cu/Zn superoxide dismutase.

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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 $Ru(bpz)_3^{2+}$. It was clearly shown that this augmentative effect was not due to the involvement of superoxide anion and OH°. To gain further mechanistic information, sequencing experiments were performed. 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 the electron transfer was proposed. 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. The fluorescence quenching of $Ru(bpz)_3^{2+}$ by the Cu/Zn SOD allowed us to propose the involvement of an electron transfer between the protein and the ruthenium complex at the excited state. Moreover, low temperature EPR spectroscopy analysis of $Ru(bpz)_3^{2+}$, performed in the presence of SOD and under irradiation, revealed the formation of $Ru(bpz)_3^{1+}$. This last result points out the participation of a second electron transfer between the photosentizer at the excited state and the metalloprotein.

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 oxidized by SOD to generate Ru^{3+}. Subsequently, Ru^{3+} oxidizes the guanine. In a second pathway, $Ru(bpz)_3^{2+}$ at the excited state can also be reduced by SOD to generate Ru^{1+}. The reactivity of this reduced species is under investigation. The catalytic copper site of the SOD and tryptophan residues 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.

MUSCLE OXIMETRY AND FUNCTIONAL BRAIN MAPPING BY TIME-RESOLVED NEAR INFRARED SPECTROSCOPY

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A novel multi-channel time-resolved instrument for muscle oximetry and functional brain imaging was developed and applied. In a first set-up 2 sources and 8 collection channels were available, while in a second one 9 sources and 12 collection channels were implemented. Both set-ups are based on picosecond diode lasers, fiber optics for light injection and delivery, a compact multianode photomultiplier tube and a PC board for time-correlated single photon counting. They were characterized in terms of reproducibility among sources and collection channels, linearity in the assessment of optical properties (absorption and reduced scattering), and stability. *In vivo* measurements were performed on volunteers to monitor: i) spatial changes in calf muscle oxygen saturation and total hemoglobin concentration during dynamic plantar flexion exercise[1] and ii) the optical response of the brain following either motor (finger opposition, 5 Hz) or cognitive task (word generation).

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LASER-ULTRAVIOLET-A INDUCED ULTRAWEAK PHOTON EMISSION IN MAMMALIAN CELLS

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In the field of the early diagnosis of cancer the possibility of detecting biophysical changes between carcinogenic and normal cells by means of non-invasive techniques has interested the work of many researchers world-wide.

The authors have developed the idea to evidence differences between different pathological conditions by measuring the delayed luminescence emitted from mammalian cells, that is the low-level photo-induced emission which lasts longer time after the excitation light has switched off. As a matter of fact previous measurements on unicellular algae have shown the close connection between the change in DL features and the biological state of the system and experimental results well accord a theoretical model which connect the phenomenon of DL to the presence of coherent autolocalised electron states (molecular solitons) formed in the low-dimensional macromolecules constituting the cytoskeleton. On the other hand the disorganization of the cytoskeleton structure is univocally related to cancer, so the idea was to evaluate such disorganization by means of DL measurements.

In order to measure the DL of mammalian cell cultures a new advanced research equipment for fast ultraweak luminescence analysis has been realized. This equipment is able to detect single photons after UVA laser irradiation (337 nm) and is characterized by a very low background signal, a high efficiency in collecting the luminescence emitted by cell cultures, and a low delay time between the end of the illumination pulse and the beginning of signal acquisition. Broad band (due to the low-level signal) interferential filters allowed to perform a spectral analysis in the visible range.

Cell suspensions of human fibroblasts and melanoma cells from culture collection were examined. Significant differences between the two types of samples have been observed both in the temporal decays, denoting a different distributions of the decay times, and in the relative contribution to the total emission of the different spectral components.

AUTOFLUORESCENCE SPECTROSCOPY OF LIVER METABOLIC CONDITIONS DURING EXPERIMENTAL TRANSPLANTATION PHASES

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Organ transplantation phases consist in: withdrawal from the donor, preservation, grafting in the recipient. Hypoxia and nutrient depletion occurring during preservation and grafting phases strongly affect the metabolic equilibrium, inducing tissue damages. The liver main functions are biosynthesis, catabolism and detoxification, which imply rather complex and intertwined biochemical processes involving various endogenous fluorophores -NAD(P)H, flavins, vitamin A, fatty acids, lipopigments, porphyrin derivatives-. Among these, NAD(P)H and flavins exhibit fluorescence properties which are strictly dependent on their redox state and, for NAD(P)H, on the bound/free conditions, in close relationship with the energetic metabolic reactions. Autofluorescence is thus potentially very useful to provide parameters to monitor the organ metabolism. Autofluorescence analysis for real-time monitoring of liver functionality was tested in isolated and perfused rat livers, purposely submitted to standard or "damaging" preservation conditions (cold storage with conventional or "poor" cold solutions), before rewarming (37°C)-reoxygenation, or in pig livers, submitted to transplantation from donor to recipient, a procedure similar to the clinical practice. Emission spectra were recorded with PMA-11 (Hamamatsu), through a single fiber-optic probe (exc. 366nm). Autofluorescence showed changes both in fluorescence intensity (FI) and in the relative contributions of single fluorophores, evaluated by means of spectral fitting analysis. FI rised by about 80% during cold ischaemia under all the conditions applied. In rat livers two parameters were defined: i) bound/free NAD(P)H, NAD(P)H/flavins ratios, for both preservation and restoration phases; ii) FI $t^{1/2}$ during rewarming/reoxygenation, which can be directly related to the damage of the liver tissue (ATP tissue content; LDH leakage, and lipid peroxidation, evaluated in the perfusate by parallel biochemical methods). In pig liver, FI decreased already during grafting, before blood reperfusion, proving to be temperature-sensitive. After venous and arterious blood reperfusion a further decrease occurred, with faster kinetic in the latter case. In conclusion, autofluorescence analysis confirms its potential for a direct, real time evaluation of strategies for improving organ preservation control. (Supported by MIUR-COFIN2001).

MULTISPECTRAL IMAGING AUTOFLUORESCENCE MICROSCOPY OF LEUCOCYTES FROM SEPTIC PATIENTS

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Sepsis may be defined as the systemic response to severe infection. The multiple organ dysfunction syndrome (MODS) is the final stage of the disorder and may result not from infection itself, but from a systemic inflammatory response syndrome (SIRS). This is a process characterized by an abnormal host response as a generalized activation to inflammatory reaction in organs remote from the initial insult.

The main purpose of our research is to find possible correlation between the course and evolution of sepsis and phenotypic alteration in leucocytes obtained from septic patients. Multispectral Imaging Autofluorescence Microscopy (MIAM) and Autofluorescence Micro-Spectroscopy (AMS) have been used in order to analyse autofluorescence properties of leucocytes taken from septic patients at different stages during the evolution of the disease.

Both emission spectrum and autofluorescence pattern of leukocytes from septic patients showed differences in comparison with the normal ones. Increased emission intensity, alterations of cell size, cell membrane and cytoskeleton organization were observed.

In conclusion the results suggest what follows:

a) Cell autofluorescence analysis represents a new approach to the study of leukocyte morphology and function in septic disease.
b) Autofluorescence monitoring on viable leucocytes from septic patients provides information on structural and metabolic changes occurring in cells during the evolution of the septic syndrome in patients under treatment.
c) Assayes based on the intrinsic fluorescence properties of leukocytes could be, in perspective, useful tools for distinguish low-risk from high-risk patients and detecting early, subclinical sepsis in high-risk patients.

EXTERNAL PHOTOPROTECTION: APPLICATION TO THE PREVENTION OF PHOTODERMATOSES AND UV- INDUCED SKIN CANCERS

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The incidence of melanoma and non melanoma skin cancers is rapidly increasing over the past decades. It is now well established that sun exposure is an identified predisposing factor. The role of UVB in skin cancers is well known by a direct genotoxicity mediated by cyclobutane type dimers between adjacent pyrimidine residues and p53 mutations. The role of UVA mediated by immunosuppression and free radicals has been under evaluated for a long time. Reactive oxygen species can be induced by UVA irradiation and are supposed to induce p53 mutations as UVB irradiation does. Therefore assessing DNA damage and oxygen reactive species in sunscreen protected areas could help to find biological endpoints to further evaluate sunscreen formulations for total protection against the whole spectrum of UV.

The role of sunscreens as skin protectors against cancers is still debated. The results of case control studies are controversial. Concerning non melanoma skin cancers, sunscreen use can reduce the rate of apparition of actinic keratosis

Sunscreens have been recently proposed to induce melanoma . The arguments in favour are a decrease of anti free radical defences (dibenzoyl methane),titanium oxyde can increase the free radicals production and alter the antigen presentation by langherans cells. On the other hand there are arguments for a protective role of sunscreens: the decrease of p53 mutations, a decrease of pyrimidin dimer production, a decrease of sunburn cells in the epidermis and the decrease of the UV induced immunosuppression.

Epidemiological arguments are also controversial The use of sunscreens can allow a prolonged sun exposure with out the erythemal signal and artificially suggest to patients that they are well protected and avoid other sun protective measures. Therefore in Australia epidemiological studies a have suggested that the large use of sunscreens by more than 70% of the population have decreased the incidence of melanoma

PROGRESS IN HARMONIZATION OF METHODS TO TEST THE EFFICACY OF SUNSCREENS: THE INTERNATIONAL SPF TEST METHOD 2003

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Ultraviolet (UV) radiation is an etiologic factor for the development of nonmelanoma skin cancers and also possibly melanoma. There are considerable data to suggest that protection from solar ultraviolet (UV) radiation will reduce the risk of acute and chronic skin damage in humans. The sun protection factor (SPF) numerical rating is an in vivo, standardized measure of sunscreen efficacy. Whereas the SPF provides an index of protection against erythemally effective solar UV, largely confined to the UVB (290-320 nm) and short-wavelength UVA (320-340 nm) region, there is currently no agreed-upon method to measure broad-spectrum protection against long-wavelength UVA (340-400 nm). The physical and biologic properties of UVA radiation are relevant as UVA contributes to photoimmunosuppression and photocarcinogenesis. An ideal sunscreen assessment system would address both UVB and UVA protection. The methods that are most widely used nowadays to determine SPF and UVA protection are briefly outlined and discussed. However, the SPF rating should continue to serve as the major determinant of sunscreen efficacy, as terrestrial sunlight damage to DNA is principally caused by the UV-B wavelengths. More than 90% of the lesions are cyclobutyl pyrimidine dimers or pyrimidine (6-4) pyrimidone photoproducts. These lesions are largely repaired by a process called *nucleotide excision repair* (NER) The use of topically applied DNA repair enzymes to prevent UVB-radiation induced damage seems to be a promising approach to increase the effectiveness of sunscreens. The development of antioxidants and active agents that can be used in combination with or in addition to UV filters to provide better photoprotection can represent an important outcome. It is very important that a single method is used in all countries to evaluate the efficacy of sunscreens. The first effort has been the publication of the "International Sun Protection Method" by the COLIPA Task Force on Sun Protection Measurement in 2003. This new method represents an improvement of the pre existing COLIPA European SPF Method (1994). The method is presented and discussed and the perspectives for a harmonization of SPF methods in all countries are outlined.

PHOTOAGING PREVENTION BY ORAL SUPPLEMENTATION : PHARMACO-CLINICAL STUDIES

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Sunlight ultraviolet radiation is involved in skin damages including sun burn, photoaging and photocarcinogenesis. The oral supplementation by "photoprotective preparation" (PP) could be a new way for photoprotection. Two clinical protocols, developed in order to evaluate an oral photoprotection preparation (PP), were supported by pharmacological studies. First, the effect of the oral PP was evaluated by systemic parameters analysis on healthy volunteers. The biological markers analyzed and the experimental conditions were validated *in vitro*. After oral treatment, the peripheral blood lymphocytes of volunteers were treated and analyzed. Results showed the antioxidant properties of the oral PP supplementation: it significantly decreased the UVA-induced-8-oxo-7,8-dihydro-2'-deoxyguanosine level and limited also the TNFα-induced activation of the NFκB transcription factor. As regards skin immunosuppression, we investigated the oral PP effect on monocyte-derived Langerhans cells generated from volunteers purified monocytes. We showed the immunoprotective properties of the oral PP that limited the UVA-induced inhibition of the CD86 co-stimulatory molecule. The purpose of the next approach was to evaluate the oral PP supplementation in a skin photoaging context. In this way, healthy volunteers were treated with the oral PP (12 weeks), their back were UVA-irradiated (10 weeks=200J/cm² total) and finally punch biopsies were obtained from UV-irradiated skin site, at the beginning of the study (T0), at the end of the oral treatment and the UVA irradiation (T3months) and, three months later (T6months). In order to analyze photoaging markers, like metalloproteinases (MMPs), mRNA were isolated from biopsies and analyzed by using real time RT-PCR technique. *In vitro* preliminary studies determined the specific UVA-induced MMPs to be analyzed on volunteers. We clearly showed that oral PP supplementation decreased the expression of the MMP-1 collagenase at "T3months" and at "T6months" and the MMP-2 gelatinase expression at "T3months" essentially. These results suggest the anti-aging property of the oral PP supplementation. By limiting the deleterious effect of UV and, in combination with UV filters, this oral supplementation could provide better systemic and cutaneous photoprotection relevant to prevent photocarcinogenesis and photoaging.

INTERNATIONAL PROPOSAL FOR UVA PROTECTION METHOD AND ITS LABELING

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The need for UVA protection is strongly recognized. Consequently there is a need to have a harmonized UVA test method to evaluate efficacy of sunscreen products and to inform the consumers about this efficacy.

Since the four last years different approaches have been proposed.

In USA, a conference was organized in 2000 by the AAD with international experts. After this meeting the AAD group proposed to combine in vitro (Critical Wavelength, λ_c) and in vivo (PPD or PFA) methods to best determine the level of UVA protection provided by sunscreens. This group suggested having proportionality between UVB and UVA protection.

At the same time, a group of manufacturers sent a petition to FDA to also support the same combination of methods and to propose a way of labeling. This proposal of labeling is based on the need to increase the UVA protection when increasing SPF thus to have a proportionality through the use of defined ratios of UVA to SPF protection.

More recently in France, health authorities have asked to photo-dermatologists, industry experts and consumers associations to work with them to define the sun protection rules. For the UVA protection this group proposed to determine the UVA protection factors by the in vivo PPD method (Japanese method. The labeling is under discussion using a proportionality of the UVB and UVA protection.

In Europe, the cosmetic industry (COLIPA) is presently working on an in vitro UVA method validated against the in vivo PPD method. For labeling it will be certainly proposed a calculation based on ratios and a labeling which should help consumers to select products.

BIOLOGICAL EFFECTS OF SUBERYTHEMAL UVA-IMPLICATION IN SKIN AGING

S. Seité

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There is now considerable evidence that UVA wavelengths (320-400nm) in sunlight may contribute to the clinical changes commonly observed in photodamaged skin. We examined the effects of repetitive suberythemal doses of UVA radiations on human skin in order to identify the epidermal and dermal changes indicative of early tissue injury. For these purpose, two areas of the back of fourteen female volunteers, phototype I to III, 20 to 40 years old, were exposed thrice weekly during 13 weeks, with increasing doses of UVA (330-440nm) for a cumulative dose of 1200J/cm^2. During the exposure period, biophysical and clinical changes were followed. After the last irradiation, a serie of epidermal and dermal parameters were analyzed and quantified on biopsied tissue sections.

This regimen of UVA exposure induces intense pigmentation with no erythema. Skin hydratation and elasticity decrease, whereas total skin thickness, assessed by echography, remains unchanged.

Irradiated epidermis reveals a significant thickening of the stratum corneum, an absence of hyperplasia and an increase in the expression of the protective iron-storage protein ferritin. No significant alteration are seen using antisera against type IV collagen or laminin, suggesting that the dermal epidermal junction (D.E.J) is mainly preserved. In the dermis, enhanced expression of tenascin is seen just below the D.E.J but type I procollagen, which is localized at the same site, is unaltered. Although we were unable to visualize any changes in elastic network organization using Luna staining or specific antiserum directed against human elastin, we notice an increased deposition of lysozyme or alpha-1 antitrypsin on elastin fibres.

These findings suggest that chronic suberythemogenic doses of UVA, resulted in morphological and histological skin alterations.

VIRUSES AND UV RADIATION

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Virus infections and UVR exposure are related mostly via immune suppression. In fact both Ultraviolet radiation A and B influence the immune system with alteration and depletion of Langerhans cells, inactivation of T helper 1 cells and activation of specific T suppressor cells and cytokine release (IL-10, TNF-α) from keratinocytes.

In addition, UVR may induce the transcription of HIV-1, stimulate HPV promoter activity and act as cofactor in cutaneous transformation. The progression towards malignancy depends on the oncogenic potential of the virus, but also on the local or general immune deficiency of the patient.

THE SONIC HEDGEHOG SIGNALING PATHWAY IN SKIN CANCERS FROM DNA REPAIR DEFICIENT XERODERMA PIGMENTOSUM PATIENTS

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The UV component of the solar spectrum can induce DNA lesions in skin cells which can result in the accumulation of genetic changes in genes involved in signaling pathways implicated in the control of cellular proliferation, differentiation and survival. Ultimately this can lead to the development of skin cancers amongst which the basal cell carcinomas (BCC) and squamous cell carcinomas (SCC), are the most common human cancers in Caucasians and increasing in incidence. BCCs are characterized by aberrant activation of Sonic-Hedgehog (SHH) signaling and mutations in the receptor of SHH, the patched gene, have been characterized in sporadic BCCs and in patients with the rare genetic syndrome nevoid BCC. To elucidate the role of UV in the deregulation of the SHH pathway, we have analyzed for alterations of the SHH signal transducers, *PATCHED* (*PTCH*), *SMOOTHENED* (*SMO)*, as well as the patched ligand, SHH, in BCCs and SCCs from UV hypersensitive xeroderma pigmentosum (XP) patients. XP, deficient in the repair of UV-induced DNA lesions and predisposed to cancers in sun-exposed skin, presents a unique model for studying photocarcinogenesis. In our study, higher levels UV-induced mutations, in particular the UV signature tandem mutations, CC->TT, of the patched tumor suppressor gene (73%), and the proto-oncogene, smoothened, (30%) are found in XP BCCs compared to sporadic BCCs. *SHH* mutations are rare and only one has been identified in 74 sporadic BCCs. We found 4 UV-specific *SHH* mutations in 33 XP BCCs. These missense *SHH* alterations are not activating mutations for its postulated proto-oncogene function, as the mutant SHH proteins do not show transforming activity and induce differentiation or stimulate proliferation to the same level as the wild-type protein. Modelisation studies show they are all located on one face of the compact SHH protein suggesting they may have altered affinity for different partners which may be important in altering other functions. Further, functional analysis of the *SHH* mutations found *in vivo* in XP BCCs will help shed light on the role of SHH in skin carcinogenesis. Finally no alterations of SHH, PTCH or SMO was found in XP SCCs, confirming the involvement of the SHH pathway specifically in the development of BCCs.

THE NEED FOR PHOTOSAFETY TESTING OF DRUGS

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Many systemic therapeutic agents photosensitize human skin to solar or artificial sources of UV radiation. The phototoxic potential of an a agent is often only noted during clinical trials late in product development: since withdrawal of an agent at this stage is extremely costly, there is increasing pressure to develop rapid and sensitive alternative methods to animal systems. An integrated approach using photostability data, phototoxicity and photomutagenicity tests has been used to evaluate the toxic reactions evoked by exposure of terfenadine and labetalol to UV radiations. Upon irradiation the molecules led to several photodegradation products. Cell damage was quantified with neutral red assay on 3T3 mouse fibroblast and a photo-irritancy factor (PIF) calculated. It is based on a comparison of two equally effective cytotoxic chemical concentrations (IC_{50}) obtained in experiments in the presence of 5000-161 mJ/cm^2 UVA-UVB and in absence of UV irradiation. PIF up to 5 is indicative of phototoxic potential. PIFs obtained with terfenadine and labetalol were <2. The photomutagenic potential was assessed using *S. typhimurium* TA102 strain. Both labetalol (up to 500 μg/plate) and terfenadine (up to 30 μg/plate) in absence of light are neither mutagenic nor toxic for the *Salmonella* strain. The product of the light-labetalol reaction did not induce mutations whereas terfenadine showed a clear mutagenity ($p<0.01$). The magnitude of the photomutagenic response was proportional to the amount of precursor compound concentration as well as the UVA-UVB dose (212-7 to 339-11 mJ/cm^2). The realization of this photogenotoxic potential established for terfenadine *in vitro* will have to be assessed under in vivo circumstances. This *in vitro* approach may be useful to predict in a screening-mode the photosensitivity potential of a new drug easily in product development and enable differentiation between those compounds to be excluded for future clinical use or those for which further clinical testing is appropriate.

DIC MICROSCOPY USED TO STUDY DNA-PROTEIN INTERACTIONS

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Differential Interference Contrast (DIC) microscopy has been used to perform a single molecule experiment, in which a DNA double helix interacts with the Lactose Repressor protein (LacR).

LacR modulates transcription of the E. Coli lactose operon. Its action is performed by loop formation in the DNA molecule, due to sequence specific recognition, with a dynamic equilibrium between the looped and unlooped states. LacR is representative of those class of proteins which modulate genetic expression by modifying the spatial configuration of double stranded DNA in prokaryotes.

The experimental setup consists of an inverted microscope (Nikon), coupled with a cw Nd:YAG laser (1064nm) focalized into the sample by a water immersion objective (N.A.=1.4) in a DIC configuration. Sample consists of a 60µm deep microchamber, comprised between two glass slides. A single DNA molecule (1Kbp) is anchored to the glass surface, while the other end is attached to a polystyrene bead (460nm diameter). Laser light is used to perform particle tracking on the bead, by measuring its position in the horizontal plane with a temporal resolution of some ms and a spatial resolution of some nm. After passing through the sample, laser light is collected by a couple of intensity photodiodes, whose signal is proportional to the bead displacement respect to the beam center.

DNA loop formation and breakdown induced by LacR modulates the mean amplitude of the bead’s Brownian motion, which is measured by the laser particle tracking technique in real time. This allows the measurement of the kinetics of DNA-protein interaction. By modulating laser power locally, it is possible to apply controlled forces on the bead, thus using the laser as an optical tweezers. The presented setup shows the possibility of a real time measurement of a molecular interaction at a single molecule level.

USING PHOTONS AND NEUTRONS TO PROBE THE STRUCTURE/FUNCTION/DYNAMICS OF MEMBRANE PROTEINS

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The three-dimensional structure of a protein is an essential step towards the understanding of its activity. However, it seems more and more clear that the enzyme function is intimately coupled to its internal dynamics. In particular, the energy landscape leading from a non-active biological state to an active one is governed by thermal fluctuations associated with localised or extended vibration modes of the protein. Incoherent quasi-elastic neutron scattering technique is especially well adapted to probe these events in the ps-ns time range. Since the cross section of hydrogen atoms are dominating this type of neutron diffraction, the observed fluctuations arise from all parts of the protein.

Data concerning the bacterial reaction center protein will be presented. This protein serves as a model for membrane redox proteins involved either in respiratory or photosynthetic chains.

Functional data of electron transfer kinetics, obtained by laser-induced absorbance changes will be presented in parallel with neutron diffraction data, as a function of temperature, in the wild type and in two mutant reaction center proteins.

Our results show:

1. that there is a clear correlation between the "open" or "softer" 3D structures (X-rays diffraction) of the mutants and their "flexibility" as measured by the mean square displacements of neutron diffraction.
2. Contrary to current hypothesis, there is no obvious correlation, at least in this protein, between its functioning (electron transfer data) and its thermal fluctuations as detected by its anharmonic movements above the dynamical transition temperature (neutron diffraction).

Probably, the relationship between functioning and dynamics is more subtle.

One of the challenges for the future is to probe for "dynamic signature" of enzymes as regards to their function and, in terms of Evolution, to their phylogeny.

PHOTOPERCEPTION IN THE MARINE ENVIRONMENT: MOLECULAR INSIGHTS FROM DIATOMS

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Marine diatoms are the most succesful group of photosynthetic eukaryotes in the oceans, and contribute close to one quarter of global primary productivity on Earth. As such, they are likely to possess sophisticated systems for optimizing their photosynthetic capacity under changing light conditions. We have characterized the expression of photosynthetic genes from marine diatoms and have found evidence for perceptory mechanisms for blue, green and red light. Whole genome sequencing of the centric diatom *Thalassiosira pseudonana* indeed revealed the presence of putative genes encoding cryptochromes and phytochromes. These photoreceptors are now being studied at the molecular and biochemical levels, and their function is being explored by reverse genetics approaches in the pennate diatom *Phaeodactylum tricornutum*. Furthermore, a putative cryptochrome-encoding gene (*CPF1*) has also been isolated from this diatom. We have found that cpf1 protein levels increase upon exposure to light and that both mRNA and protein levels are strongly regulated by a circadian rhythm, which results in alternative splicing of *CPF1* mRNA and increased protein levels during subjective days. Interestingly, we have also found that *P. tricornutum* cells are phototactic, moving towards blue light and away from red light. The photoreceptors involved in this process are currently being examined.

THE SPATIO-TEMPORAL CHANGES OF THE $InsP_3$-INDUCED Ca^{2+} RELEASE DURING STARFISH OOCYTE MATURATION

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Prior to the encounter with the sperm, the endoplasmic reticulum Ca^{2+}-releasing system (ER) that will generate a proper Ca^{2+} response in the egg must be prepared for the event. This occurs during the so-called maturation process of oocytes from different species. One prominent aspect of the process is the development of an increased sensitivity of the ER that is linked to its reorganization and that correlates with the generation of a normal sperm-induced Ca^{2+} response. These findings have been studied in starfish oocytes, in which the sensitivity of the Ca^{2+} stores to inositol 1,4,5-trisphosphate ($InsP_3$) increases in oocytes challenged with the maturing hormone 1-methyladenine (1-MA). The spatio-temporal changes of the $InsP_3$-induced Ca^{2+} release during starfish oocyte maturation has been investigated in oocytes co-injected with the Ca^{2+} dye Oregon Green 488 BAPTA-1 and $InsP_3$ caged to inhibit its activity prior to photoliberation (UV irradiation). The agonist was liberated by photoactivation at different times after the application of 1-MA. Following global $InsP_3$ photoliberation, the increased sensitivity of the Ca^{2+} stores to $InsP_3$ starts in the perinuclear area at the animal emisphere where the nucleus is located and propagates to the entire oocyte along the animal/vegetal axis. We have found that the change in the response to $InsP_3$ is not linked to the redistribution of the $InsP_3$ receptors or to the increase in their expression but to the modulation of their sensitivity to the ligand by the actin cytoskeleton.

A PROTEOMIC APPROACH TO THE INVESTIGATION OF THE EFFECTS OF UV-B IRRADIATION ON *FABREA SALINA*

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We investigated UV-B-induced perturbation on protein expression in the marine ciliate *Fabrea salina* by means of mono- and bi-dimensional electrophoresis techniques and mass spectrometry.

Specific irradiation and protein extraction protocols have been designed for this organism.

Mono-dimensional electrophoresis (SDS-PAGE) has been used to evaluate the protein content before and after irradiation, in order to have a first comparison between irradiated and non-irradiated samples.

The analysis of the SDS-PAGEs shows that irradiated samples have a larger protein content in the 66-30 KDa range that is proportional to the total UV-B dose received by the sample.

The 2D-PAGE gels also show differences between irradiated and non-irradiated samples: in particular, different over-expressed and neo-expressed UV-induced spots have been identified.

Mass spectrometry analysis, effectuated by MALDI-TOF technology, allowed the identification of the proteins implicated in the molecular light-induced pathway.

ULTRAFAST SPECTROSCOPY OF THE PHOTORECEPTOR FOR THE PHOTOPHOBIC RESPONSE OF *BLEPHARISMA JAPONICUM*

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Blepharisma japonicum is a ciliate protozoan displaying a strong step-up photophobic response to red light. The photoreceptor at the origin of this response has been identified to be a non-rhodopsin, hypericin-like chromophore (blepharismin) complexed to a 200-kDa non-soluble protein.

The present work deals with the primary phototransduction steps of *Blepharisma japonicum*'s light perception, as studied by subpicosecond transient absorption spectroscopy. First, the free chromophore of the light-adapted form of the cell (oxyblepharismin) was studied in various solvents and compared to known and new results on the parent molecule, hypericin. In a second step, we measured the primary excited-state reactivity of the corresponding pigment-protein complex, extracted by phosphate-concentration-step chromatography on a hydroxyapatite column.

Although showing very similar steady-state spectra, free oxyblepharismin and its protein complex have noticeably different excited-state behaviors. In particular, the protein complex exhibits a pronounced short-lived spectral feature in the 640-750 nm range, decaying biexponentially in 4 ps and 60 ps. Those decays, also observed in other spectral regions, are not found in the corresponding kinetics of the isolated pigment in solution. This early behavior of the protein complex might be the signature of the primary phototransduction process, possibly an electron transfer from the pigment to a neighboring protein acceptor residue as previous studies had suggested.

This work was done within the CNRS-CNR French-Italian Cooperation Program (2002-2003)

PHOTOSENSITIZING ACTION OF ZN(II)-PHTHALOCYANINE ON SMOOTH MUSCLE CELLS: FLUORESCENCE AND TRANSMISSION ELECTRON MICROSCOPY STUDIES.

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Experimental evidence has demonstrated that hydrophobic photosensitizers, such as porphyrins and phthalocyanines, are accumulated in significant concentrations for long periods of time by hyperproliferating cells. This is why photodynamic therapy (PDT) has recently been applied experimentally in prevention of hyperplasia and artery restenosis after percutaneous transluminal angioplasty (PTA).The long term success of PTA is limited by the occurrence of hyperplasia (IH), which is a complex pathological process characterized by an abnormal proliferation of smooth muscle cells (SMC) of the vascular wall. Proliferating SMC migrate to the subendothelial area and form an hyperplastic lesion, which causes stenosis and obstruction of the vascular lumen.

This study investigates the efficacy and mechanism of PDT effects on SMC. First of all, we performed cellular uptake studies as a function of Zn(II)-phthalocyanine (ZnPc) concentration (from 1 to 15 μM) and incubation time (from 15 min to 8 hr), and then intracellular localization studies using fluorescence microscopy. Moreover we investigated the cell photoinactivation as a function of irradiation time (600-700 nm, from 1 to 15 min at a fluence rate of 10 mW/cm^2) and the mechanisms involved in cell photoinactivation. In particular, we determined the expression of caspase-3 in photosensitized cells. Our data suggest that ZnPc is an efficient photosensitizer: it accumulates in the cells at a concentration as high as 0.8 nmoles/mg cell protein (8 hr incubation, 10 μM) and the SMC survival decreases by at least 80% after 3 min irradiation (1μM ZnPc). The photosensitizer localizes preferentially in the Golgi apparatus and probably induces cell death via apoptosis. At present, we are planning further studies, such as fluorescence and trasmission electron microscopy, in order to confirm the role of the apoptotic pathway.

MODULATION OF ADHESION MOLECULES EXPRESSION IN ENDOTHELIAL CELLS TREATED BY PHOTODYNAMIC THERAPY.

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Photodynamic therapy (PDT) is a treatment for cancer and several non cancer proliferating cells diseases. PDT depends on the uptake of a photosentizing compound by the diseased tissue to be treated followed by selective irradiation with visible light. In the presence of molecular oxygen, the irradiation process leads to the production of reactive oxygen species (ROS). A large production of ROS induces a direct cancer cells death by apoptosis or necrosis. By opposition, a small production of ROS is known to occur in the vessels associated with the tumour and activates various cellular pathways leading to gene transcription and an inflammatory response against the tumour. Here, we show that in human microvascular endothelial cells (HMEC-1), photosensitization by pyropheophorbide-a methyl ester (PPME) induced the activation of the nuclear factor kappa B (NF-κB). We have shown that NF-κB is active since it binds to the NF-κB sites of both ICAM-1 and VCAM-1 promoters. We have also shown that NF-κB is functional since we detected the transcription of several NF-κB target genes such as IL-6, ICAM-1 and VCAM-1. In contrast we were not able to detect the expression of ICAM-1 and VCAM-1 proteins in response to PDT in HMEC-1, although we measured an IL-6 secretion. Using specific chemical inhibitors we showed that this inhibition of ICAM-1 and VCAM-1 expression is the consequence of their degradation by the lysosomal proteases. The proteasome and calpain pathways were not involved in the degradation. The photodynamic induced oxidation of cellular membrane seems to be important in the degradation of both ICAM-1 and VCAM-1 since a treatment with BHA and alpha-tocopherol acetate induces a slight expression of the two adhesion proteins after PDT. Moreover, we showed that PPME photosensitization induces a transient dowregulation of several integrins and plasma membrane proteins that are constitutively expressed. The absence of expression of ICAM-1 and VCAM-1 at the surface of PDT treated endothelial cells although the corresponding genes are transcribed, revealed that PDT can efficiently disturb the correct addressing of proteins to their target sites.

INTRAPROTEIN ELECTRON TRANSFER AND PROTON DYNAMICS DURING PHOTOACTIVATION OF DNA PHOTOLYASE FROM *E.COLI*

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DNA photolyase is a 55 kDa soluble flavoprotein that serves in a variety of organisms to repair UV-induced lesions in DNA (for recent reviews, see [1, 2]). The main type of this lesions, a cyclobutan dimer of neighbouring pyrimidines, can be split by light induced electron transfer from the FAD cofactor of photolyase towards the damaged DNA. In order to perform this function, the FAD has to be doubly reduced ($FADH^-$), a state that can be obtained from the semireduced form FADH° (a radical that is stable in isolated photolyase) by a separate photoreaction, the so-called photoactivation. In *Escherichia coli* photolyase, excitation of FADH° induces an electron transfer over 15 Å from tryptophan W306 to the flavin. It has been suggested, that two additional tryptophans are involved in an electron transfer chain FADH° <= W382 <= W359 <= W306. This putative three-step electron transfer is completed in less than 10 ns. Subsequently, the W306 cation radical is deprotonated in about 300 ns, followed by either rereduction by external electron donors or, in their absence, charge recombination with the flavin anion $FADH^-$ on a millisecond timescale [3]. Using an approach combining site directed mutagenesis with ultrafast transient absorption spectroscopy and isotope exchange, and based on the X-ray structure resolved to 2.3 Å [4], we can obtain a quite detailed picture of the interplay of different factors governing the kinetics of electron transfer reactions during the photoactivation of *E. coli* photolyase.

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Photobiological properties of 5,10,15,20-tetrakis(*m*-hydroxyphenyl)chlorin (*m*-THPC) and 5,10,15,20-tetrakis(*m*-hydroxyphenyl)bacteriochlorin (*m*-THPBC) in cells.

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The aim of the present *in vitro* study was to investigate the photobiological properties of two parents photosensitizers, the *m*-THPC and *m*-THPBC, which are considered as some of the most active compound studied to date. Photobleaching kinetics and phototoxicity of *m*-THPC and *m*-THPBC in tumor culture cells were studied after irradiation with laser light at 652 nm and 739 nm, respectively. We also investigated phototoxicity of both dyes under the same experimental conditions (photosensitizers concentration and applied light fluences).

m-THPBC photobleaching derived from fluorescence measurements, follows a fast first order kinetics, unlike that of *m*-THPC which fits to the second order process. The initial rate of photobleaching of *m*-THPBC was about 4 time that of *m*-THPC under similar conditions. Phototoxicity assessed by clonogenic assay demonstrated that under the same experimental conditions *m*-THPBC is much less toxic than *m*-THPC (LD_{50}= 345 mJ/cm² and LD_{50}= 85 mJ/cm² respectively). However, a rather different situation was observed when phototoxicity was computed by taking into account the respective dyes photobleaching. Cell phototoxicity, plotted against the number of emitted photons of fluorescence for each photosensitizers, demonstrated a greater toxic effect in cells subjected to *m*-THPBC-PDT. The plausible explanation could be a different intra-cellular localization pattern of both sensitizers, as evidenced from fluorescence microscopy analyses. The fact that *m*-THPBC is quite photolabile and that the photobleaching kinetics follow a first order process may be an advantage for PDT, since the bleaching may limit the photodamage to normal tissues. Also, together with the advantage of a near-infrared activation (large volume of necrosis, opportunity to treat pigmented neoplasic tissues), a higher photoinactivation yield displayed by *m*-THPBC compared to *m*-THPC, illustrate the potential of using *m*-THPBC-PDT.

APOPTOSIS INDUCED BY mTHPC PHOTOSENSITIZATION IN MONOLAYER CULTURE OR SPHEROIDS

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The ability of PDT to generate apoptotic cells is acknowledged to be an important factor i the PDT treatment efficacy. mTHPC (meta-tetra(hydroxyphenyl)chlorin, Foscan®) is a secon generation photosensitizer which appears to be one of the most active sensitizers to date.

The present study addresses the contribution of mTHPC photosensitized (650 nm) apoptos in overall cell death in HT29 monolayer cells and tumour multicell spheroids.

Mitochondria events implicated in apoptotic and/or necrotic cell death were assessed i monolayer cells immediately, 4h and 24 h post-PDT. Immediately after mTHPC-PDT, parti cytochrome c release concomitantly with mitochondrial membrane depolarization wa observed, whereas by 24 h post-PDT the fluence dependency was strikingly similar for bot mitochondrial alterations and cell death. Apoptosis was measured through the activity c caspase-3 and the binding of the fluorescent conjugate Ca^{2+} dependent protein annexin-V o membrane externalized phosphatidylserine.

The maximum caspase-3 activation (12-fold more than control) was reached 24 hours afte irradiation at fluence inducing 90 % cell death (LD_{90}). Highest fluence applied resulted in th lowest level of caspase-3 activity, suggesting the inhibition of apoptotic process in favour c necrosis. The corresponding measurement of apoptotic cells (12 % of annexin-V bound cells confirmed the mild and delayed apoptotic response of HT29 cells to mTHPC-PDT. Multice tumour spheroids were irradiated at two fluence rates and different fluences. At 90 mW/cm^2, 1 J/cm^2, irradiation conditions consistent with the clinical use, no caspase-3 activity was detecte whereas at 10 mW/cm^2, significant caspase-3 activation was measured at the fluence inducin 65 % cell death. Consistent with the results obtained in monolayer cells, the highest fluenc applied inhibited caspase-3 activation.

In conclusion, we have demonstrated that mitochondria membrane damage is strongl implicated in an apoptotic and necrotic cell death mediated by mTHPC-PDT. Based on th results with multicell tumour spheroids, moderate fluences of irradiation together with lo fluence rate protocols favouring apoptotic cell death may be preferable in the clinical context.

New prospects in Photodynamic Therapy (PDT)

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Photodynamic therapy (PDT) has received increasing attention as a new treatment modality of tumours and other nonmalignant disorders. PDT is based on the interaction of a light-activated drug (photosensitizer) followed by visible light illumination of the pathological area. Upon photoactivation of the sensitizer, reactive oxygen species are produced, leading to irreversible destruction of the treated tissues. General considerations on both physical and biological processes will be introduced. In 1978, Dougherty reported the first clinical utilization of hematoporphyrin derivatives (HpD) in the PDT of human cancers. The purified version of HpD, Photofrin®, was first approved for PDT of bladder cancer in Canada in 1993. Since then, it has been approved all around the world for the treatment of lung, esophageal, bladde or gastric cancers. However, Photofrin® has an heterogeneous chemical composition, a poor tumoral selectivity and induces a long-term skin sensitivity. The design of new photosensitizers having well-defined stucture, great selectivity for tumour cells, high photobiological efficiency, fast elimination from healthy tissues and strong light absorption in the red region of the visible spectrum is thus an important challenge for the chemist community. A number of second generation photosensitizers have been developed and evaluated for their potential in PDT (porphyrins, chlorins, phthalocyanines, benzoporphyrin derivatives...).In this context, the search for optimized delivery systems also knows an important development. Aminolevulinic acid (ALA), as a prodrug for endogenous porphyrin has also been extensively examined for PDT. In Europe, several important milestones include the recent regulatory approval given in 2000 for Visudyne ®(BPD-MA) for the treatment of age-related degeneration (AMD) and for Foscan® (mTHPC, 2002) as an agent for palliative treatment of head and neck cancer. However, continued research is still necessary to develop new sensitizers with a high photoactivity and increased therapeutic index. Third generation photosensitizers are now being designed whereby the absorbing chromophore is linked to a targeting system to increase the potential in PDT. A survey of the strategies developed in the field of new sensitizers along with the most relevant clinical results will be given.

ANTIMICROBIAL PHOTODYNAMIC THERAPY: MECHANISTIC ASPECTS AND POTENTIAL PHOTOTHERAPEUTIC APPLICATIONS

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The recent discovery of photosensitising agents, which exhibit a fast and highly preferential interaction with a large variety of microbial cells, is paving the way for the development of a novel promising application of photodynamic therapy (PDT) in the treatment of microbial infections. In particular, selected Zn(II)-phthalocyanines bearing quaternarized ammonium peripheral substituents show a very fast (< 5 min.) uptake by a number of Gram-positive (e.g., *Staphylococcus aureus*) and Gram-negative (e.g., *Escherichia coli*) bacteria, as well as by yeast cells (e.g., *Candida albicans*) and parasites (e.g., *Acanthamoeba palestinensis*); the cationic phthalocyanines appear to undergo a fast binding with negatively charged groups at the surface of the outer wall surrounding most types of microbial cells. A proper control of the irradiation conditions (excitation wavelength, fluence rate, total light fluence) allows one to achieve an extensive (ca. 5 log) decrease in the survival of microbial pathogens even in the presence of phthalocyanine concentrations as low as 0.05 μM with no appreciable concurrent effects on human cells which are the main constituents of possible host tissues, such as fibroblasts and keratinocytes. All the available evidences indicate that (a) the photoinactivation of microbial cells is irreversible with no appearance of repair processes; (b) the plasma membrane represents the earliest and predominant site of cell damage, while no involvement of the genetic material can be detected; (c) as a consequence, the photoexcited phthalocyanines do not appear to promote the onset of mutagenic events; (d) the photoprocess is equally effective on both wild and antibiotic-resistant bacterial and fungal strains (e.g., meticillin-resistant *S. aureus,* or MRSA); (e) no apparent selection of PDT-resistant microbial strains takes place after repeated photosensitization cycles; (f) combination between PDT and antibiotic treatment is also possible. The PDT approach is particularly suited for the treatment of localized infections, thus avoiding any significant damage to the endogenous benign microbial flora. In this connection, the ZnPc selected for toxicological studies and subsequent clinical trials has been formulated for topical deposition in the infected lesion avoiding any appreciable diffusion into the general blood circulation which could lead to generalized photosensitivity. The PDT protocol has been successfully tested in dogs affected by spontaneous dermatitis caused by antibiotic-resistant Gram-positive bacteria: in all cases, curative results were obtained by using a mild irradiation protocol and no ZnPc leakage into the blood stream was detected.

HYPERICIN: A NOVEL DIAGNOSTIC TOOL FOR BLADDER CANCER DETECTION

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Invasive bladder cancer has a high mortality rate (< 50% 5 Year survival despite intensive surgery and radio-chemotherapy). The origin of invasive bladder cancer is flat carcinoma *in situ*, which cannot be detected by conventional diagnostic tools since it is not accompanies by architectural changes. Large multicenter studies have shown that the recurrence rate of bladder cancer is reduced by 60% when endoscopies are performed under blue light, seeking the red fluorescence of PpIX induced by previous intravesical instillation of ALA. Although the sensitivity of this technique is close to 100%, the specificity is very low with about 50% of false positive results. Hypericin, which is present in St John's wort (*Hypericum perforatum*) is known to induce hypericism (sunburns) in cattle. It is indeed a potent photosensitizer with a singlet oxygen yield of 0.73. It is also a very good fluorophore (λ_{max} 594 / 642). Those characteristics make it possible to use the same fluorescence detection equipment as the one used for PpIX detection. *In vivo* rat fluorescence spectroscopy studies have demonstrated that an intravesical instillation of a 30 µM solution of hypericin induces a tumor / normal ratio of 12. Human studies with an 8 µM solution for 2 hours show very selective fluorescence from bladder cancer, including flat carcinoma *in situ* with a sensitivity of 94% and a specificity of 95%. Besides avoiding false positive results, without loss in sensitivity) hypericin has other advantages as opposed to ALA or its hexyl derivative. Whereas the endoscopic procedure with ALA has to be very short due to photobleaching, almost no photobleaching of hypericin is observed. The extracellular efflux of PpIX is very fast (± 2 H) but hypericin fluorescence can still be observed 123 hours after instillation. Endoscopic images of clinical cases will be shown.

PHOTOSENSITIZING PROPERTIES OF FOSCAN® *IN VITRO* ET *IN VIVO*

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Photodynamic therapy efficacy (PDT) depends on three successive thresholds: photochemical threshold, which consists in the production of the reactive oxygen species, including singlet oxygen ($^1O_2$); biological threshold, which includes a death of the malignant cells directly via necrotic or apoptotic pathways and finally therapeutic threshold, which implies tumor eradication through different mechanisms, including microvascular injury and/or an antitumor activity of inflammatory cells.

Meta-tetra(hydroxyphenyl)chlorin (mTHPC, Foscan®) is a second-generation photosensitizer with a strong absorption in a far-red region (652 nm) that appears to be one of the most effective sensitizers in the clinical context.

The present study addresses the mechanism of photochemical, biological and therapeutic action of Foscan® in the models of cultured tumor cells and xenografted tumors.

We demonstrated that Foscan®-sensitized cell photoinactivation proceeds through $^1O_2$ mediated reactions. Considering very short lifetime of singlet oxygen, the primary sites of damage should be closely related to the specific sensitizer distribution. By using fluorescence microscopy techniques we further demonstrated that the endoplasmic reticulum and the Golgi apparatus are preferential sites of Foscan® accumulation. The same organelles are the primary photodamage sites. Delayed mitochondria membrane PDT-induced damage was also observed and was strongly implicated in apoptotic cell death.

Foscan® distribution between tumor and tumor vessels was monitored by confocal microscopy coupled to microspectroscopy. The short time intervals after Foscan injection (3h and 6h) were characterized by the predominant photosensitizer localization in tumor vessels, whereas with time progression (24h, 96h) we observed a gradual shift of Foscan from tumor cells located in a proximity to vessels to tumor areas remote from the vessels. This pattern of Foscan distribution together with the study of Foscan-based PDT efficacy provided an important indication for the mechanism of action of this photosensitizer.

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INTEREST OF CHLORINS FOR BRAIN TUMORS : *IN VITRO* AND *IN VIVO* STUDIES.

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The optimal treatment for high-grade gliomas remains undefined, and patient survival has little changed during the past 25 years, despite advances in surgery, chemotherapy and radiotherapy. As many as 95% of malignant gliomas are reported to regrow locally after excision and aggressive adjuvant treatment leading to an overall survied of less than one year.

New animal models are needed to investigate in vivo new therapies for this type of tumors.

In the first part of our study, we determined the graft procedure and the growth of tumor on sprague-Dawley rat brain, using C6-9 cells.

In the second part we investigated the usefulness of our model and evaluated photodynamic therapy using 2,3-dihydro-5,15-di(3,5-dihydroxyphenyl)porphyrin (SIM01), a new diphenylchlorin photosensitizer, compared to reference sensitizers, hematoporphyrin derivated (HPD) and m-THPC. We determined the photosensitizers pharmacokinetics with two methods, Optic Fiber Spectrofluorimetry and reactive oxygen species determination and then the effectiveness of photodynamic treatment by analysis of median survival time.

SIM01 maximum concentration was reached in the tumor only 12 hours after the administration, and the bratio tumor/normal brain was 2,3.

The median of the survival time observed with the SIM01 treatment was 20 days, whereas the one of untreated animals was 15 days.

With this study, we obtained an easily reproductive, simple and rapid model of glioma. SIM01 appears to be a very promising photosensitizer to treat glioma, compared to other substances.

THE NEW PORPHYRIN DERIVATIVE XF-73 IS AN EFFICIENT ANTIBACTERIAL PDT AGENT

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The increasing epidemic of infectious diseases caused by antibiotic-resistant bacteria represents a major health problem worldwide. Thus, the development of nonantibiotic modalities for killing bacteria is urgent for treating antibiotic resistant infections and limiting the development of additional antibiotic resistant strains. Photodynamic Therapy (PDT) with selected cationic photosensitizers appears to be a very promising approach for the treatment of localized microbial infections caused by both Gram-positive and Gram-negative bacteria. In our studies we evaluated the antibacterial activity of a new porphyrin derivative, namely XF-73, against the Gram-positive *S. aureus, S. epidermidis, S. pyogenes,* which are important pathogens of skin infections in man, and the Gram-negative *E. coli* and *P. aeruginosa*. The bacteria in the stationary phase of growth (10^8 cells/ml), were incubated with various XF-73 concentrations (0.001-5 µM). Afterward, the cells were washed, exposed to blue light (13.7 J/cm^2) and their survival was measured by the CFU assay. The presence of both XF-73 and light caused an exceptional reduction of cell viability for both Gram positive and Gram negative bacteria. XF-73 at 0.005 µM induced 6 log reduction of survival of *S. epidermidis* and caused sterilization of the culture at 0.01 µM. Slightly higher XF-73 concentrations were necessary for obtaining similar results with *S. aureus* and *S. pyogenes* . In any case, 0.01 µM XF-73 induced 5 log reduction of survival of *S. aureus* while 0.1 µM induced 6 log reduction of survival of *S. pyogenes.* As expected, Gram-negative bacteria were less sensitive than Gram-positive to the PDT treatment with XF-73. However, complete killing of *E. coli* and *P. aeruginosa* cells could be obtained with 5 µM XF-73. The potential development of resistance to PDT was investigated using *S. aureus* cells submitted to repeated PDT treatments. After 10 repeated PDT sessions, the cells were as sensitive as those which did receive only one treatment suggesting no development of resistance to PDT.

Based on our observations, it appears that XF-73 is characterized by a very high antibacterial activity and represents a potential useful PDT agent for the treatment of superficial and localised bacteria infections.

STRUCTURE – ACTIVITY RELATIONSHIPS OF A SERIES OF METALLO-NAPHTHALOCYANINES IN THE PHOTOTHERMAL THERAPY OF TUMOURS

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The photothermal sensitization can be promoted by both endogenous (melanin, haemoglobin) and exogenous (metallo-porphyrinoids, cyanines, azo dyes) sensitizers when they are irradiated with laser sources, operated in a pulsed regime at high fluence rates. The decay of the initially excited photosensitizer to its ground state has the end result of the transduction of the photon energy to heat in the matrix around the chromophore with the generation of an acoustic shock wave causing extensive mechanical and chemical damage. An efficient photothermal sensitizer should thus be characterized by a very short lifetime (ps) of its electronically excited states and high photostability.

We studied the photothermal activity of different metallo-naphthalocyanines in an amelanotic melanoma cell line (B78H1).

A very high photothermal efficacy was observed for 5.1 μM Pd and $Ni(OBu)_8Nc$, which caused an about 80–85 % decrease in cell survival after 20 min irradiation with a pulsed Ti:Sapphire laser (30 ns pulses, 10 Hz, 200 mJ/pulse), whereas with other derivatives such as Zn and Rh – samples, very little cell mortality was observed. The efficiency of photothermal cell sensitization appeared to be affected by the sub-cellular distribution of the photosensitizer and its aggregation state. Most active naphthalocyanines are localized in specific subcellular districts, at least for $Ni(OBu)_8Nc$. Similarly aggregated $Ni(OBu)_8Nc$ appears to be more active than its monomeric counterpart. However, different metallo-naphthalocyanines appear to give different patterns of cell response.

Moreover photothermal sensitization with naphthalocyanines appears to open new perspectives for therapeutic applications , as suggested by preliminary *in vivo* studies with C57/BL6 mice bearing a subcutaneously implanted amelanotic melanoma treated with NiNc at different light doses.

Development of a method to analyze monolayer cells by MALDI-TOFMS - Applications for Photodynamic therapy -

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Among different treatments of cancer, Photodynamic Therapy is an original, recent and efficient modality to reduce, even to destroy tumors. These studies consist to contribute to elucidation of PDT mechanisms by working with colo HT29 cells by Mass Spectrometry. Unfortunately, indium tin oxide coated is maybe inadequate for culture of monolayer cells. So, a glass slide was used as holder, in order to follow the *in situ* photobleaching of foscan® (*m*-THPC, temoporfin), a photosensitizer used in PDT. The present work presents a method to control photosensitizer behavior in situ by MALDI-MS, with the possibility to realize the analyses with different laser wavelengths.

Human colon adenocarcinoma cells were deposed on a slide into multidishes to be maintained in RPMI 1640 medium supplemented with proteins and antibiotics. At the time of logarithmically growing cells, these ones were put into contact with photosensitizer during 3h30. Then, they were illuminated by laser diode (λ=652nm). For MALDI experiments, 1µL of matrix were applied on cells and finally, selltaped to stainless steel MALDI target to be investigated with a Bruker Reflex IV mass spectrometer.

Methylen blue was used to localize these cells and to control the good adhesion of cells on support. Cells were finally impregnated by *m*-THPC at three concentrations (5, 10 and 20 µg/mL). For each experimental condition, *m*-THPC protonated ion at m/z 681 was detected. Classical matrices α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitril/ethanol (50/50, 0.1% TFA) solution and sinapinic acid (SA) in acetonitril/water (70/30, 0.1% TFA) solution were employed and the calibration of sample was achieved thanks matrix ions and external references. In order to verify that detected *m*-THPC corresponded to cellular *m*-THPC, the solutions of washing were analyzed by MALDI-TOFMS. It appears that two washing of PBS would seem necessary. Lastly, cells containing *m*-THPC, were illumined by Laser diode (652 nm) with four fluences (1, 5, 10 and 20 $J \cdot cm^{-2}$). These experiments show that the most important the signal fluence is the weaker the m-THPC signal. We noticed that proteins had an influence on the photobleaching of m-THPC. Nevertheless, the results concerning photoproducts will be developed during this communication. Moreover, two other cellular types (MCF 7 and fibroblasts) were also analyzed with this protocol and their proteinic finger printings were characterized.

COMBINED EFFECTS OF PHOTODYNAMIC THERAPY AND CHEMOTHERAPY IN HUMAN LUNG TUMOR CELLS: PHOTOFRIN AND CIS-PLATIN OR GEMCITABINE.

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The antineoplastic current therapies involve association of different drugs that have a cytotoxic action or the utilization of mixed strategies (i.e., surgery, radiotherapy) and antiblastic drugs. The latter very often give rise to side effects, that significantly reduce the quality of neoplastic patience's life. The possibility of recognizing new approaches that simultaneously maintain the therapeutic effectiveness while reducing the negative side effects, is certainly a very important result. To this purpose, we have associated the photodynamic therapy (PDT) to chemotherapy. PDT is a bimodal therapy having two components (a photosensitizer that is potentially concentrated in tumour tissue and light - irradiation) that individually are non-toxic, but tumoricidal in combination. This combination causes a photoactivation leading to an oxidative cellular damage (generation of reactive oxygen species). In this regard, we have estimated the effects caused either from single treatments (*PDT or chemotherapy*) or their combination (*PDT and chemotherapy*). The experiments have been conducted utilizing: a) Photofrin, a photosensitizer already employed in the human therapy; b) a light source set at 630 nm; c) Cisplatin or Gemcitabine, as cytotoxic drugs; d) human non small cell lung cancer cells (NCI-H1299, p53 -/-), as cellular model system. First we have measured cell viability following each treatment. The curves dose-response obtained from Trypan Blue Test that have used to establish either the concentration of each chemotherapeutic agent and the conditions of the fluence that cause a cellular mortality of near 50%. Those conditions (2.5 μM Cisplatin or 4 nM Gemcitabine respectively and the fluence of 0.72 mW/ cm^2) have been used in combination experiments of. The curves dose-response in the experiments of combination have demonstrate that cell viability is reduced up to 5% about with 2.5 μM Cisplatin and about 10% with 4nM Gemcitabine. These studies have been finally completed with FACS analysis (that enlighten alterations of distribution of sub-cellular populations in every conditione used), and with densitometric evaluation (Western Blots) of expression changes of proteins involved into pro/anti apoptotic processes and in the control of the cell-cycle. Up today, the data obtained by combined experiments seem to satisfy our working hypothesis. In fact, the combination of two approaches effects in a therapeutic efficacy which is at least additive. Current experiments indicate that is possible to obtain even synergistic combination; this hypothesis, however, deserves further investigation.

AUTOFLUORESCENCE DIAGNOSIS AND PHOTODYNAMIC THERAPY OF EARLY OESOPHAGEAL CANCER

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The early diagnosis of esophageal cancer results in more effective treatment, considerably improving the patient survival rate. Moreover, local endoscopic therapies, such as PDT, can be undertaken, which are simpler and do not present the possible adverse effects associated with surgery. Therefore, research in PDT and photodiagnosis are closely related. We present here the results of two studies conducted during the same clinical trial.

Ultraviolet-induced autofluorescence diagnosis of early esophageal neoplasia

The aim of the study was to assess the potential of autofluorescence spectroscopy for early diagnosis with special focus on Barrett's esophagus. *Patients and methods:* Measurements were performed on 22 patients under 330 nm light excitation using an optical fiber spectrofluorimeter. ***Results:*** The spectral distribution of normal esophagus mucosa and specialized columnar Barret's mucosa were similar. A strong modification of the spectral distribution was observed for high grade dysplasia and intramucosal carcinoma. Statistical analysis indicated that the spectral shape modification associated with neoplastic transformation was greater than intra and inter-patient spectral variations. The ratio I_{390nm} / I_{550nm} allows differentiation of neoplastic tissue from normal esophageal mucosa and Barrett's mucosa with a sensitivity and a specificity of 86% and 95% respectively.

Photodynamic therapy in early neoplasia in Barrett's esophagus

The aims were to use Foscan® (m-THPC), with a less penetrating green light and to test the efficacy and tolerability of this method in a series of patients with early neoplastic lesions in Barrett's esophagus. *Methods*: Four days after intravenous injection of Foscan®, (0.15 mg/kg), lesions were illuminated at a wavelength of 514 nm, through windowed diffusers during endoscopic procedures. Fluence was 75 J/cm^2 and irradiance was 100 mW/cm^2. *Results*: Fourteen lesions (7 high-grade dysplasia and 7 intramucosal adenocarcinoma) were treated in 20 sessions. Efficacy was 100% for all patients. Moreover the Barrett's mucosa disappeared. There was complete squamous re-epithelialisation in the treated area. Side effects were moderate, with only one stricture. The mean follow-up was 37 ± 16 months.