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PHOTOCHEMICAL PROPERTIES AND MEDICAL APPLICATION OF MEROCYANINES

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The recent biomedical application of merocyanine dyes exploits the differential affinity and selective phototoxicity of merocyanine for different types of normal and neoplastic cells and viruses To be an efficient membrane probe, the molecules have to be incorporated into the lipid bilayer and their chromophores have to be oriented in this system It has been shown that stilbazolium merocyanines are highly oriented in nematic liquid crystals and in polyvinyl alcohol (PVA) film which is a good model of the biological membrane since some domains with various degrees of hydrophilicity are present We report here the electron paramagnetic resonance (EPR) measurements of the stilbazolium merocyanines with four different substituents in powder state and embedded in PVA films EPR studies in these systems revealed the existence of paramagnetic species in the dark condition and the signal enhancement by illumination Observed g values suggest that these spin species are Π electron radicals of dye molecules Stilbazolium merocyanine with protons in 3,5 position create stable complexes and its EPR spectra of powder and the PVA film are identical. Methyl and di tert butyl substituted compounds create less stable complexes which are observed in the powder state but not in PVA Perchlorate salt of di tert butyl substituted merocyanine does not create complexes, either in PVA nor in the powder state

Merocyanine is a group of heterocyclic chromophores which has the chemical structure shown in Fig.1. Merocyanine 540 (5-[3-sulfopropyl-2(3H)-benzoxazolylidine)-2-butenylidene]-1,2- dibutyl- 2-thiobarbituric acid, MC540) (Fig 1A) is a negatively charged chromophore which in water absorbs maximally at 533nm

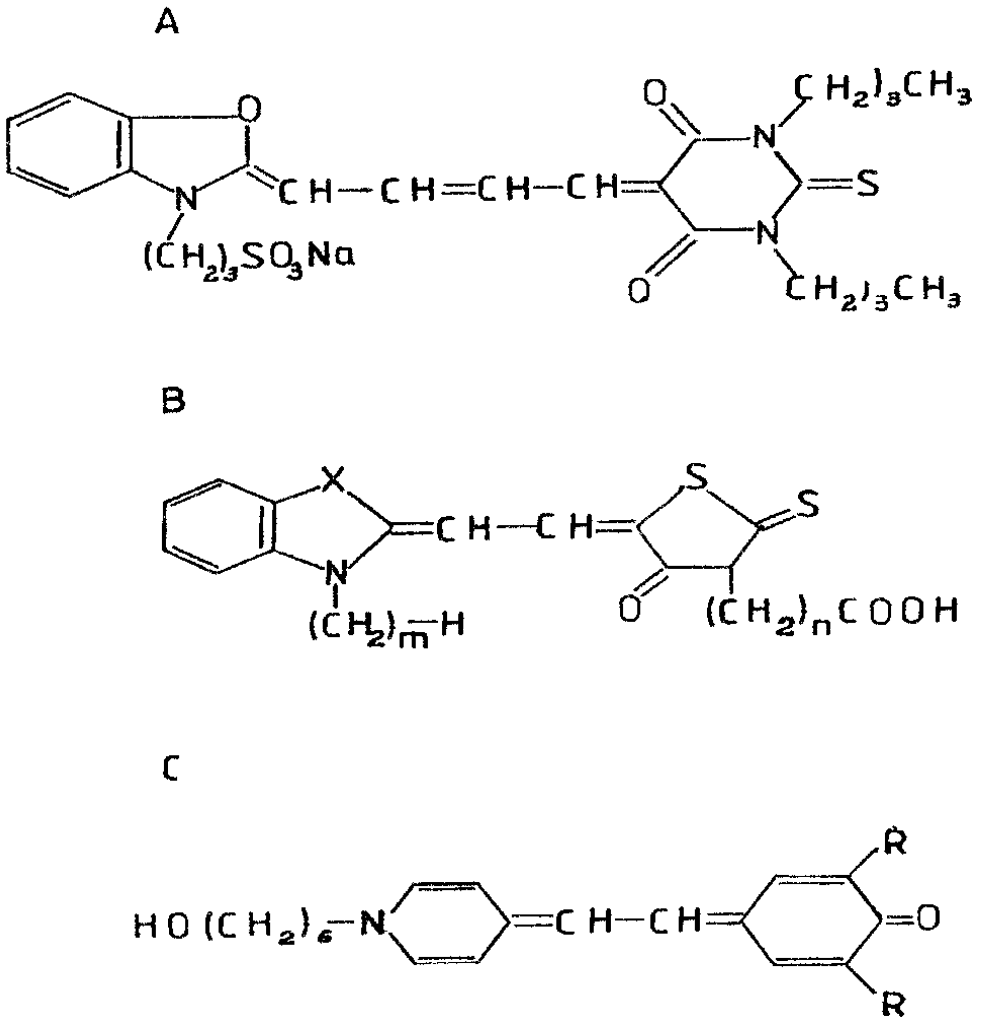

Fig 1 General characteristic structure of the merocyanine dyes A Merocyanine 540 (MC540) B Surface active merocyanine (X=O, S, Se, m=2 to 20, n=1 to 3) C Stilbazolium merocyanine (R=H, CH_3, tert butyl)

[8] In alcohols and in microemulsions, both the absorption and the fluorescence emission spectrum are red-shifted by 10-30nm Like other dyes with a polymethine chain MC540 in aqueous solution, it degrades rapidly in light. In microemulsions, the dye is stable [8] Surface-active and photoresponsible homologous series of merocyanine are shown in Fig 1B [51] with X=O, S or Se, the values of m are 2 to 20 and the values of n are 1, 2 and 3 In Fig 1C the chemical structure of several derivatives of stilbazolium merocyanines (4-[4-oxo-cyclohexa-2,5 dienylidene) ethylidene]-1,4 dihydropyridine) (R=H, CH_3, tert-butyl) [19] is presented.

Merocyanine dyes were originally developed as sensitizing additives for photographic emulsions and have been used by the photographic industry for

several decades [23] Biomedical applications of merocyanine dyes began in the mid-seventies with their first use as non-invasive probes for the recording of the transmembrane potential changes in cells and liposomes [10,39,57,58] The main advantage of the optical probes is that they allow measurements in cells or cell processes the size and geometry of which make the use of microelectrodes very difficult They also make it possible to obtain simultaneous recordings from large numbers of cells Its mechanism of action is still under investigation [2,22,62]

Among the merocyanines, dyes of the stilbazolium betaine type (Fig 1C) have aroused much interest because of their extreme solvatochromic properties [6,9,19,25] The merocyanine chromophore responds to the polarity and hydrogen ion concentration in the vicinity of the surfactant head groups in micelles Introduction of two strongly hydrophobic radicals (tert-butyls) to the immediate vicinity of the polar carbonyl group (Fig 1C) makes it possible to locate the merocyanine chromophore at varying depths in the membrane These modifications seriously affect the chemical and spectral properties of the molecule [19]

In the absence of serum the amphipathic polymethine dye MC540 (Fig.1A) binds indiscriminately to the plasma membrane of all intact cells. In the presence of serum, which is thought to bind MC540 with intermediate affinity, the dye binds preferentially to cells with high affinity dye binding sites; i e leukemia cells, certain classes of immature normal blood cells, and electrically excitable cells, such as nerve cells, striated and smooth muscle cells, and a number of ciliated protozoa [11,55] Non excitable cells such as fibroblasts, myoblasts, hepatocytes and mature blood cells are stained to a much lesser degree. Dead cells take up large amounts of dye

It is not fully understandable why electrically excitable cells, leukemia cells, and certain classes of normal hematopoietic and lymphopoietic cells bind MC540 more avidly than other cells. Experiments with artificial liposomes showed that the dye binds preferentially to liposomes made from unsaturated lipids [59,61] More dye is bound above than below the phase transition temperature [59,61] and if liposomes of identical chemical composition but of different dimensions are exposed to MC540, the dye binds preferentially to the smaller liposomes, i e the liposomes with the higher curvature and thus more widely separated polar groups [61]. These observations are the main experimental evidence in support of the hypothesis that MC540 binds preferentially to the disordered domains in the lipid bilayer [61]

Valinsky, Easton and Reich [55] were the first to recognize the potential usefulness of MC540 as a diagnostic agent. These authors examined peripheral blood leukocytes from normal individuals and patients with acute or chronic lymphocytic/nonlymphocytic leukemia or lymphoma in the leukemic phase after staining with MC540 in the presence of autologous plasma [55,56] Analysis by means of fluorescence microscopy and flow cytometry showed that the

leukocytes from patients with active disease contain a large percentage of intensely fluorescent cells while the specimens from healthy subjects consist almost exclusively of weakly fluorescent cells The observation that a rebound of MC540-positive cells can precede the first clinical manifestations of a relapse by up to 15 weeks [56] has potentially important implications for the monitoring and treatment of leukemia patients during remissions

If MC540-reactive cells are simultaneously exposed to the dye and light of a suitable wavelength, more dye is bound by the cells The average cell volume increases gradually and large surface protrusions are formed. The cells lose the capacity to proliferate, they become trypan blue-positive and disintegrate [46,55] The rate of photolysis depends on the concentration of serum and dye, the type and lot of serum used, and on the intensity and spectral properties of the light source [35,41,46,55]

The cellular targets of the phototoxic effect of MC540 have not been unequivocally identified yet The binding of MC540-mediated photosensitization of cells are oxygen-dependent processes [28] Since singlet oxygen is highly reactive and short-lived, it is reasonable to speculate that it does most damage close to where it is generated, i e. at plasma membrane When sensitive cells are exposed to MC540 and light, one of the earliest morphological changes recognized is a gradual increase in cell volume. One plausible explanation for this phenomenon is that MC540-mediated photosensitization affects transmembrane ion fluxes. Artificial liposomes which lack sophisticated membrane specializations such as ion channels or pumps become leaky when they are exposed to MC540 and light [43], most likely because of photodynamic damages to the lipid bilayer

The antiviral potential of photosensitizing dyes was recognized more than fifty years ago by Perdrau and Todd [38] and Shortt and Brooks [40] The acquired immunodeficiency syndrome (AIDS) epidemic, the realization that donor screening alone cannot make the blood supply entirely safe, and the growing demand for virus-free blood products for the treatment of immunosuppressed patients have renewed the interest in blood sterilization procedures [18]

MC540-mediated photosensitization also inactivates enveloped viruses. Significant virus killing could be achieved without excessive damage to mature blood cells, pluripotent hematopoietic stem cells, and at least some components of the clotting system [47] MC540 reacts primarily with the lipid portion of plasma membranes [11,60] and, presumably, with the lipid portion of viral envelopes It shows little or no affinity for proteins, carbohydrates, or nucleic acids. MC540 thus appears inherently less likely than many other sterilization procedures to alter the antigenicity of viral antigens or to be / mutagenic.

There are also preclinical data on the use of MC540-mediated photolysis for the selective inactivation of tumor cells in autologous remission marrow grafts [42]. The photosensitivity of the morphologically recognizable cells which

constitute the bulk of the bone marrow has not yet been investigated in detail The prevailing notion is that mature marrow cells have a low affinity for MC540 and are therefore rather resistant to MC540-mediated photolysis Unlike normal pluripotent hematopoietic progenitor cells, several experimental leukemias and some solid tumors of both human and murine origin are highly sensitive to MC540-mediated photolysis [41,44,45,48,49] This differential sensitivity of normal and neoplastic cells suggests that MC540-mediated photolysis might be used to purge remission marrow grafts of residual tumor cells without unacceptable damage to the normal pluripotent hematopoietic stem cells compartment This hypothesis has been tested with succes by transplanting lethally irradiated mice with simulated autologous remission marrow grafts [42]

In analogy to the absorption shift of dissolved dyes in different polar solvents, changes of absorption of merocyanine (Fig 1B) are possible by mixed evaporations of dyes with colourless organic or inorganic materials [4] The absorption spectra of merocyanine are strongly dependent on the polarity of the second component In the weakly polar paraffin the position of the absorption spectrum corresponds to the merocyanine spectrum in the gaseous phase [5] With an increasing dielectric constant one can observe strong batochromic shift In this way a wide shift of absorption maximum is realizable (from 442nm in paraffin to 625nm in PbI_2) The correlation λ_{max} versus dielectric constant shows in solid layers a much greater solvatochromic effect than that of merocyanine in different liquid solvents [4] Evaporated in this way dye layers are useful as optical filters in optical memories [53] or as multicolour filters in CCD sensors [15]

Merocyanine has been applied recently as a organic photoconductive compound in the photovoltaic mode also Merocyanine dyes as in Fig 1B, were vacuum evaporated on film with transparent electrodes [27]. It is possible to control the absorption spectrum of such a photodiode by placing it in an aqueous solution, probably due to self-reaggregation of the merocyanine-dye molecules This process is time-dependent.

Also Langmuir-Blodgett films of merocyanine dyes diluted with arachidic acid are known to be anisotropic photoconductors [51] The photoelectric character of these systems shows a strong correlation with the state of dye aggregation The film of a dye having benzoselenazole as a donor nucleus, for example, shows enhanced lateral photoconductivity when the film has a J-band in the optical absorption spectra [51] Information about the structure and electronic states of dye aggregates in Langmuir assemblies is of fundamental importance in understanding the nature of the system

Despite the various applications of merocyanine in photobiology, photomedicine and technique, only very little is known about the photophysics of the dye The structures shown in Fig.1 do not take into account the various possible structures of the dye in different environments. The relative weight of those structures is not known at present

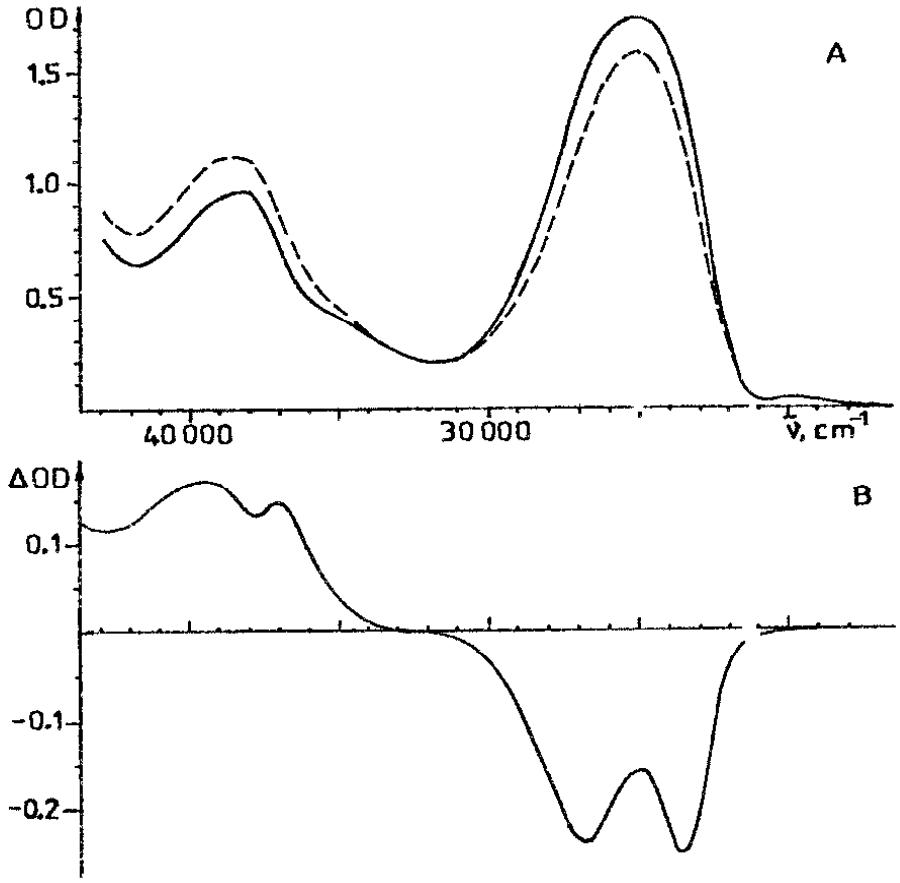

Fig 2 The spectra of stilbazolium merocyanine (structure as in Fig.1C, R = H) A Absorption spectra before (continuous line) and after (dashed line) 10 s illumination of 150W halogen lamp B Differential spectrum of the same sample (light minus dark)

In acid media the molecule is protonated on the oxygen and in protonic polar solvents there is presumably specific solvent interaction with both the oxygen and nitrogen atoms The solvent shifts of the merocyanine dyes are only substantial when such solvation can occur, in other words the bulk dielectric effect of the solvent has a small effect on the spectrum Dipolar solvent attaches with its positive end to the oxygen and its negative end to the nitrogen, making the oxygen more and the nitrogen less electron-attracting [3,7,50]. The spectra are strongly dependent on pH and also to external electric field [7,13,19,20] Systematic study on the effect of substitutents on the spectral properties of stilbazolium betaines have been carried out recently [12,14,21,37]. Structural modifications allow to obtain molecules absorbing in a very large region of the visible spectrum. Strong electron-withdrawing substitutents shift this presumably specific solvent interaction with both the oxygen and nitrogen atoms. The solvent shifts of the merocyanine dyes are only substantial when such solvation can occur; in other words the bulk dielectric effect of the solvent has a small effect

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Illumination of MC540 with visible light causes photodegradation of artificial and natural membranes, cells and microorganisms [42,43,47]. Mechanism of this action is not known in detail, although several photoprocesses have been observed in model systems: isomerization, triplet state formation, singlet oxygen evolution, changes in aggregation, free radical creation

Since to the development of picosecond laser spectroscopy it has become possible to observe directly the isomerization process in the singlet excited state of merocyanine in aqueous solution [26], in ethanol [1,24] and in octane [29] Recently we have observed photoisomerization of stilbazolium merocyanines in plyvinyl alcohol films [33,34] Absorption spectra before and after illumination and differential spectrum are ahown in Fig.2. Cis and trans isomers of merocyanine generally absorb in the same wavelength region but with different extinction coefficients [26]. Lifetime of cis isomer in polyvinyl alcohol is in the order of minutes. In degassed samples together with photoisomerization of merocyanines, triplet state formation have been observed [1,29]. It has been shown that singlet oxygen plays a major role in the phototoxic effect of the dye [29] Involvement of singlet oxygen in the modifications of membrane has been proved [54], and quantitative measurements of singlet oxygen production by MC540 have been made recently [24].

The application of electron paramagnetic resonance (EPR) techniques to these systems may provide information about their electronic and structural properties if they contain paramagnetic species In this case the behaviour of EPR signals on illumination of the system can also help in understanding the photochemical properties of the systems. Single dye molecule of merocyanine is diamagnetic and a charge transfer process should be considered for the generation of radical species [30,32]. The existence of two radical species with different spin-lattice

relaxation times and the light-induced enhancement of their concentration by an identical fraction for both radicals may indicate the generation of cation and anion radical species [31] There is no evidence for the existence of diradical species, and such radicals would be separated by a finite distance. A merocyanine dye molecule consists of donor and acceptor nuclei connected by a conjugated chain. A possible model process for production of radicals is that enhancement of intramolecular charge transfer may occur via intermolecular charge transfer for a certain structure of dye aggregates, at the expense of radical formation at both ends of the aggregate if it is one-dimensionally aligned [31,33].

It is very important to understand the photochemical and photophysical properties of merocyanine because several features make this dye very attractive not only in technique but also in medicine· its low systemic toxicity, the fact that it appears to be well tolerated by at least some clotting factors, by pluripotent hematopoietic stem cells and the majority of mature blood cells, its low oncogenic potential and its effectiveness against intracellular viruses, leukemia and lymphoma cells always show a high affinity for MC540

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