Tumour localisation kinetics of photofrin and three synthetic porphyrinoids in an amelanotic melanoma of the hamster

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Summary In this study the localisation of porphyrinoid photosensitisers in tumours was investigated. To determine if tumour selectivity results from a preferential uptake or prolonged retention of photosensitisers, intravital fluorescence microscopy and chemical extraction were used. Amelanotic melanoma (A-Mel-3) were implanted in a skin fold chamber in Syrian Golden hamsters. Distribution of the porphyrin mixture Photofrin and three porphycenes, pure porphyrinoid model compounds, was studied quantitatively by intravital fluorescence microscopy. Extraction of tissue and blood samples was performed to verify and supplement intravital microscopic results. Photofrin accumulated in melanomas reaching a maximum tumour:skin tissue ratio of 1.7:1. Localisation of the different porphycenes was found to be highly tumour selective (3.2:1), anti-tumour selective (0.2:1), and non-selective (1:1) with increasing polarity of the porphycenes. The two non-tumour selective porphycenes had distinctly accelerated serum and tissue kinetics; serum half time being as short as 1 min. The specific localisation of the slowly distributed, tumour selective photosensitiser, occurred exclusively during the distribution from serum and uptake into tissues. For the most selective porphycene, the tumour selection process had a half time of 260 ± 150 min and led to a strongly fluorescent tumour edge edema. Accumulation of porphycenes by the amelanotic melanoma (A-Mel-3) can be attributed to an enhanced uptake rate for lipophilic molecules in this subcutaneously growing neoplasm. The slow distribution of the two tumour specific photosensitisers and the strong fluorescence of these hydrophobic molecules in the tumour compartment with a high water content indicate a carrier role of serum proteins in the selection process. Enhanced permeability of the tumour vasculature to macromolecules appears to be the most probable reason for the tumour selectivity of these two sensitisers.

In the first half of this century it was shown that systemically administered porphyrines preferentially localise in neoplasms of tumour-bearing animals (Polardic, 1924; Auler et al., 1942). This phenomenon found diagnostic application as a method for tumour detection utilising the red fluorescence of the porphyrines (Lipson et al., 1961a,b; Baumgartner et al., 1987). The photosensitising properties of these molecules (Meyer-Betz, 1913) were exploited for therapeutic purposes and allowed the establishment of photodynamic therapy (PDT) as a new treatment modality for tumours (Dougherty et al., 1978).

The mechanisms leading to the tumour specific localisation of the porphyrines are still under investigation (Moan et al., 1992). Experimental progress has been complicated by the fact that even the most purified photosensitiser, Photofrin (Pi), is a complex mixture of molecular species with very similar spectral characteristics (Dougherty, 1987; Pandey et al., 1990).

In the present investigation, the localisation of three chemically pure synthetic porphyrinoids in an amelanotic melanoma grown in a transparent hamster skin chamber (Endrich et al., 1980) was studied fluorometrically and compared with the localisation of Photofrin. This tumour model has been used before for the study of microcirculation in neoplastic tissue (Asaishi et al., 1981; Endrich et al., 1982). Porphycenes (Vogel et al., 1986) were selected as pure porphyrin model compounds for their high absorption and fluorescence yields (Arandemila et al., 1986; Kreimer-Birnbbaum, 1989) and their well-established chemistry (Vogel, 1990). The three porphycenes employed varied in the number of polar substituents and thus in lipophilia. The ether, ester, hydroxy and carboxy groups present in these porphycenes are those functionalities found in the analysis of Photofrin (Pandey et al., 1990). Two of the porphycenes employed (the trietherporphycenes HEPn and CBPn) have already been shown to eradicate amelanotic melanomas of hamsters when irradiated at a dose level where photofrin had no phototherapeutic effect (Dellian et al., 1992).

The aim of our study was to distinguish between uptake and retention type mechanisms in the tumour localisation process by measuring the time dependence of drug content in blood, skin tissue, and a melanoma in the hamster.

Methods

Photosensitisers

9-Acetoxy-7,12,17-tetrapropylporphycene (ATPPn) was prepared from tetrapeptroporphycene (Vogel et al., 1987), 2-hydroxyethyl-7,12,17-tris(methoxyethyl)porphycene (HEPn) and 2-carboxy-2'-methoxyccarbanbone[2,3]-7,12,17-tris(methoxyethyl)-porphycene (CBPn) were prepared from tetakis(methoxyethy1)porphycene (Vogel et al., manuscript in preparation). Structural formulae of the porphycenes employed in this study are shown in Figure 1. Porphycenes were characterised by NMR, mass-, IR- and UV-Vis spectroscopy and had a purity >97% as monitored by HPLC. The polarity of the porphycenes employed rises in the order ATPPn < HEPn < CBPn as demonstrated by the polarity of solvents necessary to elute each molecule from silica gel columns (0.063-0.2 mm, E. Merck, Darmstadt, Germany) that were run at ambient pressure with 20 g silica for each 10 mg porphycene sample. Additives (1:1 vv) to dichloro-

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A further finding supporting this hypothesis is that the water content in A-Mel-3 tumours (200–300 mm3) is about 81%, whereas the water content of skin tissue in hamsters is only 54% (Leunig, M., unpublished data).
methane were hexane for ATPPn, ethylacetate for HEPn and methanol for CBPn for comparable retention times. The polarity of these additives as defined by the polarity index P' of Snyder (1974) is 0.0 for hexane, 4.3 for ethylacetate and 6.6 for methanol.

Photoserin (Lederle, Wolfrathshausen, Germany) and L-α-dioleylphosphatidylcholine, purity >99%, and 9,10-diphenylanthracene, purity >99% (Sigma, Deisenhofen, Germany) were used without modifications. Organic solvents were at least analytically grade and PBS was Dulbecco's without Ca2+ and Mg2+.

**Liposomes**

Porphycenes were incorporated in small unilamellar vesicles of dioleylphosphatidylcholine (DOPC; diameter: 110 ± 30 nm) on a molar ratio 1:200 to allow a quantitative follow-up and to avoid deviations in fluorescence intensity by aggregation as detailed elsewhere (Richert, in press). Shortly, a film produced by co-evaporation of phosphatidylcholine and porphycene solutions in chloroform/methanol (9:1, both puriss., Merck, Darmstadt, Germany) was taken up in PBS to yield a 0.1% lipid suspension. Samples (1.8 ml) were sonicated with a Branson probe sonicator and subjected to 0.2 μm sterile filtration after 24 h annealing time. Accuracy of preparation was monitored by UV/Vis spectroscopy of diluted aliquots. All steps were carried out under argon protective gas and samples were stored at room temperature in the dark. DOPC small unilamellar vesicles incorporate the porphycenes employed in monomeric, photoactive form, as monitored by time resolved fluorescence spectroscopy.

Liposomal porphycene formulations were tested for their ability to release the sensitisers *in vivo*. To this end, after injection porphycenes were extracted from erythrocytes by the same procedure as described for serum.

**Animals and tumour model**

Experiments were performed on 23 male Syrian Golden hamsters (60–70 g b.w.) fitted with titanium chambers (for technical details see Endrlich et al., 1980). Following implantation of the transparent access chamber and a recovery period of 48 h from anaesthesia and microsurgery, preparations fulfilling the criteria of an intact microcirculation were utilised for implantation of 2 × 10⁶ cells of the amelanotic hamster melanoma A-Mel-3 into the chamber preparation. Fluorescence microscopy was performed after 6–7 days of growth (mean tumour diameter of 4–5 mm) when functioning tumour microcirculation was established. Permanently indwelling catheters (PE10, inner diameter 0.28 mm) were implanted into the right jugular vein and/or carotid artery 24 h prior to sensitisers injection. The animals tolerated the catheters and chambers well and showed no signs of discomfort.

After i.v. sensitisers injection, animals were housed in absolute darkness in single cages under carefully controlled temperature conditions with free access to water and standard pellet food.

**In vivo fluorescence measurements**

For fluorescence microscopy the awake, chamber bearing hamsters, laying in the perspex tube on a specially designed stage (Effenberger, Munich, Germany), were positioned under a modified Leitz microscope (Type 307-143003/514660, Leitz, Munich, Germany) and monitored with a 14-fold magnification (Figure 2). During the experiments, the animal temperature was kept constant using a feed back controlled heating pad (Effenberger, Munich, Germany). Epillumination was performed with a 100 W, XBO mercury lamp attached to a Ploemopak illuminator.

For visualisation of the photosensitiser fluorescence, the tissue was illuminated 2–5 s at a power density of 200–300 μW cm⁻². Porphycene fluorescence was excited at a wavelength of 340–380 nm, PFS fluorescence at 355–425 nm. The emission fluorescence of the porphycenes and PFS was detected above 610 nm. Fluorescence images were recorded by means of a silicon intensified target (SIT) (100–0.1mLux) video camera (C2400-08, Hamamatsu, Herrsching, Germany). Digitisation was performed using an image analysis system (IBAS 2000, Kontron, Eching, Germany) and images were later stored on a hard disc. This procedure was performed prior to photosensitisers application to record the tissue auto-fluorescence and repeated at defined time points (30 s, 1, 3, 5, 15, 30 min, 1, 3, 6, 24, 48, and 72 h) after i.v. injection of 1.4 μmol kg⁻¹ b.w. of either porphycenes (ATPPn n = 7, HEPn n = 6, CBPn n = 4) or 5 mg kg⁻¹ b.w. Photofrin (n = 6). The photosensitisers doses were based on pilot studies, where concentration-fluorescence linearity was determined and are in the therapeutic range for the porphycenes and Photofrin (Dellian et al., 1992).

Photosensitisers fluorescence intensities were measured densitometrically off-line by means of the image analysis system (IBAS, Kontron, Eching, Germany) and tissue auto-fluorescence was digitally subtracted. Changes of the camera sensitivity or the light intensity during the experiments were corrected using solid fluorescent reference signals (Impregum F, Seefeld, Germany) inserted into the observation field of the chamber preparation. Photostability of solid references was proved using a 5 nmol ml⁻¹ tetraprropylporphycene toluene solution, known to be photostable (Aramendia et al.,

![Figure 1](image-url)  
*Figure 1* Structural formulae of porphycenes.
All fluorescence values are given in percent of the reference fluorescence signal (% Ref.). The geometric resolution of the digitised images was 512 × 512 pixel by a densitometric resolution of 255 grey values. Photosensitiser fluorescence in tumour and adjacent tumour-free tissue was determined in defined areas (250 μm²) by digital light measurement. These defined areas did not overlay blood vessels with a diameter > 30 μm. The image analysis was used for an interactive frame positioning. Thus areas of measurement were identical for all time points. Spatial inhomogeneities of the light source and the camera were compensated by shading correction performed with the image analysis system.

**Chemical extraction**

After the final intravital microscopic measurement, animals were sacrificed by an overdose of anaesthesia (Pentobarbitatal, 300 mg kg⁻¹ b.w.) i.p. and tissue specimens of the tumour and adjacent tumour-free tissue were immediately excised and frozen in liquid nitrogen for chemical extraction.

To enable measurement of rapid serum kinetics for the three porphycenes (dose: 1.4 μmol kg⁻¹ b.w.), a catheter blood sampling technique was applied to a second group of 21 hamsters (ATPPn n = 8, HEpn n = 9, CBPn n = 4). The minimum interval between injection and sampling was 30 s. Blood samples (40 μl) from anesthetised hamsters bearing a venous catheter as described above and an additional catheter implanted in the right carotid artery were taken in heparinised capillaries. The samples were centrifuged to determine the hematocrit and to isolate serum used for chemical extraction.

Serum and tissue specimens were extracted by a procedure that recovered 85 ± 5% of every porphycene employed from serum and erythrocytes, and extracted <10% of the initially extracted amount of dye from the tissue sediments in re-extraction experiments. Ten μl serum samples or weighed amounts of tumour and tumour-free tissue (5–20 mg), cut in small pieces, were treated with methanol. Serum probes were briefly agitated and tissue specimen homogenised in a Potter-Elvehjem vessel followed by subsequent additions of acetonitrile containing Diphynylanthracene (DPA) reference standard. Samples were sonicated, and centrifugation yielded a supernatant that was subjected to spectrofluorometric analysis. The efficiency of the extraction procedure was determined by comparing the fluorescence of extracts from porphycenes incubated either with erythrocytes, serum or buffer alone (control).

Emission spectra were recorded on a spectrofluorometer equipped with a red sensitive phototube at a 5 nm slide width. Light source intensity was calibrated against 10⁻⁴ M tetraphenyldiazeni diene solid standard in a polymethylmethacrylate matrix (Starpa, Pfungstadt, Germany). Peak intensities were read against DPA internal standard intensities at 429 nm and compared to the porphycene calibration plots. ATPPn 72 h tissue extracts eventually exhibited an additional 670 ± 10 nm peak, showing porphycene excitation characteristics, whose intensity was quantified as the ATPPn 644 nm maximum.

During the sampling, some of the interstitial fluid was lost (<20%), which might have led to a slightly lower tumour selectivity of ATPPn (Table IV) compared to the microscopic measurements.

The extraction protocol was restricted to the porphycenes since no method for the quantitative extraction of Photofrin, whose chemical composition is still partly unknown (Pandey et al., 1990) could be established.

**Mathematical analysis and statistics**

Least square fits, using a Marquardt algorithm (Bevington, 1969), were employed for the serum kinetics. Serum kinetics were analysed according to the two compartment model (Benet et al., 1980). Eventual deviations of injected sensitiser dose were corrected, by factorising deviation from 1 min averages of individual serum concentrations. Loss of blood volume caused by repeated blood sampling from the animals was corrected for by adjusting serum concentrations to the actual hematocrit.

For nonparametric one-way analysis of variance and multiple comparison of ranks of several independent samples, the Kruskal-Wallis test was utilised. Single comparisons of unpaired samples were performed using U-test and of paired samples by the Wilcoxon-test. Data are given as mean ± standard deviation (SD) or standard error of the mean (SEM), respectively. Tumour:skin tissue fluorescence ratios (Figure 6) are means of ratios of individual animals, diverging slightly from the ratio of mean fluorescence of each tissue (Figure 5).
Results

Pharmacokinetics of the porphycenes showed a strong dependence on the chemical properties of the sensitisers (Figure 1 and Figure 4). For each single porphyrinoid, time constants for uptake and elimination from melanomas were similar to those found for skin tissue and blood. The rates of accumulated or eliminated molecules, however, differed distinctly between the tissues monitored.

Halflife of distribution from serum decreased in the order ATPPn > HEPn = CBPn (Figure 3, and Table I). HEPn additionally exhibited a redistribution phase in its serum kinetics prolonging its full distribution and reflecting the HEPn > CBPn order in tissue uptake. Actual serum halflives of distribution were determined to be 7 h for the alkylporphycene ATPPn and 1 min for both etherporphycenes by the fit procedure (Table I). The redistribution process had a halflife of 1 h for HEPn (Table I). Elimination halflife also decreased in the order ATPPn > HEPn > CBPn, with values of more than 1 day for ATPPn, about half a day for HEPn, and 3 h for CBPn. Thus, the acceleration of the pharmacokinetics followed the polarity of the porphycenes.

One minute after injection, the most lipophilic alkylporphycene ATPPn had not penetrated erythrocytes effectively, whereas both trietherporphycenes were found in considerable amounts in red blood cells. At that time, dye concentration ratio of serum to red blood cells was 130:1 for ATPPn, 2.3:1 for HEPn, and 1.4:1 for CBPn (Table II). After 10 h, less than 10% of the serum concentration of ATPPn was found in erythrocytes.

Tissue uptake, defined as the time interval to reach maximum tissue fluorescence, was shortened in the order CBPn > HEPn > Pf > ATPPn. Maximum fluorescence intensity in tumours was detected 30 s after injection for CBPn, 3 h after injection of HEPn, and 24 h after injection of ATPPn (Figure 4). Tumour fluorescence reached a maximum 6 h after Pf injection and increased again after 24 h, though not significantly.

Figure 3 Serum-kinetics of porphycenes. a, ATPPn; b, HEPn; c, CBPn. Serum-levels were spectrofluorometrically measured from extracted samples. Bars indicate typical errors, taken from the s.d. of repeated extraction of one sample in every concentration range. Lines give results of fit procedure. The insert is a bilogarythmic plot of identical values. Note the x-axis of the CBPn plot being 1/10 of ATPPn and HEPn plots.
Fluorometric measurements in the tumour bearing chambers demonstrated that uptake and elimination in the skin tissue was coincident with uptake and elimination in the melanoma for all sensitisiser tested, however, the amount of photosensitizers detected in both tissues differed distinctly. Both fluorescence and extraction measurements yielded this result independently (Figure 4, Table III and IV). Only two of the photosensitisers (ATPPn and Pf) were found to localise preferentially in tumours. ATPn was delivered far more effectively to tumours than to non-neoplastic tissue. Photofrin also accumulated in the melanoma, though in a less pronounced way. HEPn only showed slight tumour selectivity 30 s after injection, but later reached higher concentrations in subcutaneous host tissue than in tumours. To our knowledge, HEPn is the first photosensitiser that localises ‘anti-tumour-selectively’. The most polar porphycene, CBPn, showed no specificity for the fluorometrically monitored tissues.

The time courses in tumour specificity are given as the fluorescence ratio of tumour:skin tissue in Figure 5. These ‘selectivity functions’ showed a strong time dependence for ATPn and HEPn and to a lesser degree for Photofrin. The maximum tumour selectivity ratio was 3.2 for ATPn, 1.7 for Pf, and 1.3 for HEPn (Table IV). Increases in tumour selectivity occurred during the uptake phases of tissue kinetics for the tumour-selective sensitisers ATPn, Pf, and HEPn. The halflife for the tumour selection process of ATPn equalled the serum distribution constant within experimental error. The time constant for selectivity of HEPn was between the halflives for redistribution and elimination from the serum. No increase in tumour selectivity was seen during elimination periods from tissues and serum for any of the sensitisers tested and decreased for both the highly tissue specific porphycenes ATPn and HEPn.

Fluorescence pictures taken of tumour chambers 24 h after dye injection (Figure 6) showed peak emission intensities at tumour edges for ATPn. The anti-tumour selective porphycene, HEPn, was located in large, diffuse spots and stripes in the subcutaneous tissue and was not localised in tumours and/or the vasculature as evidenced by fluorometric measurements.

Discussion

The elucidation of the tumour localisation of porphyrinoids presents a challenge to PDT researchers, since understanding of the underlying mechanism(s) might allow the design of not only improved photodynamic, but also non-photodynamic drugs. Intravital microscopy of tumours grown in skin chambers has proved to be a valuable tool for the study of microcirculation (Endrich et al., 1989; Asaishi et al., 1991; Leung et al., 1992). It appears to be a suitable technique for the study of porphyrinoid localisation since these PDT drugs can be directly visualised by their fluorescence. Thus pharmacokinetics can be determined at the microscopic level without artifacts caused by sacrifice of the animal with high experimental accuracy, since the local pharmacokinetic can be measured from one individual. Additionally fast diffusional processes, not observable by ex vivo techniques, can be monitored. However, care must be taken to ensure a linear correlation between fluorescence intensity and drug concentration in the tissue, for well known aggregation and quenching phenomena can lead to a deviation from linearity. In the present study intravital fluorescence microscopy was compared to chemical extraction of microsurgically obtained tissue samples from monitored areas. The agreement of results within experimental errors demonstrates the validity of the approach (Table IV).

The results of the present investigation demonstrate that preferential localisation in tumours is not a feature common to all porphyrinoids. Among the three pure model compounds tested, only the most lipophilic dye, ATPn, showed a strong positive affinity for the amelanotic melanoma (A-Mel-3). The tissue kinetics of this tumour selective porphycene (ATPPn) and Photofrin appear strikingly similar (Figure 4a and d). Both sensitisers show the increase in tumour-selectivity (Figure 5a and b) during the uptake phase into tissues. For ATPn the uptake nature of the tumour localisation is additionally demonstrated by the similarity of time constants for the rise in tumour-specificity (260 ± 150 min) and the distribution from the serum (430 ± 30 min). The prolonged uptake of Photofrin compared to ATPn can be rationalised in terms of the long persistence of a distinct hydrophobic fraction of the porphyrin mixture in the serum (Bellnier et al., 1989).

The observed accumulation of ATPn and Photofrin during their delivery from the bloodstream strongly suggests that uptake and not retention-type tumour-localisation mechanisms lead to the tumour preferential localisation of porphyrines. If compromised lymphatic drainage (Gullino, 1975) and/or prolonged retention of protogenic molecules in low pH tissues (Brault et al., 1986; Barret et al., 1990) were the underlying localisation mechanisms, an increase of the tumour selectivity should appear during the elimination phases of tissue kinetics. The decrease in tumour:skin tissue ratio during the elimination of HEPn and ATPn (Figure 5a) points to the absence of a specific dye retention in the tumour model used in this study.

From our results, a model can be proposed to explain the localisation kinetics of Photofrin. Assuming that the Photofrin mixture contains a fraction of anti-tumour selective molecules like HEPn besides an ATPnP-like localising fraction its lower selectivity can be explained. This assumption seems reasonable because the dihydroxethylporphyrine hematoporphyrine, is known to be present in Photofrin (Dougherty et al., 1987). Since the time constant for the process leading to the anti-tumour selectivity of HEPs is similar to the time constant of the positive process of the model (Table III), the two Photofrin fractions corresponding to these porphyrines might localise coincidently in opposite ‘directions’. Thus, the weak time dependence in the tumour-selectivity of Photofrin (Figure 5b) is understandable.

Photofrin is known to be lipophilic (Dougherty et al., 1983; Kessel & Chou, 1983; Dougherty, 1987) and ATPnP is the most lipophilic sensitisser among the porphycenes tested. ATPnP remains within lipoprotein serum carrier particles for hydrophobic molecules or residual liposomes as indicated by its low concentration in erythrocytes (Table II). This finding is in accordance with earlier studies with a similar porphycene (Guardiano et al., 1989). The porphyrins in photofrin will probably form aggregates in watery solutions or, upon injection, become associated with serum proteins (Cohen & Margalit, 1990). The negligible uptake of ATPnP

Table I Serum distribution (D), redistribution (R), and elimination (E) halflives of the porphycenes

| Porphycene | tD (min) | tR (min) | tE (min) |
|------------|----------|----------|----------|
| ATPnP      | 430 ± 30 | –        | 1700 ± 1300 |
| HEPn       | 0.91 ± 0.1 | 62 ± 8 | 500 ± 160 |
| CBPn       | 1.1 ± 0.3 | –        | 170 ± 30 |

Values are derived from fits to serum-kinetics, determined by chemical extractions; errors are s.d.

Table II Porphycene concentration ratios serum to erythrocytes 1 min after i.v. injection

| Sensitisser | Ratio_{D/R} | Ratio_{R/E} |
|-------------|-------------|-------------|
| ATPnP       | 130:1       |             |
| HEPn        | 2.3:1       |             |
| CBPn        | 1.4:1       |             |

TUMOUR LOCALISATION OF PORPHYRINOIDs
Figure 4  Tissue fluorescence-kinetics in the melanoma and skin tissue. a, ATPPrn; b, HEPn; c, CBPn; d, Pf. Closed circles indicate tumour, open circles tumor-free subcutaneous tissue. Fluorescence intensities of selected areas in tumour and tumour-free tissue are given relative to solid fluorescence reference signals as mean ± s.e.m. Tumour fluorescence was significantly higher compared to the tumour-free subcutaneous tissue for ATPPrn and Pf (P<0.001), not significantly different for CBPn, and significantly lower for HEPn (P<0.001). Note the x-axis of the CBPn plot being 1/3 of other plots.
Figure 5 Tumour to skin tissue ratios. a, ATTPn and HEPn; b, CBPn and Photofrin. Tumour to tumour-free tissue ratios in fluorescence intensities of monitored areas as determined by intravital microscopy. Values are mean of the ratio of individual animals ± s.e.m.

Table III Time to reach half maximum (t<sub>i</sub>) and maximum (t<sub>max</sub>) tumour selectivity ratio

| Sensitizer | t<sub>i</sub> (min)* | t<sub>max</sub> (h)* |
|------------|-------------------|-------------------|
| Pf         | (a)               | 1                 |
| ATTPn      | 260 ± 150         | 24                |
| HEPn       | 170 ± 30 (b)      | 0.01              |
| CBPn       | (c)               | 6                 |

Values are mean ± s.d. (a) Not unambiguously evaluable, (b) process leading to skin tissue selective localisation, and (c) no time dependence. *Determined by a monoexponential fit process to data of Figure 5. †Directly obtained from acquired data.

The macromolecules can accumulate in tumours either by endocytotic processes or by extravasating from the blood through holes in the endothelial lining. Both an enhanced number of LDL receptors on tumour cells (Gomer et al., 1981; Norata et al., 1985; Maziere et al., 1991) and high vascular permeability of tumours for macromolecules (Jain, 1987) are known. If the endocytotic activity of the tumour cells was the main accumulation mechanism, the interstitial fluid of tumours should be impoverished in sensitizer compared to the surrounding tissue and tumour cells should show bright fluorescence. The microscopic pictures revealed, however, strongly fluorescent tumour edge edema in (Figure 6b) as expected for vascular permeability as major reason for the tumour localisation.

The permeability hypothesis might also explain the selectivity found for a wide range of different photosensitisers (Gomer, 1991), many of which were shown to have an affinity to macromolecular carrier particles in the bloodstream, like lipoproteins or albumin (Cohen et al., 1990). In accordance with this ‘vascular’ localisation process, no difference in sensitizer uptake had been found between malignant and non-malignant cell lines in vitro (Moan et al., 1981).
Figure 6 Photomicrographs of the amelanotic melanoma. a, Trans-illumination, photomicrograph of an A-Mel-3 tumour (diameter: 4 mm) localised at the left lower edge of the chamber preparation, 6 days after implantation. b, Epi-illumination 24 h subsequent to i.v. injection of 1.4 μmol kg⁻¹ b.w. ATPn as recorded by intravitral microscopy. Note the strong fluorescence within the tumour surrounding edema. c, Epi-illumination of a tumour chamber 24 h after i.v. injection of 1.4 μmol kg⁻¹ b.w. HEPn. Note the non fluorescent tumour and tumour surrounding edema. The two fluorescent squares at the top of the chamber represent the reference signals. (The bars represent 2 mm).

### Table IV (a) Tumour selectivity ratio at 6 h (r₆) and 72 h (r₇₂), and maximum tumour selectivity ratio (rₘ₅) as evaluated by fluorescence microscopy

| Sensitiser | r₆ (h) | r₇₂ (h) | rₘ₅  |
|------------|--------|---------|------|
| Pf         | 1.6 ± 0.2 | 1.7 ± 0.4 | 1.7 ± 0.3 |
| ATPn       | 2.3 ± 0.7 | 2.7 ± 1.3 | 3.2 ± 1.1 |
| HEPn       | 0.6 ± 0.2 | 0.3 ± 0.1 | 1.3 ± 0.2 |
| CBPn       | 1.1 ± 0.3 | –       | 1.1 ± 0.3 |

Values are mean ± s.d.

(b) Tumour selectivity ratio at 6 h (r₆) and 72 h (r₇₂) as evaluated by chemical extraction

| Sensitiser | r₆ (h) | r₇₂ (h) |
|------------|--------|---------|
| Pf         | –      | –       |
| ATPn       | 1.8 ± 0.4* | 2.0 ± 0.6 |
| HEPn       | 0.64 ± 0.1* | 0.4 ± 0.2 |
| CBPn       | 1.1 ± 0.2* | –       |

Values are mean ± s.d. except for those marked *, these are mean ± error of two extractions.

Further evidence for the 'selective macromolecule filtration assumption of macromolecules comes from the localisation of the non-tumour selective porphycenes HEPn and CBPn. Unlike macromolecular bound molecules (Nugent & Jain, 1984), HEPn and CBPn are fast distributed from the blood (Table I) as seen for small molecules (Gerlowski & Jain, 1983; Gibaldi & Perrier, 1982) and penetrate erythrocytes effectively (Table II). Small standard deviations of the tissue distributions (Figure 4b and c) support distribution processes driven by physical gradients for small molecules.

The almost instantaneous tissue uptake of CBPn, which reached its maximum 30 s after the end of dye injection favours free diffusion as possible transport mechanism. The distribution of the less polar hydroxyphorphyene HEPn probably proceeds via fast membrane uptake as it has been observed for the hydroxysteroid cholesterol (Barclay et al., 1990). HEPn reaches its maximum tissue concentrations relatively slowly, probably via diffusion along hydrophobic structures. The equilibrium distribution pattern seems to be governed by the amount of hydrophobic sites within a tissue. Highly fluorescent fat cells found in the subcutaneous host tissue at high magnification and the high water content of the
amelanotic melanoma support this explanation. Hence, the pharmacokinetics of HEPn do not seem to favour earlier assumptions of a general accumulation of lipophilic molecules by sensitiser retaining tissues (Freetas, 1990).

In conclusion, our study demonstrates that intravitinal microscopy is a valuable tool for the study of porphyrinoid pharmacokinetics. Investigating synthetic porphyrinoids of variable lipophilicity, it was shown that polar functional groups accelerate the pharmacokinetics of photosensitisers. The most lipophilic porphyrine studied accumulated in tumours twice as much as Photofrin. Local tumour selectivity of sensitisers was found to originate in uptake and not elimination processes in the body compartment monitored. Increased permeability of the tumour vasculature to carrier macromolecules probably plays a governing role in the localisation process. Extension of these experiments to other physiological compartments, tumour models and photosensitisers will be needed to support these conclusions.

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Abbreviations
ATTPn, Acetoxytetraproporphyrine: CBPn, Carboxymethoxycarbononylbenezotris(methoxyethyl)porphyrine; DPA, Diphenylanthra-
cene; DOPC, Dioleylphosphadieylcholine; HEPn, Hydroxypyt-
ris(methoxyethyl)porphyrine; n, Number of animals; PBS, Phos-
phate buffered saline; PDT, Photodynamic Therapy; Pf, Photofrin; SIT, Silicon intensified target.

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