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Cytotoxic Effects and Physicochemical Properties of Marycin: a New Hematoporphyrin Derivative

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Abstract—*This paper reports some physico-chemical properties and cytotoxic activity of marycin, a new hematoporphyrin derivative. The data show that marycin is a new compound, different from the other porphyrins tested. This product appears to be pure by adsorption or reversed phase thin-layer chromatography and high performance liquid chromatography and has chromatographic behavior different from those of other porphyrins tested. It does not appear to link to some tested metals and has a UV-visible absorbance spectrum different from that of a hematoporphyrin methylester. Furthermore, marycin has cytotoxic activity against K-562, ZR-75, MCF-7, HT-29, LOVO, human tumor cell lines and the MRC-9 human lung embryonic cell line. The new radiometric assay was used for all cell lines. Marycin decreases the growth index, measured by the radiometric assay as $^{14}CO_2$ production. The cytotoxic activity is dose-dependent. Marycin is active at low doses but the activity varies with the cell line studied. The compound has low toxicity on the normal cell line MRC-9. Marycin is very liposoluble and would be expected to have high affinity and toxicity for tumors. The compound is active without light activation. How marcyin acts is still a matter for speculation.*

INTRODUCTION

HEMATOPORPHYRIN derivatives are taken up by tumors and released slowly from neoplastic tissues [1–5]. The tumor localizing capacity of hematoporphyrin derivatives has been successfully utilized for tumor phototherapy [6–11]. However, the hematoporphyrin preparations used for phototherapy are mixtures of compounds [12, 13]. The structure of the active hematoporphyrin compound that causes tumor photodamage is still under discussion and several structures have been proposed: the active compound has been reported to be a dimer, trimer or polymer with ether, ester bridges [14–16].

Consequently, we have prepared a semisynthetic derivative of hematoporphyrin and tested it on human tumor cell lines cultured *in vitro*. This paper describes some physico-chemical properties and the cytotoxic activity of marycin, a new hematoporphyrin derivative. We describe some chromatographic properties and solubility in solvents, absorbance spectra and molar extinction coefficients and some cytotoxic effects against the tumor cell lines K-562, MCF-7, HT-29, LOVO or the normal cell line MRC-9, cultured *in vitro*.

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Non-standard abbreviations: hematoporphyrindimethylester = HEPDE; hematoporphyrin derivative = HPD; absorbance = A; high-performance liquid chromatography = HPLC; thin-layer chromatography = TLC.
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MATERIALS AND METHODS

Reagents of analytical grade were obtained from BDH and Carlo Erba (Milano, Italy). Hematoporphyrin HCl was ordered from Fluka (Buchs, Switzerland). Solvents for HPLC were purchased from E. Merck (Darmstadt, FRG).

Preparation of hematoporphyrin derivatives

Marycin was prepared from hematoporphyrin by a method including esterification of hematoporphyrin with methanol–sulfuric acid 19 : 1, condensation of hematoporphyrin dimethylester in presence in *p*-toluene–sulfonic acid and subsequent reduction of the product with lithium aluminum hydride (Pinelli *et al.* paper in preparation).

Marycin has also been acetylated with acetic anhydride–pyridine 2 : 1 [14]. Hematoporphyrin

itself was also reduced with lithium aluminum hydride [17].

Hematoporphyrin was also esterified with methanol–sulfuric acid 19 : 1 [18]. Reduced hematoporphyrin or methylester hematoporphyrin were used as chromatographic reference standards.

Chromatography

High performance liquid chromatography: a Perkin–Elmer (Norwalk, CT, USA) high-performance liquid chromatograph, series 2/2, equipped with an LC-75 variable wavelength UV-visible detector was used. A Lichrosorb CN (Merck) column (10 μm particle size, 250 × 4.6 mm) was operated at room temperature. The mobile phase was methanol–0.05 M phosphate buffer, pH 3.5, 70 : 30 and the flow rate was 2 ml/min. The column effluent was monitored at 398 nm. Samples were injected by a Rheodyne 7105 (Berkeley, CA) injection system with a 150 μl loop.

Thin layer-chromatography: for adsorption chromatography precoated TLC plates, silica gel 60, or reversed phase chromatography RP18 plates, layer thickness 0.25, were obtained from E. Merck. The plates were developed in Camag tanks.

Atomic absorption spectrophotometry

A Perkin–Elmer spectrophotometer for atomic absorption, model 603 was used. Two mg of marycin, dissolved in methanol–water 1 : 9 mixture, were analyzed under lights with wavelengths for detecting zinc, aluminum, vanadium metals.

UV-visible spectroscopy

The samples were dissolved in dioxane at a concentration of 3 μg/ml. Absorbance spectra from 300 to 700 nm were recorded on a Perkin–Elmer UV-visible spectrometer, model lambda 5.

Partition between organic and aqueous phases

Hematoporphyrin and marycin were dissolved in chloroform (0.1 mg/ml). The solutions were shaken with equal volumes of phosphate buffer, 0.1 M, pH 7.4. After centrifugation, aliquots of the organic and aqueous phases were injected into the liquid chromatograph and compared with unwashed samples as reference standards.

Cytotoxic activity

Marycin was screened for cytotoxic activity against ZR-75, MCF-7 (human breast cancer), HT-29, LOVO (human colorectal cancer), K-562 (myelogenous leukemia) and MRC-9 (human embryonic lung fibroblast) cell lines. All the cell lines were maintained in specific media plus 10% heat inactivated fetal calf serum.

The cell lines ZR-75, MCF-7 and MRC-9 were gifts from Dr. C. Kent Osborne (University of Texas, Health Science Center at San Antonio) and were maintained in minimal essential media. The LOVO cell line, maintained in Ham's F_{12}, HT-29, maintained in McCoys' medium and K-562, maintained in RPMI-1640 medium, were gifts from Professor Daniel D. Von Hoff (University of Texas, Health Science Center at San Antonio).

A new radiometric method for screening anticancer drugs was used [19]. Marycin was dissolved in sterile 5% DMSO and diluted with sterile buffered 0.9% NaCl solution to give several concentrations, expressed as μg/ml, as reported in Fig. 5, when mixed with cell suspensions containing [^{14}C]glucose. Controls were in the medium without drug. All samples were run in triplicate. There were 20,000 cells/ml tissue culture media for all lines except K-562, for which there were 120,000 cells/ml. The suspension mixtures were injected into rubber-stoppered vials. The sealed vials were incubated at 37°C in the dark. The labelled CO_2 produced by surviving cells was measured on day 6 of incubation by a Bactec Instrument, model 460, to give the growth index.

The percentage of survivals was calculated as follows:

$$\frac{\text{growth index of drug-treated vials}}{\text{growth index of drug-untreated vials (controls)}} \times 100 = \% \text{ of survival.}$$

Furthermore, hematoporphyrin HCl was screened like marycin, against the tumor and normal cell lines mentioned above and under the operating conditions previously described.

Statistical analysis

The mean values ± S.E. for triplicate determinations for controls and groups of treated cells (percentage of controls) were calculated and Student's t test was used to determine the significance of differences [20]. The linear regression of the data (percentage of controls vs. log doses) was calculated by the least square method. The ED_{50} values for 50% of survivors with their 95% confidence limits were calculated [20].

RESULTS

Chromatography

Thin-layer chromatography: marycin had a single spot on silica gel 60, with an R_f different from those of hematoporphyrin, reduced hematoporphyrin and protoporphyrin IX (Fig. 1).

Marycin also had a single spot in RP_{18} reversed phase, with an R_f value different from those of hematoporphyrin HCl, hematoporphyrin dimethylester and acetyl-marycin. Acetyl-marycin also had

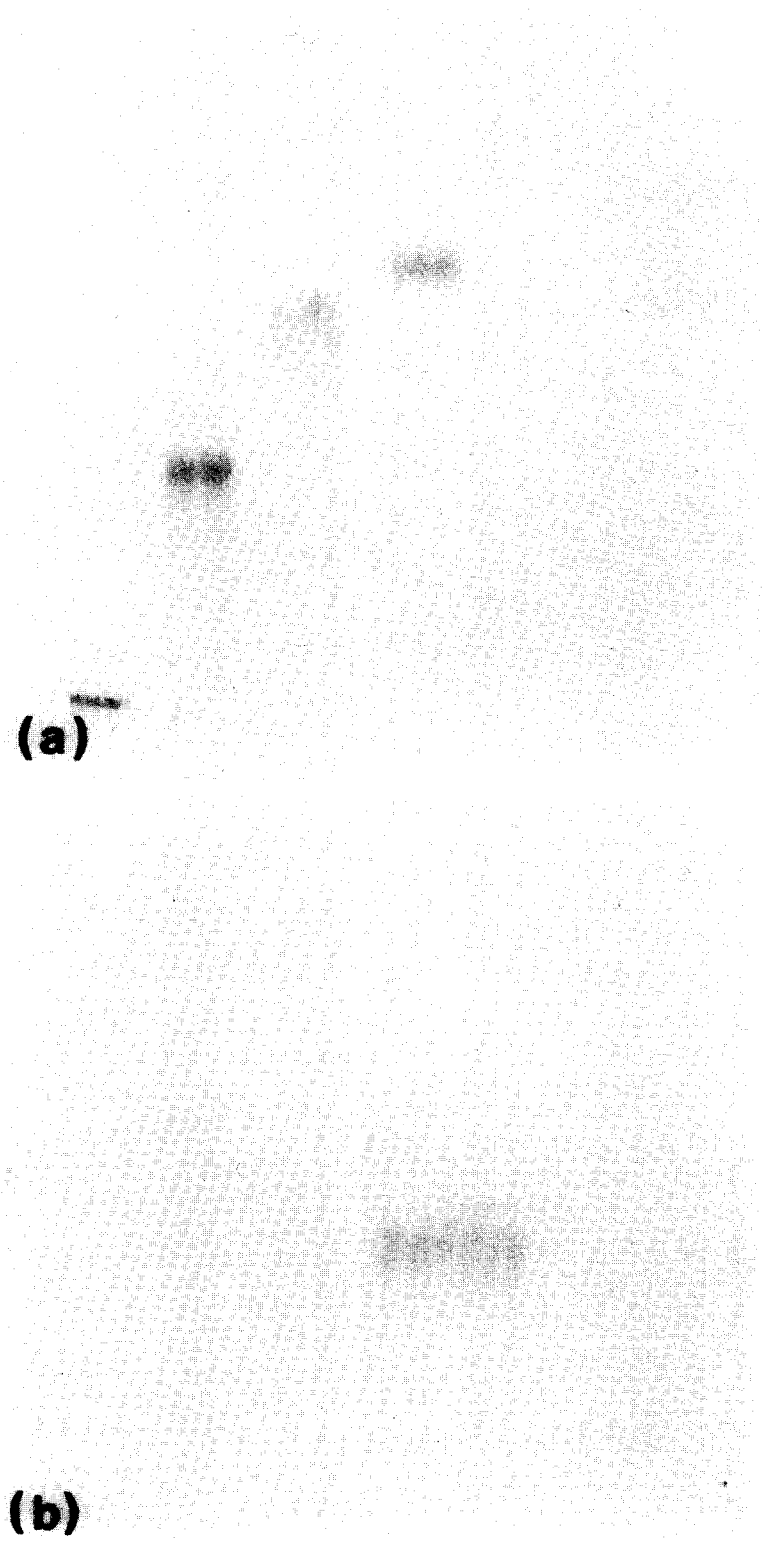

Fig. 1. *Separation of porphyrins on silica gel* 60 *thin-layer plates.* (*A*) *The porphyrins were spotted from left to right: marycin, hematopoprhyrin, protoporphyrin IX, reduced hematoporphyrin; the plate was developed with ethyl acetate–methanol–acetic acid,* 90 : 10 : 1. *Marycin is well separated from other porphyrins.* (*B*) *Marycin, spotted alone, and developed with ethylacetate–methanol–acetic acid,* 70 : 30 : 1. *Marycin shows a single spot. All porphyrins were spotted in amounts of* 30 μg *and are visible by day-light: marycin has a violet colour, the other porphyrins have brownish colours. Under UV light* (366 nm), *marycin emits orange fluorescence, other porphyrins give red fluorescence.*

Table 1. *Relative mobilities* (R_f) *of marycin and other porphyrin compounds on RP*18 (*reversed phase*) *thin-layer plates, with acetonitrile–acetone–water* 60 : 20 : 20 *as mobile phase. The* R_f *values are the means* ± *S.E. for* 4 *individual determinations*

Hematoporphyrin HCl	0.475 ± 0.032
Marycin	0.342 ± 0.039
Acetyl-marycin	0.152 ± 0.011
Reduced hematoporphyrin	0
Hematoporphyrin dimethyl ester	0.29

Fig. 3. The ε values (mM extinction coefficients) are also given. The values for marycin were calculated using the molecular weight of marycin as it appears to be from mass spectrometry which exhibits a significant intense peak at *m/e* 1122 [M^+] (Pinelli *et al.*, paper in preparation).

Percentage distribution

As seen in Fig. 4, marycin was highly concentrated in the organic phase (74%), while hematopor-

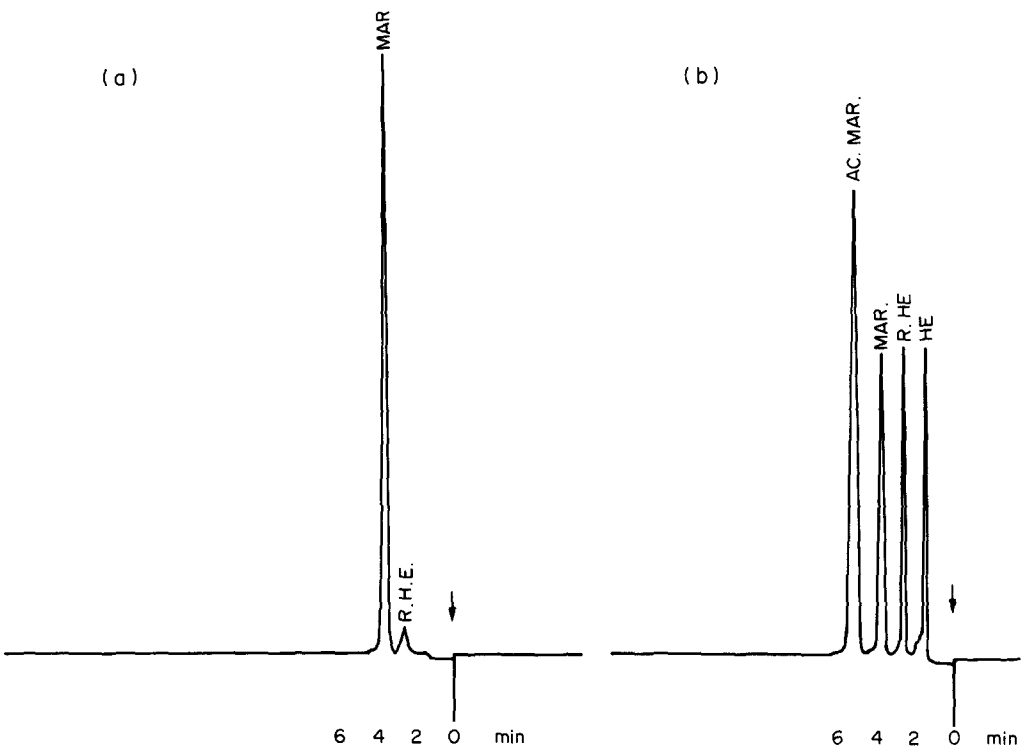

Fig. 2. *HPLC chromatograms of various hematoporphyrin derivatives.* (*A*) *Marycin*; (*B*) *He* (*hematoporphyrin*), *R-He* (*reduced hematoporphyrin*); *MAR* (*marycin*), *Ac.Mar.* (*acetyl-marycin*); *the separation was carried out on a Lichrosorb CN* (10 μm, *250 mm* × 46 mm *I.D.*) *column, with methanol–0.05 M phosphate buffer, pH* 3.5, 70 : 30, *as mobile phase, at a flow rate of* 2 ml/min. *The eluate was monitored at* 398 nm. *Marycin has a retention time of* 3.6 min *and appears to be homogeneous and pure* (94%). *The trace contaminants are reduced hematoporphyrin and hematoporphyrin. Marycin and its acetyl derivative were separated from other porphyrin compounds.*

only one spot (Table 1).

High-performance liquid chromatography: marycin had a retention time of 3.6 min, different from those of other porphyrins tested (Fig. 2).

Marycin was 94% pure by HPLC. The trace contaminants were mostly reduced hematoporphyrin (tetraol) (3.4%).

Atomic absorption spectrophotometry

Zinc, vanadium, aluminum, looked for in samples of 2 mg/ml of marycin, were not present in concentrations > 0.5 μg/ml.

UV-visible spectroscopy

The absorbance spectra of hematoporphyrin dimethylester and marycin in dioxane are shown in

phyrin was contained for the most part in the aqueous layer (83%).

Cytotoxic activity

The effects of marycin on tumor cell lines (K-562, ZR-75, MCF-7, HT-29, LOVO) and on the normal cell line MRC-9 are shown in Fig. 5.

Marycin decreased the growth index, measured as $^{14}CO_2$ production in the radiometric assay, for all the tumor cell lines. The regression lines for the effect (percentage of control) vs. log dose show that the dose–response relation was linear (Fig. 5).

The ED_{50} values (μg/ml), concentrations leaving 50% survivors, are all in the range of a few micrograms/ml, although they vary for the different cell lines. Note that the cytotoxic effect against MRC-9,

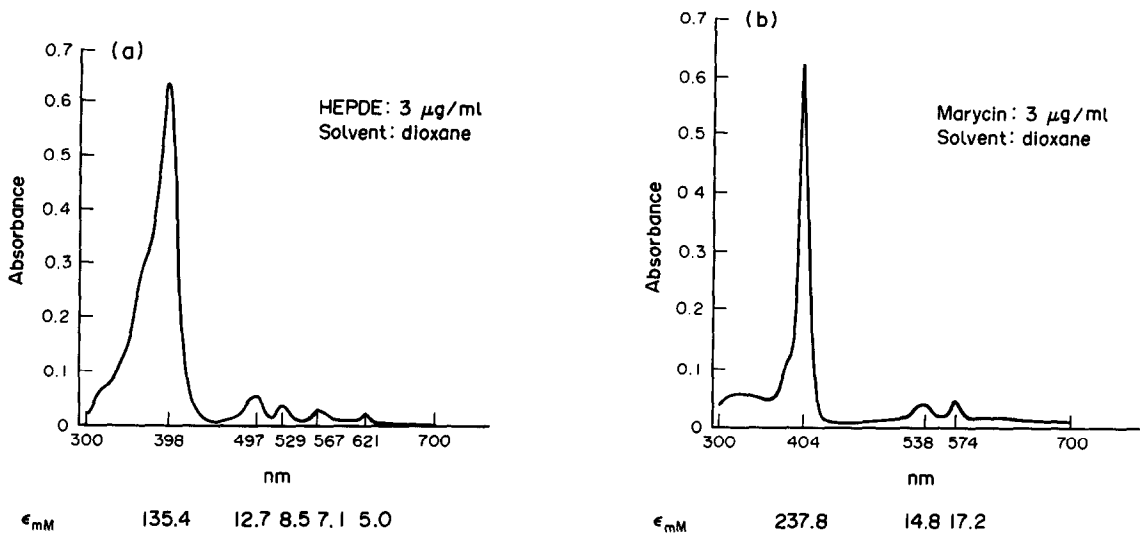

Fig. 3. Absorbance spectra of hematoporphyrin dimethylester (HEPDE) (starting material) and marycin (final product), dissolved in dioxane at a concentration of 3 μg/ml, *in a Perkin–Elmer UV-visible spectrophotometer, model lambda* 5. *The molar extinction coefficients* (ε) *are reported.*

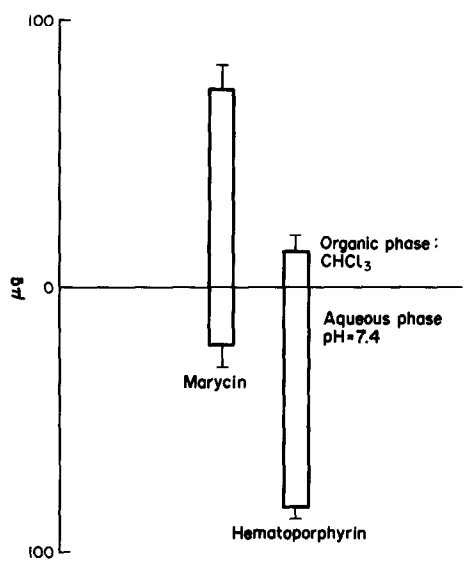

Fig. 4. Partition between organic phase (chloroform) and aqueous phase (0.1 M *phosphate buffer, pH* 7.4) *of marycin and hematoporphyrin. The samples were quantified by HPLC, monitored at* 398 nm. *The samples are expressed as a percentage of unextracted material as reference standards and are the means ± S.E. for* 4 *individual determinations. While hematoporphyrin is concentrated in the aqueous phase, marycin is largely extracted into the organic lipophilic layer.*

the normal cell line, was much lower (ED_{50} = 9.21) than the effects on the K-562, ZR-75, MCF-7, HT-29 tumor cell lines.

Hematoporphyrin HCl had ED_{50} values > 50 μg/ml for all tumor cell lines, which indicates very low toxicity.

DISCUSSION

From the TLC and HPLC experiments, marycin and also its acetate appear to be chromatographically pure. This is important, because the hematoporphyrin derivatives currently used for tumor phototherapy are mixtures of many compounds and several structures have been proposed for the active principles of hematoporphyrin [14–16].

Several of the experimental data reported here show that marycin is a new compound, different from other porphyrins tested. Marycin has TLC and HPLC chromatographic behavior totally different from those of hematoporphyrin, reduced hematoporphyrin or hematoporphyrin dimethylester. Additionally, marycin is not linked to some metals, since it contained none of the metals tested for by atomic absorption spectrophotometric analysis.

Furthermore, marycin is a reduction product obtained with lithium aluminum hydride and should have hydroxyl functions instead of carboxyl groups and cannot be identical with any of the products contained in hematoporphyrin derivative mixtures [14–16]. In addition, marycin has a totally different absorbance spectrum and mM coefficient value from those of hematoporphyrin. Consequently, marycin appears to be a new structure. The structure of marycin is being further elucidated by analysis of its NMR and MS spectra (Pinelli *et al.*, paper in preparation).

Marycin has cytotoxic activity against cancer cell lines cultured *in vitro*. It depresses or abolishes the metabolic activity of tumor cell lines, measured as labelled CO_2, but it has low cytotoxicity against the normal cell line.

The cytotoxic activity cannot be attributed to light activation, since the experiments were done in the dark. How marycin exerts its cytotoxic effects is still a matter for speculation.

From the chromatographic data reported in this paper and from the partition in solvents marycin is a very lipid-soluble compound. Since the lipophilicity of porphyrins determines their toxicity to biological membranes [21], the high lipid affinity of

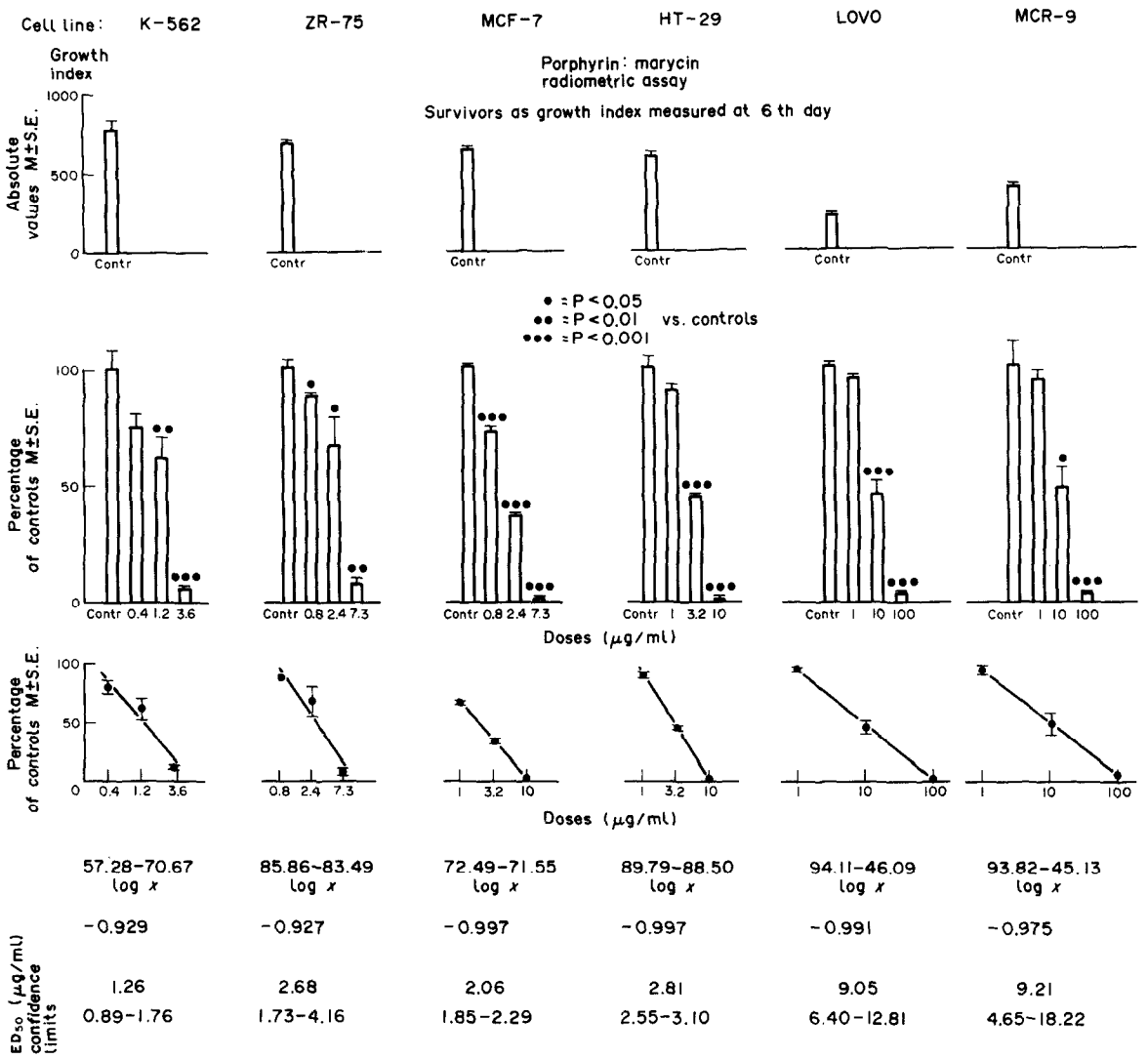

Fig. 5. Effects of marycin on tumor cell lines: K-562 (human myelogenous leukemia); MCF-7, ZR-75 (human breast cancer); HT-29 and LOVO (human colorectal cancer); MRC-9 (human lung embryonic fibroblasts) evaluated by radiometric assay as growth index on day 6. The samples are the means ± S.E. for 3 individual observations. ED_{50} *values (with confidence limits) are* μg/ml, *are also reported. Marycin appears to be more cytotoxic for tumor cell lines than for normal cell line.*

marycin could facilitate the crossing of membranes by this compound and then be toxic to the tumor cells by some unknown mechanism.

In addition, since marycin absorbs light, with a high absorbance, research is in progress to study its photosensitizing effects on tumors.

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