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Pharmaceutical Technology at the Service of Targeted Drug Delivery

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Abstract: Research in pharmaceutical technology has drifted from formulation of systems with improved drug absorption and bioavailability to systems targeting molecular sites of diseases. The research unit of Pharmaceutical Technology from the University of Geneva focuses on the development of systems for both diagnostic and therapeutic purposes. Three types of constructs for targeting are reviewed. With a fine-tuning of size and surface composition, polymeric nanoparticles are developed to improve detection of micrometastasis by fluorescence imaging. Furthermore, surface coating with specific antibodies increase the therapeutic efficiency of the encapsulated chemotherapeutic agent for tumor treatment in animal models. Constructs that are activated by remote sources of energy are investigated in the unit. For instance, microbubbles bearing specific antibody fragments at their surface are useful contrast agents for ultrasound molecular imaging. Microbubbles, if combined with a thrombolytic drug and ultrasound, improve clot lysis, which is promising for stroke treatments. Enzymatically activated prodrug scaffolds are also under development. With this approach, intrinsic enzymatic activity of a diseased tissue activates the formulations. This concept led to the development of theranostic agents that can be used for both diagnostic and therapeutic purposes.

Keywords: Drug delivery systems · Drug targeting · Molecular imaging · Theranostic agents

1. Introduction

Traditionally, the main task of pharmaceutical technology was the optimization of dosage forms for drug release at the site of absorption. Conventional dosage forms are needed, but current research primarily focuses on the delivery at the cellular level rather than at the site of absorption. The Pharmaceutical Technology research unit at the School of Pharmaceutical Sciences (University of Geneva, University of Lausanne) designs original delivery systems for diagnostic and therapeutic purposes. Targeting is central to this research. Several projects are developing systems, constructs or scaffolds that bear a targeting moiety, such as peptides and antibodies. These constructs can be designed for molecular imaging or therapeutic applications depending on the content of the systems. The research unit also designs constructs activated by remote sources of energy, such as ultrasound or light, providing synergetic effects and improved targeting. Further improvements of the drug delivery systems are achieved by designing scaffolds that can be activated by intrinsic enzymatic pathways characteristic for a particular disease. Some formulations are designed to have a dual function enabling diagnostic imaging as well as a therapeutic action; these agents are termed theranostic agents.

Three topics have been chosen for the present review: the formulation of polymeric nanoparticles evolving towards actively targeted systems, ultrasound-sensitive microbubbles for theranostic applications, and research on light-sensitive formulations and prodrugs that can be activated through enzymatic triggers.

2. Passive and Active Targeting of Ovarian Cancer, the Benefit of Polymeric Nanoparticles

Nanocarriers have been widely investigated for therapeutic applications in cancer. This concept is based on the encapsulation of chemotherapeutic agents in colloidal carriers acting as a cargo. Pharmaceutical nanocarriers are lipidic or polymeric particles of 1 to 1000 nm in diameter in which the drug is entrapped, encapsulated, or adsorbed. These carriers include micelles, liposomes, polymeric nanoparticles (nanospheres and nanocapsules), solid lipid nanoparticles, nanogels and dendrimers. Polymeric nanoparticles (NPs), which consist of a dense polymeric network, have received much attention. NPs offer several advantages, including the possibility to formulate poorly soluble drugs (*e.g.* paclitaxel) with a high drug-loading capacity. NPs also exhibit favorable distribution in cancerous tissues due to the enhanced permeability and retention effect. Furthermore, the surface of these nanocarriers may be modified with specific ligands, such as antibodies, aptamers, glycoproteins, lectins, or peptides, to favor interaction with a defined target.

Ovarian cancer is the fifth most fatal form of cancer in European and American women. The survival rate primarily depends on the disease stage at the time of diagnosis. When diagnosed at stage III or more advanced stages, the five-year mortality rate reaches 70%.[1] Combined taxane- and platinum-based chemotherapy is applied after surgery to achieve clinical remission. However, most patients suffer from recurrence within 18 to 24 months, and repeated treatments are less effective because of progressive drug resistance. Our group has developed polymeric NPs for this indication following two directions. The first strategy applies NPs to enhance the early detection of micrometastases to improve diagnosis and tumor resection. The second strategy uses tumor recognition with specific antibodies linked to drug-loaded NPs.

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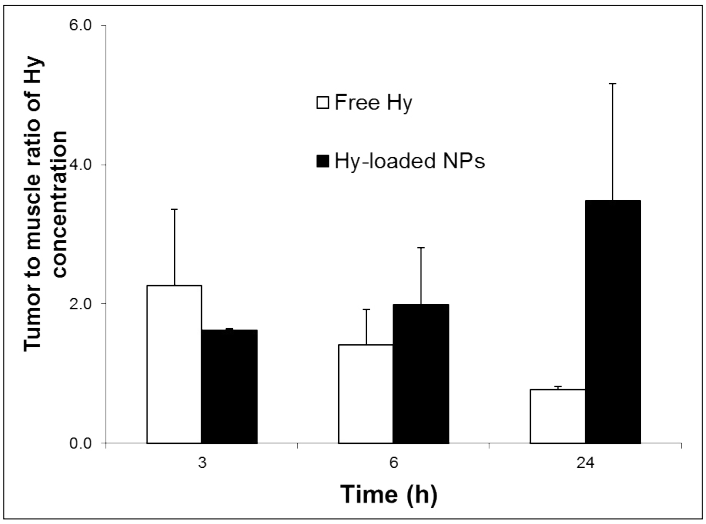

Fig. 1. Biodistribution of hypericin (2 mg/kg, iv) in Fischer F-344 rats with NuTu-19 tumors expressed as tumor to surrounding muscle ratio of hypericin concentrations at different time points of administration (n = 3).

Fluorescence photodetection (PD) using photosensitizers (PS) is a potent technique to optimize tumor resection and spare normal tissue.[2] Hypericin (Hy), a natural compound extracted from *Hypericum perforatum* is an interesting drug for PD in oncology. Hy has exhibited promising results for bladder cancer detection in the chick chorioallantoic membrane model and humans.[3,4] As Hy is a hydrophobic drug, systemic intravenous administration is challenging and restricts its medical applications. Polymeric NPs may overcome the delivery issues of Hy, as previously suggested for other PS.[5]

Hy-loaded NPs were produced, and the size and drug-loading capacity were characterized.[6] The feasibility of fluorescence PD using the encapsulated Hy was evaluated *in vivo* and compared to free Hy in Fischer 344 rats bearing ovarian cancer (NuTu-19 cell line). The fluorescence contrast after laparatomy was higher when NPs were used compared to a Hy-containing solution. An analysis of the tissue content after Hy extraction from tumors and surrounding muscle confirmed these direct observations (Fig. 1). For the free drug, tumor concentrations were maximal 3 h after administration and decreased steadily to an equal concentration in tumor and muscle at 6 and 24 h. In contrast, the maximal tumor concentrations of Hy using NPs were achieved at 24 h with a tumor to muscle ratio of 3.5. A more selective accumulation in the ovarian tumor was achieved using NPs than free drug.[7] These results are typically a good example of passive targeting, in which a higher tissue drug concentration is obtained due to a preferable distribution of the particles in the cancerous tissue, which is irrigated by a leaky vasculature.

Paclitaxel (Tx) is one of the most efficient agents against ovarian cancer, but it suffers from poor water solubility and a low therapeutic index. These features are associated with serious side effects partially due to a suboptimal biodistribution. Actively targeted therapy can be defined as using the recognition of a molecular entity specific to the disease, affected organ or associated cells to guide the otherwise toxic drug to its site of action. This strategy is particularly attractive for cancer therapy due to the intrinsically high toxicity of most chemotherapeutic drugs related to the unfavorable biodistribution to both cancerous and healthy tissues. Furthermore, this approach is appealing towards cancers that spread as micrometastases, such as ovarian cancer. Targeted therapy uses a ligand specific to a molecular target that is overexpressed by the diseased cells. Most strategies directly couple the drug to the targeting entity. However, this methodology involves a direct and often complex chemistry between these entities. The resulting entities are often too large for the active drug to find its intracellular target. The use of a drug carrier is appealing in this context. This concept uses the encapsulation of a large amount of chemotherapeutic agent in colloidal carriers. The surface of these nanocarriers may be functionalized with specific ligands, such as antibodies, aptamers, glycoproteins, lectins or peptides, to favor the interaction with a defined target.

Nanoparticles were obtained by functionalizing the surface of paclitaxel-loaded nanoparticles (NP-Tx) with monoclonal antibodies (mAbs).[8,9] Trastuzumab (Herceptin®, HER) was used as targeting moiety for ovarian cancer cells that overexpressed Her2-specific antigens.[10]

The efficacy of these immunonanoparticles (NP-Tx-HER) has been evaluated in a disseminated xenograft ovarian cancer model that was induced using an intraperitoneal (IP) inoculation of SKOV-luc-D3 cells that overexpressed Her2 antigens. The evaluation of therapeutic efficacy by bioluminescence imaging clearly demonstrated the superior antitumor activity of NP-Tx-HER compared to free Tx (Fig. 2). Moreover, a significantly longer survival rate was observed for mice treated with NP-Tx-HER compared to free Tx, Herceptin® alone or Tx-loaded nanoparticles coated with an irrelevant mAb (Mabthera®, rituximab). The biodistribu-

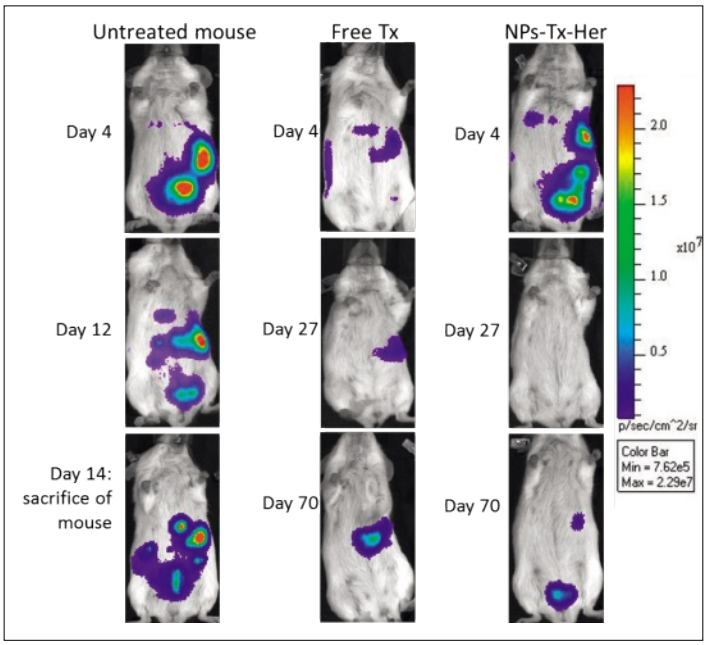

Fig. 2. *In vivo* monitoring of SKOV-luc-D3 peritoneal ovarian cancer growth using bioluminescence imaging. Treatment of alternate IP and IV administrations of either saline (controls), free paclitaxel (free Tx) or herceptin-tagged paclitaxel-loaded nanoparticles (Np-Tx-Her) every three days was initiated five days after tumor implantation and completed on day 20. The mice were imaged ventrally between 4 and 70 days after SKOV-luc-D3 cell inoculation. The images were set at the same color scale for evaluation of tumor growth over time (n = 3).

tion pattern of Tx was assessed in healthy and tumor-bearing mice after intravenous (IV) or IP administration. An equivalent biodistribution profile for Tx encapsulated either in uncoated nanoparticles (NP-Tx) or NP-Tx-HER was observed in healthy mice after IV or IP injections, except for a lower drug accumulation in lungs when the formulations were administered IP. A single dose injection (IV or IP) of encapsulated Tx in mice bearing tumors induced a higher tumor accumulation compared to free Tx. However, no difference in overall tumor accumulation between NP-Tx-HER and NP-Tx was observed.

The results of these two approaches demonstrate the interest in the development of polymeric nanoparticles for the treatment and diagnosis of cancer. Research on the targeted delivery of chemotherapeutic drugs is ongoing in systems using new recognition entities that have been developed in collaboration with Dr. Marie Cohen of the Department of Obstetrics and Gynecology of the Faculty of Medicine of the University of Geneva.

3. Ultrasound-sensitive Microbubbles: From Diagnostic Use to a Therapeutic Modality

Various ultrasound (US) imaging modalities have been used clinically for years such as Doppler and B-mode. Even more than X-ray or MRI, it is a widespread ubiquitous imaging technique. Ultrasound imaging is essentially based on the backscatter of ultrasound waves induced by variation of tissue impedance. It is used as such for most of the exams in gynecology, urology, hepatology. For more than 15 years now, this imaging modality benefits from acoustic contrast agents that enhance the vascular compartments of the body. Based on early observations of an improved image contrast in the presence of air microbubbles (MBs) in the bloodstream, research has gone towards the formulation of stabilized MBs. The rationale of MBs as ultrasound contrast agents (UCA) is based on their compressibility. MBs undergo volumetric oscillation under acoustic pressure waves, which produces an intense backscattered acoustic signal providing sharp images. Nowadays, modern medical ultrasound imaging systems are not only monitoring the acoustic backscatter of bubbles at fundamental frequency but also take advantage of the nonlinear oscillation of MBs and the generation of harmonic signals.

Several approaches for the production of stabilized MBs were investigated.[11] Stabilization of fluorinated droplets with phospholipids was the most successful, leading to several commercialized products, among them SonoVue® and Definity®. The size of these UCAs is well controlled and small enough to freely circulate in the bloodstream for the few minutes that is required to image a patient. These so-called 'blood-pool agents' are very useful for the diagnosis of a variety of diseases that are characterized by an impaired or enhanced blood circulation, such as myocardial infarction and tumor tissue, respectively.

The advent of molecular imaging led to the development of targeted MBs. UCAs with a high acoustic impedance mismatch that display targeting ligands on their surface have been conceived. UCAs that recognize and bind to vascular markers of inflammation (*e.g.* selectins), neovascularization (*e.g.* integrins) or blood clots would significantly improve the diagnosis and treatment follow-up of a variety of patients.

Stroke is the third leading cause of death and disabilities in industrialized countries.[11] However, the outcome of current treatments could be dramatically improved if an accurate diagnosis was performed very early in the first hours after the onset of the event. The detection of the precise location, size, and type of blood clots would reduce the burden of stroke. Therefore, molecular imaging using specific thrombus-targeting contrast agents was initiated. We developed perfluorocarbon-filled phospholipids-based UCA targeted to the GP IIb/IIIa receptor, which is expressed by activated platelets, using abciximab as the targeting moiety.[12] The commercially available antigen-binding fragment (Fab) of the monoclonal chimeric antibody 7E3, abciximab, prevents cardiovascular complications during or after coronary interventions. This agent was used to target platelet-rich thrombi in our studies. Abciximab was chemically grafted to the 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(*p*-maleimido-phenyl)butyramide] sodium salt-containing MBs *via* reduction of the disulfide bond that links the constant region of the heavy to light chain of the Fab. The high concentration of GP IIb/IIIa on activated platelets, which is the most important cellular component of arterial thrombi, was the rationale of the choice of this particular target. The abciximab-grafted MBs exhibited high static and dynamic binding to fixed human platelets. The ultrasonographic detection of white and red clots using targeted MBs yielded higher signals compared to commercial non-targeted blood-pool MBs. In subsequent *in vivo* studies, molecular imaging of human thrombi inserted in rat carotids was evaluated.[13] The *in vivo* ultrasonic examination was significantly improved in the clots that were targeted with abciximab-grafted microbubbles. The quantification of *in vivo* contrast image enhancement displayed a significant signal increment for abciximab-grafted MB-targeted clots compared to nonspecific immunobubble-targeted clots.

These data showing improved clot diagnosis provided the impetus towards sonothrombolysis, which is a promising adjuvant therapy for the treatment of ischemic stroke. Recent human studies have demonstrated that the outcome in patients treated with a thrombolytic drug (recombinant tissue plasminogen activator, rtPA) is improved with the combination of ultrasound and MBs. However, the exact mechanisms of the sonothrombolysis process remain unknown. Therefore, *in vitro* studies on human blood clots were initiated to understand the complex mechanisms of lysis using various sonothrombolysis protocols.[14] Scanning electron microscopy and immunostaining confirmed the presence of red blood cells, fibrin, and platelets in the clots. Ultrasound without MBs exerted no effect on clot lysis. No differences between US alone and untreated control or between rtPA + US and rtPA were observed. US + MB exhibited no effect on clot lysis in the absence of rtPA. Only the concomitant use of rtPA + US + MB significantly increased lysis compared to other treatments (Fig. 3). The most efficient combined treatment was achieved at an acoustic pressure of 600 kPa with MBs in circulation and a low dose of rtPA (0.3 µg/mL). This lysis rate was similar to the rate that was obtained with a 10-fold higher dose of rtPA alone (3 µg/mL), which indicated the possible reduction in rtPA doses in future clinical protocols.

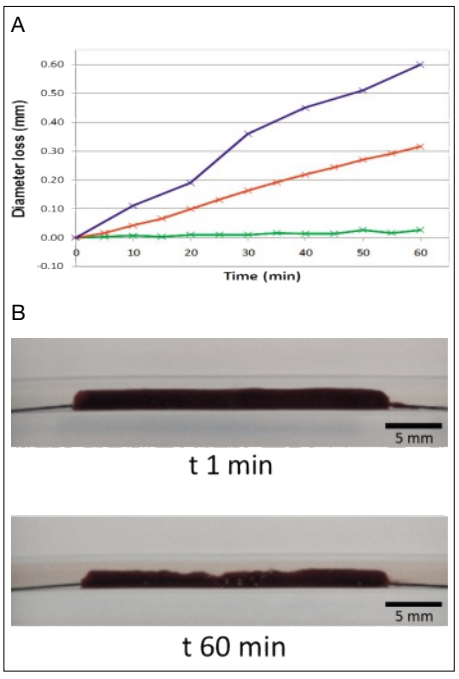

Fig. 3. A) Blood clot diameter loss (mm) as a function of time control (green), rtPA alone (red), rtPA + US + microbubbles combination (blue). B) Blood clot exposed to rtPA, + US + microbubbles combination.

4. Strategies to Address Altered Enzymatic Functions for Selective Drug Delivery

Enzymatic pathways and cascades are temporarily and spatially tightly controlled in all living organisms. However, unbalanced regulatory mechanisms are often the direct consequence of disease onset. The Warburg effect is the most prominent historical example of altered enzymatic pathways since cancer cells generate energy through anaerobic glycolysis and formation of lactic acid, which acidifies the tumor microenvironment. Needless to say that like targeting cell surface receptors, malfunctioning enzymatic cascades can be used to tackle or treat diseases by designing prodrugs that are specifically activated by these pathways. Uncontrolled proteolytic activity is the foremost known altered enzymatic function in cancer.

We have recently designed theranostic agents that are specifically activated by proteases to visualize and treat tumors using fluorescence and photodynamic therapy (PDT). Photodynamic therapy involves the simultaneous use of light, molecular oxygen, and a photosensitizer to locally produce high amounts of reactive oxygen species, which ultimately destroy the irradiated tissue.[15] Most photosensitizers also display a reasonable fluorescence quantum yield, and this fluorescence can be used to delineate the tumor margins if the compound displays reasonable tumor selectivity. We designed novel polymeric photosensitizer prodrugs (PPPs, see Fig. 4A) to improve the selectivity of conventional photosensitizers. Multiple copies of photosensitizer peptide conjugates are covalently coupled to a polymeric carrier in PPPs. These compounds are optically silent when activated by light and do not produce any reactive oxygen species due to the strong intramolecular energy transfer/and exciplex formation in the native configuration. However, the peptide spacer is specifically digested by a given protease, and single photosensitizer units are released from the polymeric backbone of the PPP when this protease is encountered. This release restores fluorescence and photodynamic activity. PPPs utilize multiple selective mechanisms. First, the high molecular weight enhances permeability and retention. Second, PPPS are only activated at sites of enhanced proteolytic activity of the target protease. Third, the photodynamic action is confined to the sites of active photosensitizers due to the short lifetime of reactive oxygen species (ROS). Finally, photodynamic therapy is restricted to the irradiation area due to the need for light for photosensitizer activation. We have previously prepared PPPs that target proteases, including trypsin, matrix metalloproteinases, human kallikreins, thrombin, and urokinease-like plasminogen activator (uPA). The latter protease is involved in the proliferation and progression of multiple cancers, including breast, colon, and prostate cancer. These uPA-sensitive PPPs were optimized *in vitro* and *in vivo*, and we demonstrated that these compounds are specifically activated in nude mice inoculated with prostate cancer cells. The colocalization of fluorescence and bioluminescence of the luciferase transfected tumor cells demonstrated the selectivity of our compounds (see Fig. 4B). Photodynamic therapy abolished the presence of any tumor-indicating bioluminescence and cured most prostate cancer-bearing mice; the drug or light alone did not improve survival. Another aspect of this study was the non-invasive control and prediction of therapeutic outcome using a bioluminescent tumor model limited the number of animals that were required to reach statistical significance.

In another recent study we have designed PPPs targeting the protease thrombin for its known involvement in rheumatoid arthritis. Thrombin-sensitive PPPs only accumulated in inflamed joints in an experimental animal model of rheumatoid arthritis (Fig. 4C). Neither a control PPP nor the free photosensitizer displayed any selectivity when applied intravenously to mice. The fluorescence was confined to the inflamed joint and remained constant for at least 48 hours in all of the tested conditions. Furthermore, the fluorescence intensities of whole animal fluorescence imaging correlated with the clinical score of the inflamed joints.

Heme biosynthesis is another metabolic pathway that is beyond control in cancer and rheumatoid arthritis. Heme biosynthesis is a conserved pathway in all nucleated

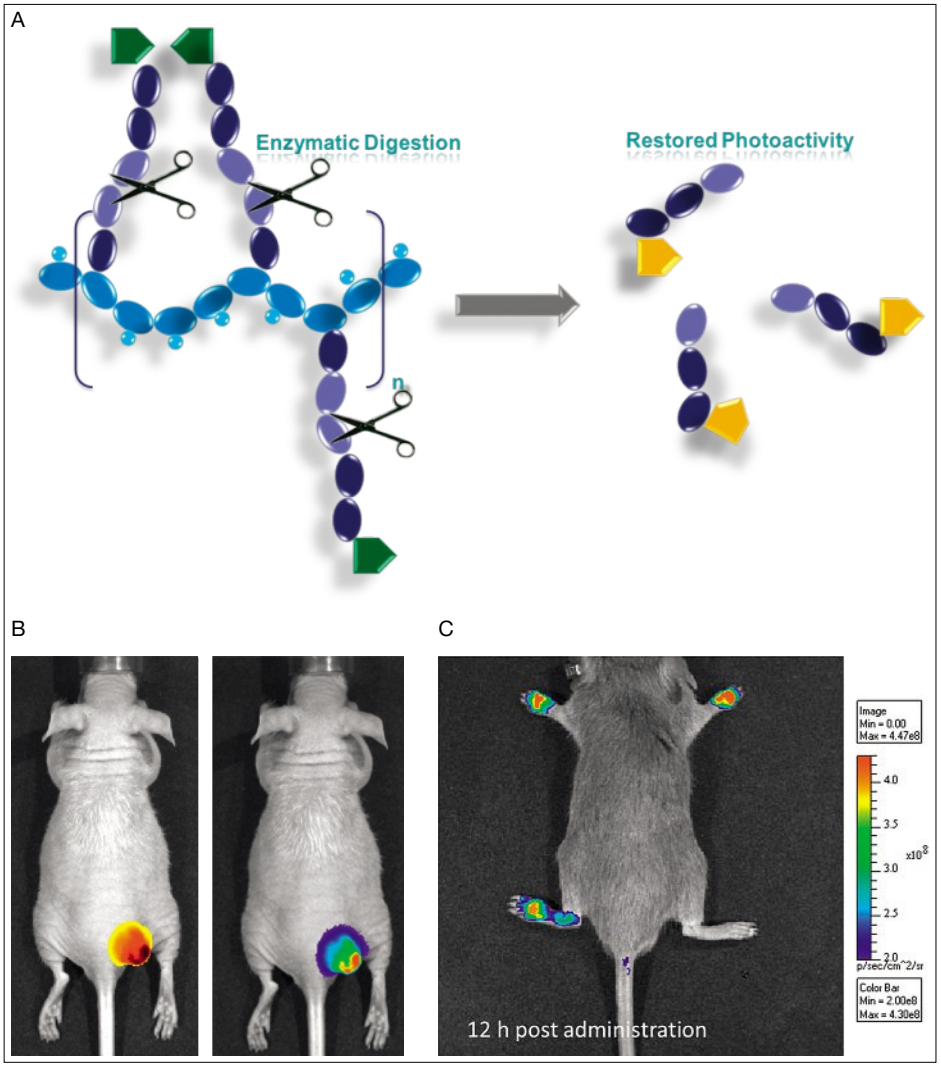

Fig. 4. A) Principle of protease-sensitive prodrugs for fluorescence diagnosis. The prodrug is inactive in its native state due to the quenching between closely positioned dyes on a polymer backbone. Proteolytic degradation of peptidic side chains releases the active drug from the copolymer and restores photoactivity. B) The uPA-sensitive prodrugs were selectively activated in prostate cancer xenografts 24 h postadministration as exemplified by the colocalization of bioluminescence (left) and fluorescence (right). C) Thrombin is a key mediator of rheumatoid arthritis. The design of thrombin-sensitive polymeric prodrugs helped distinguish between healthy and inflamed joints in an experimental animal model of arthritis.

mammalian cells. Heme is an iron chelate of protoporphyrin IX (PpIX) that serves as a prosthetic group in numerous hemoproteins, such as hemoglobin, myoglobin, redox cytochromes and the P450 class of detoxifying cytochromes. Furthermore, this pathway plays an important role in the regulation of protein synthesis and cell differentiation. Heme synthesis begins with the assembly of 5-aminolevulinic acid (5-ALA) from succinyl coenzyme A and glycine in the mitochondria. The final heme precursor, PpIX, is formed through six subsequent consecutive enzymatic steps. PpIX is the only fluorescent and photodynamically active compound in the entire pathway, but it becomes inactivated by the incorporation of ferrous iron into the tetrapyrrolic skeleton by ferrochelatase. The endogenous formation of 5-ALA is tightly controlled by heme through a negative feedback at transcriptional and translational levels. However, when provided exogenously 5-ALA boosts heme biosynthesis, resulting in an overproduction of PpIX, especially in tumors. Ever since this phenomenon has been observed in the late 1980s 5-ALA-mediated PDT and fluorescence detection has been explored clinically for the treatment and detection of various diseases.[16] 5-ALA was granted marketing authorization for some niche markets, such as the treatment of actinic keratosis and the fluorescence-guided resection of glioblastoma, but the great breakthrough in this domain was achieved with the appearance of more lipophilic derivatives.[17] Indeed, 5-ALA is a zwitterionic molecule that does not cross biological barriers with ease under physiological conditions, and must be actively transported from the extracellular space to the cytosol. Therefore, only small amounts of 5-ALA reach the cellular compartment to act as a substrate for 5-ALA dehydrase, which produces only a moderate increase in PpIX levels. Modification of the carboxylic function into an ester increases the lipophilicity, which increases the available substrate quantity for heme biosynthesis in the cytoplasm. Two of these compounds have been commercialized.

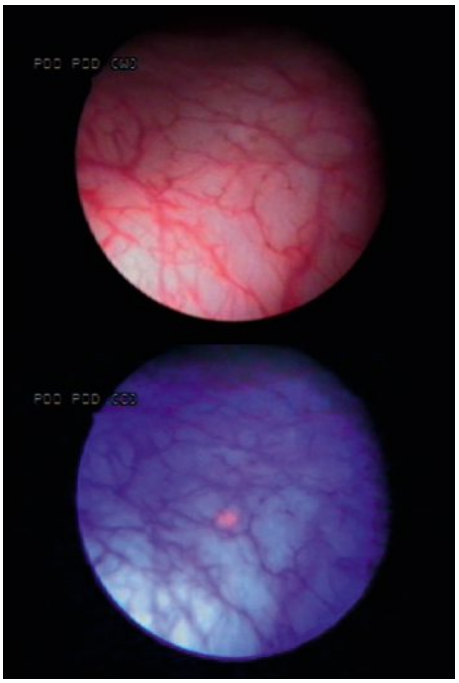

Fig. 5. White light image of a bladder wall with a carcinoma *in situ* (top). Only HAL-mediated fluorescence allowed for the identification of the otherwise invisible lesion (bottom).

5-Methylaminolevulinate is approved for the treatment of actinic keratosis and basal cell carcinoma, and the corresponding 5-hexylaminolevulinate (HAL) is used for the fluorescence photodetection of bladder cancer (Fig. 5). Clinical trials are exploring HAL use for the treatment of cervical intraepithelial neoplasia and the fluorescence detection of colon cancer. We also developed mucoadhesive formulations that are suitable for detection of Barrett's esophagus. We are currently developing formulations and derivatives of 5-ALA that are suitable for systemic administration to further exploit the outstanding selectivity of 5-ALA derivatives for other (non) neoplastic pathologies, including the treatment of bacterial infections. We recently discovered an enzyme that converts heme to PpIX, which might provide an option for the use of heme as an agent for photodynamic therapy.

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