Metabolic profile of a peptide-conjugated chlorin-type photosensitizer targeting neuropilin-1: an in vivo and in vitro study

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Non-standard abbreviations: Ahx, 6-aminohexanoic acid; a.i., arbitrary intensity; a.u., arbitrary units; DiOC₆, 3,3′-dihexyloxacarbocyanine iodide; DLI, drug-light interval; EDTA, ethylene diamine tetra acetic acid; EGM2, endothelial growth medium; Fmoc, 9-fluorenlymethoxy-carbonyl; HPLC, High Performance Liquid Chromatography; HUVEC, human umbilical vein endothelial cells; i.v., intravenous; LDL, Low-density lipoprotein; MALDI-TOF, Matrix Assisted Laser Desorption Ionisation-Time of Flight; mTHPP, 5,10,15,20-tetrakis(m-hydroxyphenyl)porphyrin; NRP-1, neuropilin-1; PBS,
phosphate-buffered saline; PDT, photodynamic therapy; PEG, polyethylene glycol, p.i., post injection; PS, photosensitizer; s.d., standard deviation; tBu, tertio-butyl; TEM buffer, Tris EDTA molybdate buffer; TPC, 5-(4-carboxyphenyl)-10,15,20-triphenylchlorin; VEGF, Vascular Endothelial Growth Factor.
ABSTRACT

Since angiogenic endothelial cells of the tumor vasculature represent an interesting target to potentiate the anti-vascular effect of photodynamic therapy, we recently described the conjugation of a photosensitizer (5-(4-carboxyphenyl)-10,15,20-triphenylchlorin, TPC), via a spacer (6-aminohexanoic acid, Ahx), to a Vascular Endothelial Growth Factor (VEGF) receptor specific-heptapeptide (H-Ala-Thr-Trp-Leu-Pro-Pro-Arg-OH, noted ATWLPPR), and demonstrated that TPC-Ahx-ATWLPPR binds to neuropilin-1 (NRP-1). Since peptides often display low stability in biological fluids, we examined the in vivo and in vitro stability of this conjugate by HPLC and MALDI-TOF mass spectrometry. TPC-Ahx-ATWLPPR was stable in vitro in human and mouse plasma for at least 24h at 37°C but, following intravenous injection in glioma-bearing nude mice, was degraded in vivo to various rates, depending on the organ considered. TPC-Ahx-A was identified as the main metabolic product and biodistribution studies suggested that its appearance in plasma mainly resulted from the degradation of the peptidic moiety into organs of the reticuloendothelial system. According to in vitro cell culture experiments, TPC-Ahx-ATWLPPR was also significantly degraded after incorporation in human umbilical vein endothelial cells (HUVEC), mainly into TPC-Ahx-A and, to a lesser extent, into TPC-Ahx-AT and TPC-Ahx-ATWLPP. TPC-Ahx-ATWLPPR mostly localized into lysosomes and, when HUVEC were treated with the lysosomal enzymes inhibitor ammonium chloride, this resulted in a significant decrease of the peptide degradation. This study provides essential information for the choice of the time of activation of the photosensitizer (drug-light interval) not to be exceeded and for the future design of more stable molecules.
INTRODUCTION

The selectivity of photodynamic therapy (PDT) as an anti-cancer treatment relies on the local generation of cytotoxic reactive oxygen species in the tumor, due to both preferential uptake of the photosensitizer (PS) by malignant tissue and subsequent localized light irradiation. Indeed, the PS, at the concentrations used for PDT, is non-toxic in the dark and becomes photocytotoxic only when light at an appropriate wavelength is applied. However, the tumor selectivity of PSs used in the clinic is limited, as they tend to accumulate in normal tissue (Dougherty et al., 1998). This can be improved by using third generation PSs, which consist of PSs to which a tumor-targeting moiety is attached (Sharman et al., 2004). Among these tumor-targeting molecules, peptides are receiving increasing interest in the field of PDT (Schneider et al., 2006). They do not only exist in a natural form but can also be designed synthetically as novel molecules. In addition, the effective tissue penetration of short synthetic peptides, in combination with their selective binding and internalizing capacity by cancer cells, make peptides ideal candidates for the delivery of therapeutic agents such as PSs (Schneider et al., 2006).

We recently reported the synthesis and in vitro efficacy of a new peptide-conjugated PS (referred to hereafter as TPC-Ahx-ATWLPPR) having affinity for endothelial cells of the tumor neovasculature by targeting the Vascular Endothelial Growth Factor (VEGF_{165}) receptor neuropilin-1 (NRP-1), and not the type 2 VEGF receptor (VEGFR-2/KDR), as previously thought, through its peptidic moiety (Tirand et al., 2006). TPC-Ahx-ATWLPPR displayed enhanced uptake and photodynamic properties in endothelial cells, compared to its non-conjugated counterpart TPC. Destruction of the neovasculature of tumors may indirectly lead to tumor eradication, following deprivation of life-sustaining nutrients and oxygen (Folkman, 1995; Dougherty et al., 1998), and this anti-vascular
effect is thought to play a major part in the destruction of some vascularized tumors by PDT (Ichikawa et al., 2005).

Despite their numerous advantages over other molecules (e.g. antibodies, proteins) as targeting agents, the main disadvantage of peptides is related to their natural structural conformation which makes them extremely sensitive to endo- and exo-peptidases present in most tissues (Adessi and Soto, 2002). As a result, they often display low stability in biological fluids, which limits their use in vivo. While the PS moiety of TPC-Ahx-ATWLPPR is responsible for the photocytotoxic activity of the conjugate, the peptidic part is responsible for its selectivity. Degradation of the peptidic moiety of the molecule would impair the selectivity of the conjugate, and would both allow it to accumulate in normal tissues, where the activated PS could exert non-desirable photocytotoxicity, and decrease the amount of PS delivered to targeted diseased tissues. Therefore, in order to optimize the in vivo use of the new conjugate TPC-Ahx-ATWLPPR that proved very effective in vitro (Tirand et al., 2006), we sought to investigate its stability after intravenous (i.v.) injection to mice bearing NRP-1-expressing tumors. The metabolic products were identified and quantified by Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) mass spectrometry and reverse phase High Performance Liquid Chromatography (HPLC), respectively. Besides, in order to gain mechanistic insight in the degradation process observed in vivo, the in vitro stability of TPC-Ahx-ATWLPPR was studied in human and mouse plasma as well as in human umbilical vein endothelial cells (HUVEC) that express NRP-1.
MATERIALS AND METHODS

Synthesis and purification. 5-(4-carboxyphenyl)-10,15,20-triphenyl-chlorin (TPC) was purchased from Frontier Scientific (Logan, Utah). Wang resin, 9-fluorenly-methoxy-carbonyl (Fmoc)-aminoacids and Fmoc-aminohexanoic acid were from Senn Chemicals International (Gentilly, France). The side chains of threonine and tryptophan were respectively protected by tertio-butyl (tBu) and N-tert-butoxy-carbonyl groups. The PS-peptide conjugate TPC-Ahx-ATWLPPR (Fig. 1) was synthesized on a multichannel peptide synthesizer, according to a classical Fmoc/ tBu solid phase methodology and purified by reverse phase HPLC, as described previously (Tirand et al., 2006). Stock solutions of 2 mM in dimethyl sulfoxide, aliquoted and kept in the dark at –20°C, were used.

Chemical characterization. Purity and chemical identity of TPC-Ahx-ATWLPPR were assessed by HPLC and MALDI-TOF mass spectrometry analyses, respectively, as described in corresponding paragraphs. Hydrophobicity/hydrophilicity properties of TPC-Ahx-ATWLPPR were determined using the distribution coefficient $D_{pH 7.4} = \frac{C_{octanol}}{C_{PBS}}$ of the compound between the two immiscible solvents n-octanol and phosphate-buffered saline (PBS), that had previously been pre-saturated with each other. TPC-Ahx-ATWLPPR was mixed with an equal volume of octanol (1.5 mL) and PBS (1.5 mL) at room temperature. The mixture was agitated during 3h. When equilibrium between the two phases was achieved, the solvents were separated by centrifugation (2,500g ; 5 min). The amount of PS contained in each phase was determined by HPLC. The distribution coefficient $D_{pH 7.4}$ is expressed as log (mean ± standard deviation (s.d.), n=6).
Cell line and culture. For in vitro experiments, NRP-1-expressing Human Umbilical Vein Endothelial Cells (HUVEC), pooled from several donors, were used (Cambrex, Verviers, Belgium) and routinely grown in endothelial growth medium (EGM2), as described previously (Tirand et al., 2006).

Animals and tumor model. Female athymic Foxn1 nude mice (nu/nu) were obtained from Harlan (Gannat, France), and used at 7–9 weeks old and weight of 20–25 g. Animal procedures were performed according to institutional and national guidelines. The model of human malignant glioma was obtained using U87 cells, as described previously (Tirand et al., 2006).

In vivo metabolism of TPC-Ahx-ATWLPPR. When tumors reached a volume of 70±30 mm³ (15±5 days after tumor grafting), TPC–Ahx–ATWLPPR (2.8 mg/kg) dissolved in polyethylene glycol (PEG) 400–ethanol–water (30/20/50, v/v/v) was injected via the tail vein, and mice were kept in the dark. After a time ranging from 1 to 48h, mice were anesthetized by intraperitoneal injection of ketamine (Ketalar®, Panpharma, Fougères, France) at 60 mg/kg and xylazine (Rompun®, Bayer Pharma, Puteaux, France) at 8 mg/kg. Blood samples were collected in heparinized tubes (BD Vacutainer, Becton Dickinson, Plymouth, UK) by cardiac puncture, cooled on ice and the plasma was separated by centrifugation (700g ; 10 min) within 30 min of collection. Mice were sacrificed by cervical dislocation and the tissues were carefully excised, rinsed with saline and blotted dry. The tissues selected for dissection were tumor, liver, kidney, spleen and skin (removed from the back of the mice). At least three animals were used per time point. All samples were protected from light. Blood, tumor and organs samples were kept at
-80°C in polypropylene tubes, until analysis by HPLC and/or MALDI-TOF mass spectrometry.

**In vitro stability of TPC-Ahx-ATWLPPR.** Human and mouse plasma were obtained from whole blood of human volunteers and nude mice, respectively. Mice and human blood samples were collected in heparinized tubes by heart puncture, or using an indwelling i.v. cannula placed in the arm, respectively. After immediate centrifugation at 700g for 10 min at 4°C, the supernatant (plasma) was withdrawn and frozen at -80°C in aliquots until required. TPC-Ahx-ATWLPPR was added to pre-warmed human or mouse plasma at a final concentration of 1 µM, and incubated up to 24h at 37°C. Samples were taken after 2, 6 and 24h, and kept at -80°C in polypropylene tubes, until analysis by HPLC.

For **in vitro** cell experiments, HUVEC were grown in T-25 flasks (initial seeding concentration: 5.10⁴ cells/mL) for 3 days. EGM2 medium (3 mL) containing 1 µM TPC-Ahx-ATWLPPR (with or without 30 mM ammonium chloride, Merck Eurolab A.A., Fontenay-sous-Bois, France) was then added. At different times, ranging from 1 to 24h, the EGM2 cell medium was collected. Subsequently, cells were rinsed twice with cold PBS, trypsinized, counted, centrifuged (220g ; 5 min) and kept at -80°C in polypropylene tubes, until analysis by HPLC and/or MALDI-TOF mass spectrometry. All incubations were performed in triplicates.

**Preparation of samples for HPLC and MALDI-TOF mass spectrometry analyses.**

Extraction of the PS from plasma, tumor, organs, cells and cell medium was carried out as described previously (Barberi-Heyob et al., 2004; Tirand et al., 2006). To remove surface blood, tissue samples (tumor, organs) were rinsed in physiological saline, blotted dry and
weighted. They were crushed in 500 µL TEM buffer (10 mM Tris, 1.66 mM EDTA, 5 mM molybdate, pH 7.4) using an Ultra-Turrax® T25 device (IKA, Labortechnik, Janke & Kunkel, Germany). Then, all samples were spiked with 100 µL of 5,10,15,20-tetrakis(m-hydroxyphenyl)porphyrin (mTHPP) (500 ng/mL in methanol) as an internal standard of extraction. Extraction involved solvent precipitation using methanol combined with dimethyl sulfoxide (5/0.1, v/v). Samples were then vortexed, homogenized for 30 min, and sonicated for 10 min (Branson 1200, Roucaire Instruments Scientifiques, Les Ulis, France). Tissue and cellular debris were removed by centrifugation (2,500g ; 15 min). The PS-containing organic phase was then concentrated by evaporation at room temperature for at least 3h with a Speedvac® apparatus (Fisher Bioblock Scientific, Illkirch, France) and re-suspended into 200 µL methanol.

Control (blank) plasma, tumor, organs, cells and cell medium (*i.e.* containing no exogenous PSs) were used in order to determine whether endogenous constituents coeluted with the PSs. Calibration samples, used to construct calibration curves, were prepared by mixing plasma, tumor, organs, cells or cell medium with appropriate concentrations of PS. For these control and calibration samples, the PS extraction procedure was identical to that described above.

**High Performance Liquid Chromatography (HPLC) analysis.** The chromatographic system was composed of a programmable solvent module (System Gold 126, Beckman), an autosample injector (507e, System Gold, Beckman) and a scanning fluorescence detector (RF-10A XL, Shimadzu). Analyses were performed by reverse phase HPLC on a C18 analytical column (250 × 4.6 mm i.d., S-5µm, YMC, Interchim, France), under isocratic elution conditions with a mobile phase of methanol-H₂O (95/5, v/v) and a flow rate of 1 mL/min. Fluorescence emission was detected at 652 nm, with an excitation
wavelength set at 416 nm. These wavelengths correspond to the maximal intensities of the fluorescence emission and absorption spectra of TPC-Ahx-ATWLPPR, respectively (Tirand et al., 2006). All chemicals were of analytical grade quality. The chromatograms were acquired and analyzed using GOLD™ Nouveau software version 1.6 (Beckman Coulter France A.A., Roissy CDG, France). Quantitations were based on peak areas and deduced from calibration curves.

Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) mass spectrometry analysis. 1 µL of a 2,5-dihydroxybenzoic acid solution (150 mg/mL in water/acetonitril, 1/1, v/v) was mixed with 1 µL of sample and spotted on the stainless steel MALDI targets. The solvent was evaporated prior to insertion in the source. Mass spectra were acquired over the range 0–2000 Da. Analyses were performed on a Bruker Reflex IV time-of-flight (TOF) mass spectrometer (Bruker-Daltonic, Bremen, Germany) equipped with the SCOUT 384 probe ion source. The system used a pulsed nitrogen laser (337 nm, model VSL-337ND, Laser Science Inc., Boston, MA) with a maximal energy of 400 µJ/pulse. The detector signals were amplified and transferred to the XACQ program on a SUN work station (Sun Microsystems Inc. Palo Alto, CA). Spectra were processed with the XMass 5.1 program (Bruker Daltonics, Bremen, Germany). External calibration of MALDI mass spectra was carried out using sodic and potassic distribution of PEG 600 and PEG 1500 mixtures.

Intracellular localization by confocal laser scanning microscopy. Exponentially growing HUVEC were plated at $10^3$ cells per well in Labtek-II® 8-chambered coverslips (Dutscher, Brumath, France). After a 48-h attachment and growth period at 37°C, the cells were incubated with TPC-Ahx-ATWLPPR (1 µM) for 24h and specific fluorescent
organelle markers (Molecular Probes-Europe, Leiden, The Netherlands), using the
procedure adapted from the experimental protocol previously reported (Di Stasio et al.,
2005). The endoplasmic reticulum was stained by incubating cells for 30 s at room
temperature with 1 μg mL⁻¹ of 3,3'-dihexyloxacarbocyanine iodide (DiOC₆). Mitochondria were identified after staining the cells for 30 min at 37 °C with 0.5 μM MitoTracker® Green. LysoTracker® Green was used at a final concentration of 0.2 μM for 30 min to identify lysosomes. To visualize the Golgi apparatus, cells were labeled with 2 μM of N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosine (BODIPY® FL C₅-ceramide, BPC) for 20 min at 4°C, and then further incubated in dye-free EGM2 medium at 37°C for 30 min. At the end of the double staining, the labeling solution was removed by gentle rinsing with EGM2 medium, and cells were imaged using a confocal laser scanning microscope (TCS SP2-AOBS, Leica microsystem, Germany) equipped with an x63, numerical aperture 1.3 oil immersion objective (Leica). A pinhole of 60.85 μm was used and each image recorded contained 512x512 pixels. An Argon laser was used as excitation light at 488 nm for all organelle probes and TPC-Ahx-ATWLPPR. Fluorescence of the organelles probes was detected on channel 1 with a 505–545 nm band pass emission filter. Channel 2 was used to detect the red fluorescence of TPC-Ahx-ATWLPPR with a 640–660 nm band pass emission filter. The fluorescence images were displayed in green and red “false” colors, for organelle markers and PS, respectively.
Statistical analysis. Unless otherwise indicated, all data are mean values ± s.d. calculated from at least three independent experiments. Mann and Whitney U test was used to test for the significant level between independent variables. The level of significance was set to p<0.05.
RESULTS

Chemical characterization of TPC-Ahx-ATWLPPR. The molecular structure of the conjugate TPC-Ahx-ATWLPPR is shown on figure 1. The peptide-conjugated chlorin was obtained with a final purity greater than 95%, as assessed by analytical reverse phase HPLC (Fig. 2A). Several peaks can be observed on the chromatogram. The peak eluted at 5±1 min corresponds to mTHPP, used as an internal standard. The double peak with a retention time of about 21 min corresponds to TPC-Ahx-ATWLPPR. The two peaks arise from the presence of two isomers of the molecule, due to the reduction of a double bond on either opposing side of the tetrapyrrolic macrocycle. These isomers could also be observed in the commercial TPC used for synthesis. Identity was confirmed by MALDI-TOF mass spectrometry (theoretical mass = 1596.81 Da, experimental mass = 1596.83 Da, Fig. 2B). The value of the octanol/ PBS distribution coefficient log $D_{\text{pH 7.4}}$ was equal to 2.6±0.2, arguing for the hydrophobic character of TPC-Ahx-ATWLPPR.

In vivo plasma stability. No degradation product of TPC-Ahx-ATWLPPR could be observed in vivo in plasma up to 1h after i.v. injection (not shown). From 2h post injection (p.i.), peaks with shorter retention times (12 ± 2 min) than those corresponding to TPC-Ahx-ATWLPPR (21 ± 3 min) appeared on the HPLC chromatograms of plasma and increased as a function of time after injection (Fig. 3A1 and Fig. 3A2). We identified the predominant metabolic product as resulting from the enzymatic cleavage of the peptide bond between the alanyl and threonyl residues (Fig. 3B). This degradation product (TPC-Ahx-A) was detected in plasma at all time points from 2h p.i. (Fig. 3C). However, the amount of degradation product in plasma was relatively low (less than 20% of the total amount of PSs), up to 4h p.i.
**In vitro stability in plasma.** In order to assess the involvement of the plasma compartment in the degradation process, we followed the *in vitro* stability of TPC-Ahx-ATWLPPR in plasma. A concentration of 1 µM was chosen, as it represents a relevant *in vivo* concentration. Indeed, after injection of 2.8 mg/kg of TPC-Ahx-ATWLPPR in glioma-bearing mice, the *in vivo* plasma concentrations of this peptide-photosensitizer conjugate were in the range of 200 to 6,000 ng/mL (0.1 – 3.8 µM), from 1 to 48h p.i. (Figure 3C). TPC-Ahx-ATWLPPR was stable *in vitro* in human plasma, up to 24h at 37°C (Fig. 4). Likewise, no degradation could be observed in mouse plasma, up to 24h in the same conditions (*not shown*). This suggested that the degradation observed in mouse plasma *in vivo* did not involve plasma peptidases.

**In vivo biodistribution and metabolism in glioma-bearing nude mice.** In order to explore the biodistribution and stability of TPC-Ahx-ATWLPPR in tumor-bearing mice, PS concentrations were determined in the tumor and organs of mice (n=3 or 4) at different times. Following i.v. injection into tumor-bearing mice, PS accumulated to the highest levels in liver and spleen (Fig. 5A). It should be noted that part of the PSs quantities estimated in the liver, a highly vascularized organ, is due to PS present in the blood circulation, as our values have not been corrected for blood content of the tissue. Moreover, endogenous porphyrins (*e.g.* heme) are present at significant levels in the liver. These porphyrins could not be completely separated from our PSs; indeed, part of their elution peaks superimposed to the ones of our PSs (*i.e.* similar retention times). As a result, the real PS quantities in the liver are in fact lower than those presented on figure 5A, but would remain much higher than the PS quantities in all the other organs considered. Total PS levels in the tumor were higher than in the skin at all time points (Fig. 5A).
Whereas TPC-Ahx-ATWLPPR was stable in vitro in plasma for at least 24h, a degradation product could be observed in vivo in plasma (Fig. 3A2), as well as in other organs (Fig. 5A). In the organs of the reticuloendothelial system, TPC-Ahx-ATWLPPR was rapidly degraded, and the major part of the hydrolysis of the peptide bond(s) occurred in the liver (Fig. 5A). Indeed, the degradation product represented more than 85% of the total amount of PS present in the liver as early as 2h p.i. TPC-Ahx-A was characterized as the main metabolic product from liver by MALDI-TOF analysis (Fig. 5B). On the contrary, the percentages of degradation measured at 2h and 4h p.i. in the tumor were inferior to 25% and 45%, respectively (Fig. 5A). Likewise, the extent and rate of degradation in the skin were very low, with no degradation observed up to 4h p.i. (Fig. 5A). Therefore, the degradation observed in vivo in the plasma likely resulted from a release from some organs (i.e. liver, spleen), where the degradation took place.

**In vitro stability in cell medium and endothelial cells.** TPC-Ahx-ATWLPPR was stable in EGM2 medium up to 24h at 37°C (Fig. 6A). No degradation product could be observed in the cell medium, and the supernatant was progressively depleted in TPC-Ahx-ATWLPPR, due to its high rate of uptake in HUVEC (Fig. 6A). On the contrary, TPC-Ahx-ATWLPPR was progressively degraded inside HUVEC into products that were still fluorescent and, therefore, potentially photocytotoxic (Fig. 6B). The amount of degradation increased as a function of incubation time. TPC-Ahx-ATWLPPR was significantly degraded (p<0.05) from 6h after incorporation by HUVEC. By MALDI-TOF mass spectrometry, a main degradation product (TPC-Ahx-A) and two secondary metabolites (TPC-Ahx-AT and TPC-Ahx-ATWLPP) were identified (Fig. 7).
**Localization of TPC-Ahx-ATWLPPR.** Intracellular localization in HUVEC following incubation with TPC-Ahx-ATWLPPR (1 µM, 24h) was studied using fluorescent organelle probes, and confocal fluorescence microscopy. Yellow color on merged images from intracellular localization of the PS (red) and the probe (green) indicates colocalization of both (Fig. 8). TPC-Ahx-ATWLPPR mostly localized into lysosomes (Fig. 8A), compared to Golgi apparatus, mitochondria and endoplasmic reticulum (Fig. 8B, C and D, respectively).

**In vitro stability of TPC-Ahx-ATWLPPR in HUVEC in presence of an inhibitor of lysosomal enzymes.** Ammonium chloride is a lysosomotropic weak base that, as such, inhibits the action of lysosomal enzymes by increasing lysosomal pH. As shown on figure 9, the addition of this inhibitor to the cell medium significantly decreased the percentage of intracellular degradation of TPC-Ahx-ATWLPPR, arguing for the role of lysosomal enzymes in the degradation process.
DISCUSSION

It is well known that, following its in vivo administration, a drug is progressively cleared and/or metabolized into active or inactive metabolites. The photocytotoxic activity of the TPC-Ahx-ATWLPPR molecule is brought by the PS moiety, while selectivity for the tumor neovasculature is due to the presence of the peptidic component. Therefore, inactive metabolites would result from the breakdown of the macrocycle, while sole degradation of the peptide would be detrimental to selectivity but would still give rise to active molecules. Indeed, one of the main disadvantages of peptides is their sensitivity to proteolysis, which limits their use in vivo (Adessi and Soto, 2002). In this work, we first investigated the in vivo stability of TPC-Ahx-ATWLPPR following i.v. injection into tumor-bearing mice. A significant degradation could be observed in plasma from 4h p.i. We identified the main metabolic product as being TPC-Ahx-A. This metabolite would very probably have lost any selectivity for angiogenic endothelial cells that line the neovessels of the tumor, since the arginyl residue at the C-terminus of the ATWLPPR peptide has been shown to be essential for affinity for NRP-1 (Tournaire et al., 2001). Therefore, for future in vivo assessments of photodynamic activity of TPC-Ahx-ATWLPPR, the drug-light interval (DLI, i.e. the time interval between PS injection and its subsequent activation by light) should preferably, according to this study, be inferior or equal to 4h. Indeed, after that time, more than 20% of the PSs present in plasma is TPC-Ahx-A, which does not possess any specificity for endothelial cells lining the tumor vessels, contrary to TPC-Ahx-ATWLPPR. Using a DLI longer than 4h would certainly allow PS accumulation in non-targeted tissues (e.g. skin), with potential skin photosensitivity problems, but would probably not improve much its uptake in target tissues (i.e. endothelium of tumor vessels).
The PSs (TPC-Ahx-ATWLPPR and TPC-Ahx-A) accumulated at much higher levels in the liver than in the tumor. This has also been observed for other hydrophobic photosensitizers, including Foscan®, with tumor to liver ratios of PS concentrations as low as 0.03 (Jones et al., Br J Cancer, 2003), but only PSs present in the tumor will be activated, due to localized light irradiation. It should be noted that PSs present at the time of irradiation in plasma and endothelial cells lining the tumor vessels, and not only in tumor cells, also play a major part in tumor eradication by PDT, by inducing vascular damage and occlusion of tumor vessels, which leads to tumor deprivation in nutrients and oxygen (Cramers, Br J Cancer, 2003).

A variety of proteases are present in human plasma, including both exopeptidases and endopeptidases (McDonald and Barrette, 1980; McDonald and Barrette, 1986). Therefore, we assessed the in vitro stability of TPC-Ahx-ATWLPPR in mouse and human plasma and did not observe any degradation during at least 24h at 37°C, thus discarding any role of the plasma peptidases in the degradation phenomenon. The conjugated PS accumulated to high levels into organs of the reticuloendothelial system (i.e. liver, spleen), in agreement with its hydrophobic character, like other lipophilic PSs, e.g. Foscan® (Jones et al., 2003; Jori and Fabris, 1998). Indeed, upon administration into the bloodstream, most PSs associate with various serum proteins including both high and low density lipoproteins (LDLs) and albumin (Hopkinson et al., 1999). LDLs are believed to be important in the transportation of hydrophobic PSs. α2-Macroglobulin/LDL receptors are widely found in cells of various types, with LDL uptake most pronounced in adrenals, liver and spleen (Rosenkranz et al., 2000).

The conjugated PS was rapidly degraded in organs of the reticuloendothelial system, as soon as 1h p.i. Some degradation also occurred quite rapidly in the tumor, less than in metabolic organs but more than in plasma. All these results strongly suggested that the
degradation product TPC-Ahx-A identified in plasma resulted from the degradation of the peptide in organs of the reticuloendothelial system, mainly liver, and subsequent release into the bloodstream.

In order to gain mechanistic insight into the degradation phenomenon, we undertook *in vitro* studies in HUVEC. TPC-Ahx-ATWLPPR was not degraded into the cell culture medium, thus allowing it to accumulate at high levels into cells through active targeting of NRP-1, as confirmed by the high depletion rate of the PS observed in the medium. On the contrary, following internalization by HUVEC, the peptide moiety of TPC-Ahx-ATWLPPR was progressively degraded, mostly into TPC-Ahx-A, as observed *in vivo*, but also, to a lesser extent, into TPC-Ahx-AT and TPC-Ahx-ATWLPP. Degradation of the peptidic moiety inside cells is not detrimental to our strategy, since the peptide has already played its targeting role, and that, at this stage, only the PS moiety is involved in the photocytotoxic effect.

The distribution of a PS within cells depends on the route by which it enters, as well as on its physicochemical properties, *e.g.* its hydrophobicity/hydrophilicity; the type, number and arrangement of its charged groups; the presence of a central atom in the tetrapyrrole structure; its aggregation state *etc* (Rosenkranz et al., 2000). For example, LDL-bound PSs can enter target cells *via* receptor-mediated endocytosis. Chlorin e6 covalently bound to LDLs has been detected in enzymatically active lysosomes (Schmidt et al., 1992). We showed that TPC-Ahx-ATWLPPR also mostly localized into lysosomes, in agreement with the fact that TPC-Ahx-ATWLPPR targets NRP-1 and should be incorporated into cells by a receptor-mediated endocytosis mechanism. Lysosomes are known to contain a large variety of hydrolytic enzymes, which degrade proteins and other substances taken up by endocytosis. The main proteolytic activities experienced by endocytosed peptides and proteins in the endosomal/lysosomal pathway belong to a family of papain-like proteases.
called cathepsins (Roberts, 2005). pH rate profiles indicate that the low pH of the lysosomes (approximately 5.5) is optimal for the majority of cathepsins. Treatment with the lysosomotropic weak base ammonium chloride significantly decreased the extent of intracellular degradation.

Since some peptides can be significantly stabilized by glycosylation or the addition of D-aminoacids at the N-terminus (Powell et al., 1993), the relatively slow rate of degradation observed in plasma may be due, at least in part, to the steric protection afforded by the PS moiety at the N-terminus, which may prevent the peptide from being degraded by aminopeptidases. Moreover, no oxidation into a porphyrin, that would present less interesting photophysical properties than the corresponding chlorin, could be observed on the PS moiety in any experiment, as assessed by MALDI-TOF analysis, contrary to what has been observed with other PSs (Laville et al., 2004).

In order to achieve higher selective delivery to the tumor neovessels, the in vivo stability of the peptide moiety of the conjugate may be increased through formulation and/or chemical modification. The first strategy encompasses the use of pegylated liposomes, which will impact on the pharmacokinetics of the PS and which may limit its accumulation in the reticuloendothelial system. For instance, Ichikawa et al encapsulated benzoporphyrin derivative monoacid ring A in pegylated liposomes modified with the H-Ala-Pro-Arg-Pro-Gly-OH (APRPG) pentapeptide, which had earlier been isolated as a peptide specific to angiogenic endothelial cells. Pegylation of liposomes aims to avoid opsonization in the bloodstream, which is a prerequisite for the clearance of the liposomes by the reticuloendothelial system, such as liver and spleen (Ichikawa et al., 2005). The second method implies the generation of modified peptides with improved stability properties, through e.g. cyclisation, the use of D- amino acids and reduced peptide bonds.
etc. (Adessi and Soto, 2002), for which the knowledge of the site of degradation on the peptide that were determined in this study is essential.

The ATWLPPR peptide has already been used in several *in vitro* and *in vivo* studies (Janssen et al., 2003; Rodrigues et al., 2003; Perret et al., 2004; Renno et al., 2004), but, to the best of our knowledge, none has reported on its instability. The present study draws attention to this potential problem with peptides, especially in the case of targeting strategies, and provides useful information for the choice of the DLI for *in vivo* assessments of photodynamic activity and for the future design of more stable molecules.
References


Footnotes

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Legends for Figures

**Figure 1.** Molecular structure of one of the isomers of the peptide-chlorin conjugate TPC-Ahx-ATWLPPR. Arrows indicate the two possible sites of double bond reduction during synthesis of a chlorin from a porphyrin, leading to a mixture of two isomers.

**Figure 2.** A. HPLC chromatogram of TPC-Ahx-ATWLPPR (retention time, 21 ± 3 min) after synthesis and purification, supplemented with mTHPP (retention time, 5 ± 1 min) as an internal standard. The two peaks corresponding to TPC-Ahx-ATWLPPR arise from the presence of two isomers of the molecule, due to its asymmetrical character. B. MALDI-TOF mass spectrum of TPC-Ahx-ATWLPPR after synthesis and purification (theoretical mass = 1596.81 Da, experimental mass = 1596.83 Da).

**Figure 3.** A. Representative HPLC chromatograms of plasma extracts 2h (1) and 4h (2) after i.v. administration of 2.8 mg/kg of TPC-Ahx-ATWLPPR to glioma-bearing nude mice, supplemented with mTHPP (retention time, 5 ± 1 min) as an internal standard. Peaks eluted at shorter times (12 ± 2 min) than TPC-Ahx-ATWLPPR (21 ± 3 min) appear on the chromatograms; their intensity increases as a function of the time after i.v. injection. For chromatographic conditions and extraction procedure, see Materials & Methods. B. MALDI-TOF mass spectrum of plasma extracts 4h p.i. C. In vivo plasma stability of TPC-Ahx-ATWLPPR in nude mice. Plasma concentrations, determined by HPLC, of TPC-Ahx-ATWLPPR [circle] and TPC-Ahx-A [triangle] are expressed as a function of time p.i. (data points show the mean ± s.d., n≥3 ).

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**Figure 4.** Representative HPLC chromatograms of human plasma extracts following 2h (A), 6h (B) and 24h (C) *in vitro* incubation at 37°C with TPC-Ahx-ATWLPPR (1 µM), supplemented with *m*THPP (retention time, 5 ± 1 min) as an internal standard.

**Figure 5.** A. Biodistribution of the PSs (*black*, TPC-Ahx-ATWLPPR; *light grey*, degradation product) in different organs, according to time after i.v. administration of 2.8 mg/kg of TPC-Ahx-ATWLPPR in tumor-bearing mice. The data presented are the mean percentages of injected dose per gram of tissue (*n*=3 or 4). Values were not corrected for blood content of the tissue. Some of the high levels of PSs in liver and spleen are written in brackets at the top of the corresponding bars. PSs in plasma and endogenous molecules (*e.g.* heme) account for a non-negligible part of the PSs quantities estimated in the liver, a highly vascularized organ. B. MALDI-TOF mass spectrum of liver extract 24h p.i. of TPC-Ahx-ATWLPPR.

**Figure 6.** *In vitro* stability of TPC-Ahx-ATWLPPR in EGM2 cell medium and inside HUVEC, after its incorporation. Amount of TPC-Ahx-ATWLPPR [circle] and TPC-Ahx-A [triangle], expressed as a function of time in EGM2 medium (A) and inside cells, after extraction (B), at different times, ranging from 1 to 24h (data points show the mean ± s.d., *n*=3).

**Figure 7.** MALDI-TOF mass spectrum of the PS incorporated by HUVEC, following incubation with 1 µM of TPC-Ahx-ATWLPPR during 24h, after extraction.

**Figure 8.** Confocal fluorescence microscopy images of HUVEC double stained with TPC-Ahx-ATWLPPR (*in red*) and an organelle probe (*in green*). A. LysoTracker® Green-
stained lysosomes. **B.** BPC-stained Golgi apparatus. **C.** MitoTracker® Green-probed mitochondria. **D.** DiOC6-stained endoplasmic reticulum.

**Figure 9.** Percentage of degradation product inside HUVEC following incubation with TPC-Ahx-ATWLPPR (1 µM; 6h or 24h), with (*light grey bars*) or without (*white bars*) lysosomal peptidase inhibitor (ammonium chloride 30 mM) treatment, as assessed by reverse phase HPLC.
Fig. 1
Fig. 2

A

Fluorescence intensity (a.u.)

Retention time (h)

B

TPC-Ahx-ATWLPPR

1596.83

m/z

Fluorescence intensity (a.u.)
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 9