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HEDI HUNT

Precision targeting of intraperitoneal tumors with peptide-guided nanocarriers

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Precision targeting of intraperitoneal tumors with peptide-guided nanocarriers

UNIVERSITY OF TARTU
Press

Cancer Biology Group, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia.

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I **Hunt H**, Simón-Gracia L, Tobi A, Kotamraju VR, Sharma S, Nigul M, Sugahara KN, Ruoslahti E, Teesalu T. Targeting of p32 in peritoneal carcinomatosis with intraperitoneal LinTT1 peptide guided pro-apoptotic nanoparticles. Journal of Controlled Release. 2017 Aug;260: 142–153.

II Simón-Gracia L, **Hunt H**, Scodeller P, Gaitzsch J, Kotamraju VR, Sugahara KN, Tammik O, Ruoslahti E, Battaglia G, Teesalu T. iRGD peptide conjugation potentiates intraperitoneal tumor delivery of paclitaxel with polymersomes. Biomaterials. 2016 Oct;104:247–57.

III Simón-Gracia L, **Hunt H**, Scodeller PD, Gaitzsch J, Braun GB, Willmore AA, Ruoslahti E, Battaglia G, Teesalu T. Paclitaxel-Loaded Polymersomes for Enhanced Intraperitoneal Chemotherapy. Molecular Cancer Therapeutics. 2016 Apr;15(4):670–9.

IV Ikemoto H, Lingasamy P, Willmore AA, **Hunt H**, Kurm K, Tammik O, Simón-Gracia L, Scodeller P, Kotamraju VR, Sugahara KN, Teesalu T. Hyaluronan-binding peptide for targeting peritoneal carcinomatosis. Tumor Biology. 2017 Apr;1–9.

Contribution of Hedi Hunt to each publication:

I Participated in the design of the study, performed all the experiments, analyzed the data and co-wrote the manuscript.

II Participated in the design of the study, development of methodology, performed cytotoxicity studies, participated in homing and experimental treatment studies, and took part in the analysis and interpretation of data.

III Developed methodology, performed cytotoxicity studies, participated in homing and experimental treatment studies, and contributed to the analysis and interpretation of data and review of the manuscript.

IV Participated in tumor homing experiments, performed immunofluorescent staining for confocal microscopy, and took part in the interpretation of data.

Other Publication:

I Simón-Gracia L, **Hunt H**, Teesalu T. Peritoneal carcinomatosis targeting with tumor homing peptides. Molecules. Molecules. 2018 May 16;23(5).

ABBREVIATIONS

ABX	Abraxane®
ADC	antibody-drug conjugate
AgNP	silver nanoparticle
ANOVA	one way analysis of variance
ATCC	american type culture collection
AUC	area under the curve
B/biot	biotin
CendR	C-end Rule
DAPI	4′6-diamidino-2-phenylindole fluorescent dye
DDS	drug delivery systems
DLS	dynamic light scattering
DOX	doxorubicin
EPR	enhanced permeability and retention
FAM	5(6)-carboxyfluorecein fluorescent dye
FITC	fluorescein isothiocyanate
HA	hyaluronic acid
HIPEC	hyperthermic intraperitoneal chemotherapy
IONP	iron oxide nanoparticle
IONW	iron oxide nanoworm
IP	intraperitoneal
IP3	hyaluronan targeting peptide, sequence [CKRDLSRRC]
iRGD	internalizing RGD, prototypic tumor penetrating peptide, sequence [CRGDKGPDC]
IV	intravenous
linTT1	linear TT1, p32-directed tumor penetrating peptide, sequence [AKRGARSTA]
Lyp-1	p32-directed tumor homing peptide, sequence [CGNKRTRGC]
MPS	mononuclear phagocyte system
MRI	magnetic resonance imaging
NA	neutravidin
NP	nanoparticle
NRP-1	neuropilin-1
NTA	nitrilotriacetic acid
NW	nanoworm
p32	protein 32
PC	peritoneal carcinomatosis
PEG	polyethylene glycol
PET	positron emission tomography
PIPAC	pressurized intraperitoneal aerosol chemotherapy
PS	polymersome
PTX	paclitaxel
Rho	rhodamine

RPAR/R	prototypic CendR peptide, sequence [RPARPAR]
SC	subcutaneous
TEM	transmission electron microscopy
TPP	tumor penetrating peptide
TT1	cyclic TT1, p32-directed tumor penetrating peptide, sequence [CKRGARSTC]

1. INTRODUCTION

Already more than a century ago two eminent scientists, Thomas Henry Huxley and Paul Ehrlich, envisioned a future when doctors could treat diseases by using a “very cunningly contrived torpedo” (Huxley, 1881), or “Zauberkugeln” (“magic bullets”) (Ehrlich, 1908) to specifically strike diseased tissues while sparing healthy organs. Thanks to advances in the field of drug delivery systems (DDS) in the last decades, this vision is becoming a reality (Allen, Cullis, 2004, Shi et al., 2017). Recent progress in improving DDS has nothing to do with magic, but with taking advantage of the pathophysiological changes in the microenvironment of the diseased tissues.

Cancer stands out as the disease most likely to benefit from precision drug delivery. Conventional anti-cancer therapies rely on the use of low molecular weight drugs that preferentially kill rapidly proliferating tumor cells rather than normal cells. However, existing anti-cancer drugs show poor cancer selectivity and limited penetration of malignant tissue leading to low drug concentration in the tumors and limited therapeutic efficacy (Shi et al., 2017). It has been demonstrated with PET biodistribution studies using labeled drugs that small-molecule therapeutic agents do not preferentially localize at neoplastic sites (van der Veldt et al., 2010, van der Veldt et al., 2011). For therapeutic effect, large doses of a drug must be used, which causes toxicities in non-malignant cells and side effects. The development of precision anti-cancer drugs ables to distinguish normal and cancer cells and is one of the main goals of modern anti-cancer research. Malignant tissues have features that can be targeted in order to increase accumulation of drugs at the tumor site and their therapeutic efficacy. First, enhanced permeability and retention (EPR) effect, caused by increased leakiness of tumor blood vessels, results in passive accumulation of drug in the tumor tissue (Matsumura, Maeda, 1986); second, cancer-associated signature molecules on the surface of tumor neovessels, tumor cells, and tumor-associated cells can be targeted with affinity ligands such as peptides. Affinity-based drug delivery is referred to as “synaphic” targeting; it is also referred to as pathotropic or active targeting (Ruoslahti, Bhatia & Sailor, 2010).

Primary cancers of gastrointestinal and gynecological origin often disseminate locoregionally in the peritoneal cavity to give a rise to a serious condition known as peritoneal carcinomatosis (PC). Intraperitoneal (IP) cancers are treated with a combination of surgical removal and adjuvant chemotherapy. However, due to micrometastases left behind during surgery and inefficient systemic chemotherapy, many patients experience a relapse. IP chemotherapy – administration of anticancer drugs to the peritoneal cavity – is used to decrease systemic exposure and to achieve high peritoneal concentration of cytotoxic drugs without relying on the blood supply. The efficacy of IP chemotherapy is decreased by the rapid escape of conventional non-targeted chemotherapeutics from the peritoneal space into systemic circulation, especially for smaller molecular weight agents. Novel strategies such as the development of precision

nanomedicines to specifically target cancerous lesions and the development of drugs/nanoparticles with extended residence time in the IP cavity may help to improve clinical management of PC.

Tumor penetrating peptides (TPP) are a novel class of tumor targeting peptides that can be used to deliver diagnostic and therapeutic payloads deep into malignant tissue parenchyma. After selective recruitment to tumor-associated receptors on tumor endothelial cells, TPP are proteolytically processed to trigger a secondary interaction with cell- and tissue penetration receptor NRP-1 that activates a pathway of active transport into extravascular tumor parenchyma. Due to multistep recruitment and activation pathway, the TPP are highly tumor specific. TPP platform provides a solution to the problem of poor penetration of drugs, imaging agents, and nanoparticles into tumors.

The goal of this thesis was to perform preclinical studies to explore applications of peptide-mediated targeting of nanocarriers for precision delivery to IP tumors. We used different classes of nanoparticles (NP) based on polymers, iron oxide and metallic silver – each with unique advantages, such as high drug loading capacity and ability to exit endosomes upon cellular internalization (polymersomes), possession of inherent contrast for MR imaging (iron oxide NPs), and sensitivity to etching solution that allows distinguishing extracellular and intracellular nanoparticles (silver NPs). Whereas these nanoscale platforms have been reported to be compatible with systemic delivery of payloads (Toome et al., 2017, Sharma et al., 2017, Pang et al., 2010), none of the nanoplatforms had been evaluated for IP delivery to peritoneal tumors at the beginning of our series of studies. Therefore, we performed a systematic assessment and preclinical development of nanocarriers for IP PC targeting. First, we conjugated different TPP to the NPs and tested interactions of peptide-NPs with cultured malignant cells *in vitro*. Second, we studied biodistribution and tumor tropism of the IP-administered TPP-NP in a panel of clinically relevant mouse models of PC. Finally, we studied the preclinical efficacy of TPP-NPs during experimental intraperitoneal tumor therapy.

2. REVIEW OF THE LITERATURE

2.1. Intraperitoneal (IP) carcinomatosis: challenge and current treatment options

Gastrointestinal and gynecological malignancies frequently metastasize in the peritoneal cavity and lead to severe complications such as bowel obstruction and the formation of ascites. At the time of diagnosis peritoneal dissemination of tumors is present in about 50% of gastric, 30% of ovarian and 40% of colorectal cancer patients (Goodman et al., 2016). PC has no clear clinical symptoms and is typically detected at a late stage when a large number of tumor micronodules are present over the peritoneal membranes (Sadeghi et al., 2000). PC patients undergo aggressive treatment through combination of surgical resection and/or chemotherapy (Bajaj, Yeo, 2010). However, PC is almost impossible to cure as complete surgical removal of all tumor microfoci is not possible and systemic chemotherapy has limited efficacy due to the poor vascularization of tumor nodules and the presence of the peritoneum-plasma barrier which prevents effective drug delivery from systemic circulation (Jacquet, Sugarbaker, 1996, Sugarbaker et al., 1996, Kitayama, 2014). As a result, PC patients have a bleak prognosis with median survival of only a few months (Coccolini et al., 2013) and there is an urgent need for improved therapies.

2.1.1. IP chemotherapy for treatment of PC: advantages and challenges

IP chemotherapy was first explored in the 1950s as a palliative tool in patients with certain types of peritoneal malignancies to limit the formation of ascites (Weisberger, Levine & Storaasli, 1955). In the late 1970s it was hypothesized that local administration could be used to increase tumors' exposure to anticancer drugs to improve treatment efficacy and minimize systemic drug toxicity and side effects (Dedrick et al., 1978). Since then, significant efforts have been dedicated to pre-clinical and clinical research to study the advantages and disadvantages of this challenging route of administration (Lambert, 2015). The rationale behind IP administration is based on the recognition that the presence of peritoneal-plasma barrier causes the IP drugs to be cleared slowly from the peritoneal cavity, resulting in high local drug concentrations- especially for higher molecular weight agents (Hasovits, Clarke, 2012). Peritoneal-plasma barrier prevents rapid drug resorption from the peritoneal cavity into the circulation and therefore, drug exposure at the tumor site will be increased compared to other parts of the body and beyond what would be achieved through systemic delivery (Hasovits, Clarke, 2012, Jacquet, Sugarbaker, 1996). This is expressed as the area under cavity-to-plasma concentration vs time curve (AUC) (Howell, 2008).

IP drugs have a dual mode of reaching the tumor: in addition to the high local concentration of the chemotherapeutic drug and direct access to the tumor, IP administered drugs will also enter systemic circulation via small capillary blood vessels adjacent to the peritoneum and enter the tumor microcirculation, whereas IV administered drugs solely rely on systemic accessibility (Fig.1). Therefore, IP delivery route has the advantage of targeting small avascular tumor nodules left behind after surgery.

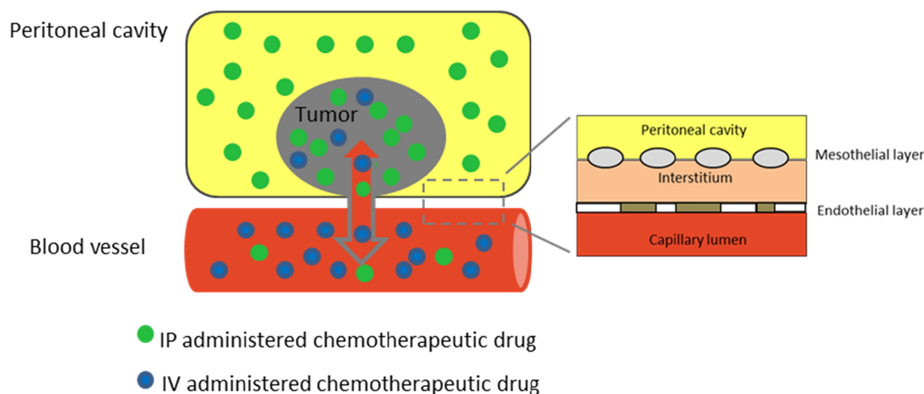

Figure 1. IP chemotherapy vs. IV chemotherapy. High concentration of chemotherapeutics can be achieved in the peritoneal cavity with intraperitoneal administration. Chemotherapeutic agents enter the tumor through direct surface contact and through systemic circulation, after crossing the peritoneal-plasma barrier and entering the bloodstream (Hasovits, Clarke, 2012). The transport of chemotherapeutics across the endothelium into the capillary lumen occurs via pores and water channels and is regarded to be the most critical barrier between the peritoneal surface and circulation (Fujiwara et al., 2007). Partially adapted from (Hasovits, Clarke, 2012).

Besides physiologically-defined parameters such as IP cavity-to-plasma AUC ratio and systemic absorption, the therapeutic efficacy after IP administration, the pharmacokinetic profile of drugs is affected by biophysical characteristics such as formulation, concentration, and size (Kitayama, 2014, Dakwar et al., 2017).

Delivery of chemotherapeutic drugs to PC lesions via the IP route has pharmacokinetic and pharmacodynamic advantages over intravenous (IV) administration. For example, it was reported that compared to the systemic administration route, IP-administered mitomycin-C has an improved AUC IP/IV ratio(Jacquet et al., 1998a, Van der Speeten et al., 2011). Comparison of IV vs. IP cisplatin in patients with stage III ovarian cancer demonstrated significantly improved survival and fewer toxic effects for the IP administration route (Alberts et al., 1996) and a recent systematic review concluded that intraperitoneal chemotherapy increases overall survival and progression-free survival from advanced ovarian

cancer (Jaaback, Johnson & Lawrie, 2016). However, none of the currently available IP chemotherapeutics has passed rigorous testing in clinical trials. At present, IP chemotherapy is used off-label with drugs approved for systemic anticancer therapy, such as doxorubicin (Jacquet et al., 1998b), paclitaxel (Kuh et al., 1999), fluorouracil analogues (Harada et al., 1995), and platinum-based drugs (van de Vaart et al., 1998).

At present, the most widely used therapeutic strategy against peritoneal tumors is the hyperthermic intraperitoneal chemotherapy (HIPEC), a procedure that includes mild heating of the chemotherapeutic solution (to 41- 42 degrees Celsius) to enhance the tumor drug penetration (Flessner, 2016). The application of HIPEC to standard treatment regimen aims to decrease the recurrence of the disease and HIPEC has become a routine strategy in the treatment of IP cancers in many cancer centers (Spiliotis, Halkia & de Bree, 2016, Flessner, 2016). In combination with cytoreductive surgery, HIPEC improves drug delivery to peritoneal tumor lesions and results in modest improvement in short- and long-term survival of the PC patients (Armstrong et al., 2006, Elias et al., 2009, Desiderio et al., 2017, van Driel, Koole & Sonke, 2018). A latest addition to IP delivery of cancer drugs is Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC) – a procedure that involves nebulization of drug solution into CO_2 pneumoperitoneum during laparoscopy (Grass et al., 2017).

Unfortunately, IP treatment approaches are far from curing PC, and face challenges related to limited therapeutic selectivity, escape of the drug into the systemic circulation, and peritoneal toxicities (Yeo, Xu, 2009). The clinical efficacy of IP chemotherapeutic drugs depends on its residence time in the peritoneal cavity. Drugs with long retention time generally show increased tumor accumulation and lower plasma concentration and less side effects (Dakwar et al., 2017). In contrast, low molecular weight drugs with short IP retention time need to be dosed frequently, with increased risk of catheterization-related issues (Poveda et al., 2007, Jaaback, Johnson & Lawrie, 2016). Generally, molecules <20kDa are rapidly cleared from the IP cavity via the direct absorption and compounds >20kDa and nanoparticles are eliminated by lymphatic drainage. Studies have shown that drug molecules with large molecular weight or water insoluble drug molecules (such as taxanes) will be retained in the IP cavity longer (Mohamed et al., 2003, Hasovits, Clarke, 2012). In recent years, nano- and microformulation of anticancer compounds has been explored as a strategy to increase peritoneal retention of drugs for an improved therapeutic efficacy and reduction of the number of IP administrations (Dakwar et al., 2017).

There is an urgent unmet need to enhance the residence time and/or tumor selectivity of the IP chemotherapeutics. The goal is to develop an IP chemotherapeutic agent that should specifically accumulate in the tumor tissue and should slowly exit the peritoneal cavity; this in turn would maximize tumor penetration and optimize cell death while minimizing pan-peritoneal and systemic toxicity.

2.2. Nanoparticles in detection and therapy of malignant disease

Nanodrugs and -contrast agents are created by encapsulation of bioactive compounds and/or imaging agents into nanoparticles with a size range of 5 to 1000 nm. Due to unique features of nanoscale carriers, nanomedicine can have a large impact of clinical management of malignant disease. Main features are listed below:

(1) Flexibility in payloads. Nanoparticles can be loaded with virtually all types of molecular payloads, including hydrophobic agents, compounds that are poorly soluble in aqueous solutions, and reactive compounds. Therefore, nanoparticles can be used as formulation aids. For example, Abraxane™, a hydrophobic microtubule-binding drug paclitaxel packaged in albumin nanoparticles, allows to circumvent the injection of hydrophobic paclitaxel in toxic Cremophor solvent (Gradishar et al., 2005). The nanoparticles can accommodate combinations of drugs for therapy, imaging agents for improved detection, or both for simultaneous treatment and detection of the disease (theranostic nanoparticles) (Shi et al., 2017).

(2) Engineered effector functions. Nanoparticles can be engineered to execute effector functions to improve pharmacokinetics and/or pharmacodynamics of their payloads. For example, nanoparticles can be programmed to disassemble and release their contents under specific conditions (e.g. at acidic pH found in endosomal compartment), or over extended periods for sustained release (Du et al., 2011). Affinity ligands can be used to target nanovehicles to normal and diseased organs and tissues, and to specific cells or extracellular structures. Importantly, low-affinity ligands are well suited for nanoparticle targeting, as the targeting ligands are present in multiple copies per particle and copy number of targeting ligands can be used to modulate the nanoparticle homing and retention at target sites (Ruoslahti, 2017).

(3) Overcoming the drug resistance. Nanocarrier loading can be used to avoid drug resistance due to plasma membrane efflux pumps. Low molecular weight drugs typically enter cells by direct penetration of the cell membrane or as a result of activity of influx pumps, and can be rapidly exported from cells by the activity of multidrug resistance efflux pumps (Goren et al., 2000, Sadava, Coleman & Kane, 2002). Drugs loaded on nanoparticles are typically released from endosomes deep inside the cell, where the drugs are better positioned to exert their activity.

Several nanoparticles have received regulatory approvals for systemic cancer treatment and imaging (Bregoli et al., 2016, Shi et al., 2017), and over 60 clinical studies on novel nanoparticle formulations are underway (www.clinicaltrials.gov, as on October 24th 2018). The first clinically approved nanodrug, Doxil® (polyethylene glycol-coated liposome-encapsulated doxorubicin), was approved in 1995, and the second nanodrug, Abraxane® (nanoformulated albumin-bound paclitaxel), in 2005. Compared to free compounds, these nanodrugs have distinct advantages. Doxil® shows reduced side cardiotoxicity (dose limiting

toxicity of doxorubicin) (Gabizon et al., 1994). Abraxane® nanoparticles can be administered in aqueous solutions, thus avoiding the use of toxic Cremophor oil used as the solvent for free paclitaxel (Gradishar et al., 2005).

In addition to lipid-based and proteinaceous NPs, polymer-based biocompatible and biodegradable nanoparticles, such as poly(lactic-co-glycolic acid) (PLGA) (Danhier et al., 2012), have been extensively used in preclinical and clinical studies. At present, different nanoparticle platforms are under investigation for the treatment of cancer including: lipid-based, polymer-based, inorganic nanoparticles, viral and drug-conjugated nanoparticles (Figure 2). In each case, the choice of NP platform depends on the physicochemical characteristics and pharmacokinetic and -dynamic profile of the payload drugs (Wicki et al., 2015, Shi et al., 2017).

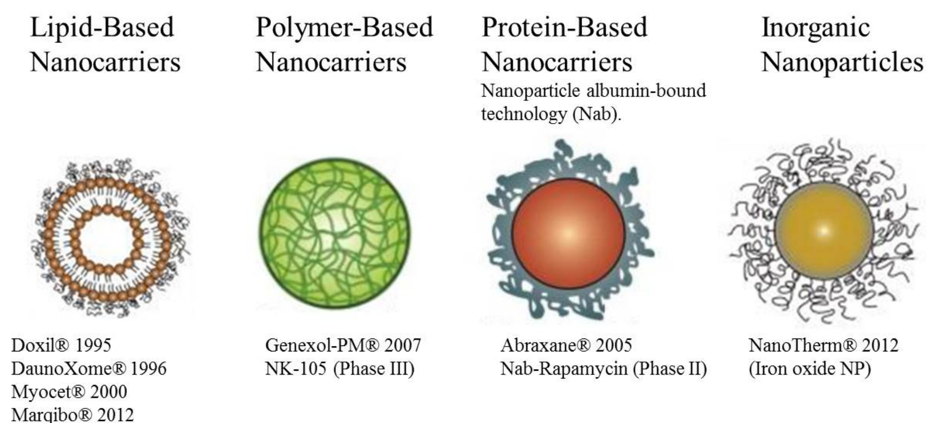

Figure 2. Schematic representation of clinical-stage nanomedicines for cancer therapy**.** A variety of nanocarriers such as lipid-based, polymer-based, inorganic nanoparticles currently used in clinical research for cancer. Partially adapted from Wicki et al., 2015.

2.2.1. Polymeric nanoparticles

Polymeric nanoparticles, or polymersomes (PS), are nanoscale vesicles formed by self-assembly of amphiphilic block copolymers in aqueous media (Gaitzsch, Huang & Voit, 2016). PS, assembled from low glass transition temperature "rubbery" polymers, are flexible and able to pass through pores up to an order of magnitude smaller than their diameter (Battaglia, Ryan, 2005, Gaitzsch, Huang & Voit, 2016, Pegoraro et al., 2014). Polymersomes are compatible with many types of cargoes, and and can be loaded with hydrophobic water insoluble drugs (e.g. Paclitaxel) in the membrane and hydrophilic drug molecules in the aqueous core. The incorporation of drugs inside the polymersome lumen can improve the therapeutic index by circumventing the use of toxic solvents. Poly(oligoethylene glycol methacrylate)-poly(2-(diisopropylamino)ethyl metha-

crylate) (P[(OEG)$_{10}$MA]$_{20}$-PDPA$_{90}$; POEGMA-PDPA) PS are pH sensitive: they are stable at physiological pH and disassemble under mildly acidic pH due to the protonation of the PDPA block (Bermudez et al., 2002, Pegoraro et al., 2014). This property of POEGMA-PDPA vesicles renders them particularly well suited for intracellular cargo delivery: after cellular internalization, the PS disassemble at endosomal acidic pH followed by endosomal rupture due to proton sponge effect and release of cargo in the cytosol (Massignani et al., 2009, Simon-Gracia et al., 2016b). PS have been used for intracellular delivery of DNA (Lomas et al., 2007), antibodies (Canton et al., 2013, Wang et al., 2012, Tian et al., 2015), antibiotic compounds (Wayakanon et al., 2013) cytotoxic drugs (Pegoraro et al., 2013, Colley et al., 2014, Simon-Gracia et al., 2016b), and bioactive peptides (Chierico et al., 2014).

2.2.2. Iron oxide nanoworms

Timely and precise detection of malignant disease and application of effective and well-tolerated therapeutic regimen are critically important for successful cancer treatment. Classic cancer detection techniques rely on imaging of anatomical features, whereas molecular imaging uses specific molecular probes to image biochemical activities, or specific receptors overexpressed in the tumor environment (Elias et al., 2008). Molecular imaging allows early detection of cancer and, at later stages, monitoring of changes in molecular behavior and host responses related to stage-specific events in disease progression in molecular and cellular levels (Weissleder, 2006).

Magnetic Resonance Imaging (MRI)-active iron oxide (IO) NPs can be engineered to have different chemical composition, size, and shape (Sun, Lee & Zhang, 2008). The biological behavior of the particles is profoundly affected by particle coating with molecules designed to decrease their nonselective uptake (e.g. PEG and dextrans) and with affinity ligands to allow precision delivery to intended target cells and -tissues (Kudr et al., 2017). The flexibility and low toxicity of IONPs have led to their applications in preclinical cancer research (Fang, Zhang, 2009). First generation of IONPs was designed for diagnostic applications and to accumulate in tumors (1) passively due to the enhanced permeability and retention (EPR) effect (discussed below), or (2) with the help of affinity ligands attached to the NP surface. The second generation of IONPs combines both therapeutic and/or diagnostic functions. For example, IONPs loaded with doxorubicin showed promising results against liver cancer in rats and rabbits (Maeng et al., 2010), peptide-guided IONPs and folic acid receptor-targeted IONPs improved MRI contrast and appeared to be promising agents for the detection of human ovarian and breast cancer, respectively (Abulrob et al., 2018, Zhang et al., 2016). IONPs can be engineered to exert anticancer effector functions: delivery of drugs, genes, and photothermal agents, or induction of magnetic hyperthermia (Li, Nejadnik & Daldrup-Link, 2017). For example, intratumorally-injected IONPs (Nanotherm®) can be heated up by an alternating

magnetic field – a procedure approved by EMA in 2012 for the treatment of glioblastoma in combination with radiotherapy and/or chemotherapy (Alphandery et al., 2015). Despite all the pre-clinical research on smart IONPs, only non-functionalized IONP formulations have been clinically approved for diagnostics (e.g. Feraheme®, Feridex®; Resovist®; Combidex®) (Zhu et al., 2017). IONPs can be imaged by MRI also in other diseases such as inflammatory and degenerative pathologies, and in demyelinating disease, multiple sclerosis (Vellinga et al., 2009, Stoll, Bendszus, 2009, Gobbo et al., 2015).

Iron oxide nanoworms (NWs) – elongated PEGylated dextran-coated paramagnetic IONPs – are a subclass of IONPs optimized for *in vivo* precision-guided delivery. Compared to spherical IONPs, the elongated form of NWs is better suited for targeted delivery applications due to geometrically-enhanced multivalent interactions between receptors and ligands (Park et al., 2009). NWs show low toxicity, long plasma half-life, and a robust ability to enhance MRI relaxivity (Park et al., 2009, Ruoslahti, 2017). Systemically administered therapeutic nanoworms carrying $_{D}$ [KLAKLAK]$_2$ proapoptotic peptide payload and functionalized with tumor homing peptides accumulate in mouse models of breast tumors and glioblastoma and dramatically improve antitumor activity and therapeutic index of proapoptotic cargo (Agemy et al., 2013, Agemy et al., 2011, Sharma et al., 2017).

2.3. Tumor-selective delivery of drugs and nanoparticles

Targeted drug delivery in cancer utilizes drug delivery systems (nanocarriers) to change the pharmacological properties of conventional drugs to modulate their biodistribution towards increased accumulation in tumor tissue for improved tumor imaging (diagnostic NPs), therapeutic outcome (therapeutic NPs), or both (theranostic NPs). The aim is to achieve tumor-selective drug delivery to improve therapeutic index of drugs – the difference in the concentration of therapeutic agent that causes the therapeutic effect and the toxic concentration.

Currently no clinically approved nanoparticle drugs have targeting ligands attached to their surface (Shi et al., 2017). Non-targeted NPs are thought to accumulate in the tumor tissue passively, due to an effect known as Enhanced Permeability and Retention (EPR). The EPR concept, introduced by Hiroshi Maeda in 1986, states that blood vessels in tumor tissue have compromised vascular wall and are hyperpermeable, and that this leakiness translates into tumor-selective delivery of circulating payloads, including nanoparticles (Matsumura, Maeda, 1986). The absence of functional lymphatics vessels in most tumors further contributes to the nanoparticle entrapment and retention at the tumor site (Fang, Nakamura & Maeda, 2011). However, the EPR effect is not universal, and shows large degree of intra- and intertumoral heterogeneity (Danhier, 2016).

In contrast to passive targeting, affinity-based targeting (also termed synaphic, pathotrophic, or active targeting) uses targeting ligands that interact with

accessible molecular markers in the tumor environment for direct drug delivery. The intended outcome of this approach, zeroing-in on the target tissue, is comparable to topical administration with high local and low systemic exposure (Ruoslahti, Bhatia & Sailor, 2010). Over the years, the most common affinity targeting approach to deliver anti-cancer drugs and imaging agents to solid tumors is using antibodies and their fragments. Various monoclonal antibodies have reached the clinical use such as Trastuzumab (for breast cancer), Bevacizumab (for colorectal cancer), Cetuximab (for colorectal cancer) and many more (Trail, King & Dubowchik, 2003, Adams, Weiner, 2005). One of the fastest growing drug classes in oncology are Antibody-Drug Conjugates (ADCs). ADCs combine the selectivity and high affinity of antibodies with potency of chemotherapeutic molecules (Perez et al., 2014). The aim thriving ADC research has been to improve the therapeutic index of chemotherapeutics by lowering the minimum effective dose and increasing the maximum tolerated dose (Beck et al., 2010). Currently the only ADCs approved by the FDA and EMA are Kadcyla®, an anti-human epidermal growth factor receptor-2 (HER2) with a maytansinoid payload for breast cancer therapy, and Adcetris® (brentuximab vedotin) with MMAE (monomethyl auristatin E) targeting CD30-positive Hodgkin's Lymphoma (Senter, Sievers, 2012, Lambert, Chari, 2014). These two approved ADCs have paved the way for ongoing clinical trials with more than 60 investigational ADC candidates (Beck et al., 2017).

In addition to ADCs, four nanoparticles with chemotherapeutic payloads and coated with affinity ligands recognizing for example HER2, PSMA (prostate specific membrane antigen), EGFR and TfR (transferrin receptor) are currently being evaluated in clinical trials for different types of solid tumors, including gastric adenocarcinoma (Shi et al., 2017). Nevertheless, antibodies have disadvantages that limit their clinical application, including high manufacturing cost, low ability to extravasate and reach parenchymal target cells due to large size and high affinity (that causes affinity site barrier) and immunogenicity (Liu, Wu, 2008). In contrast, peptides as affinity ligands are affordable and have shown good tissue penetrating ability due to their small size, low immunogenicity, multivalent presentation on a NP and can be readily coupled to different classes of molecular payloads using well-established chemistries (Ruoslahti, Bhatia & Sailor, 2010, Ruoslahti, 2012).

2.4. Nanoparticles in PC

Formulation has a profound effect on the pharmacokinetics, biodistribution, and efficacy of the drugs. Nano- and microformulation of anticancer compounds can increase peritoneal retention of drugs that translates into an improved therapeutic efficacy and reduction of the number of IP administrations (Dakwar et al., 2017). As nanoparticles enter cells via endocytosis, loading of drugs in nanoparticles can also bypass or alleviate drug resistance due to overexpression of drug efflux pumps. In the context of IP-targeted nanotherapies, the main

areas of investigation are extension of the drug residence time in the IP cavity, increasing the specificity towards cancer cells, and limiting the side effects.

2.4.1. Nanoparticles in pre-clinical development for PC therapy

Different types of nontargeted nanoparticles have been evaluated for intra-peritoneal delivery in the PC (of colorectal, gastric, ovarian carcinoma origin) bearing mice. These include lipid-based NP formulations and polymer-based NPs (such as polymersomes, polymeric microspheres and hydrogel-based systems). The NP-encapsulated drug payloads include paclitaxel, doxorubicin, 5-fluorouracil (5-FU), and docetaxel (Van Oudheusden et al., 2015). For example, Emoto et al. used in their study micellar nanoparticle platform loaded with Paclitaxel for treatment of MKN-45P gastric cancer xenografts, and observed enhanced NP penetration into tumor nodules and significant decrease in tumor nodules and tumor weight compared to free Paclitaxel-Cremphor-treatment (Emoto et al., 2012). In another study, Fan and colleagues found that IP-administered polymer-based thermosensitive hydrogel based on polylactic acid and Pluronic L64 loaded with combination of chemotherapeutic drug docetaxel and anti-tumor peptide LL37, reduced significantly the growth of colorectal cancer peritoneal xenografts in nude mice (Fan et al., 2015). In addition, this system increased the survival of the treated mice compared to the control groups. Additional polymer-based delivery systems for PC have been evaluated for IP targeting of PC (Liu et al., 2013a, Gong et al., 2012, Soma et al., 2009, Vassileva et al., 2007).

Several studies have addressed the effect of affinity targeting on the tumor distribution and efficacy of IP-administered drug-loaded NPs. Folic acid receptor -α (FR-α) upregulation is commonly seen in ovarian cancer lesions (Elnakat, Ratnam, 2006). Cerium oxide NPs have antitumor activity due to increase in production of reactive oxygen species (ROS) (Hijaz et al., 2016). Coating of the cerium oxide NPs with folic acid increased their cellular internalization and decreased cell proliferation in cultured ovarian cancer cells. In addition, when the folic acid guided-NPs were IP-administered in ovarian PC-bearing mice in combination with a known chemotherapeutic drug cisplatin, the tumor burden in mice was decreased (Hijaz et al., 2016). Similar results were reported when folic acid–guided nano-paclitaxel liposomes were ad-minstered to ovarian cancer xenograft model in mice via IP injection (Tong et al., 2014).

In another study, coupling of tumor homing peptide F3 to α-particle-emitting radionuclides was found to improve survival of PC-bearing mice compared to the untreated control mice and control group treated with non-targeted radio-nuclides (Essler et al., 2012). In another study, application of integrin-targeting tripeptide RGD coupled to fluorescent dye indocyanine green (RGD-ICG) allowed image guided surgery with shorter time for surgery and more complete

removal of malignant tissue (Cheng et al., 2017). Remarkably, the diameter of detectable tumor nodules was <2 mm and compared to conventional surgery the time required for surgery using RGD-ICG was decreased ~3 fold (Cheng et al., 2017).

2.4.2. Therapeutic nanoparticles in PC clinical trials

Two untargeted NP formulations are currently being assessed for IP tumor therapy in clinical trials (Table 1). The first study, a phase I trial, assessed the safety, tolerability and pharmacokinetic profile of IP administered Cremophor-free Paclitaxel (Nanotax®) in patients bearing solid tumors confined to the peritoneal cavity (Williamson et al., 2015). The patients received six escalating IP doses of Nanotax® over 28 days. The study concluded that compared to IV administration IP administration results in reduced systemic toxicity and improved PK profile. Nanotax®, a ~700 nm rod-shaped nanoformulation of paclitaxel is retained in the peritoneal cavity and shows minimal escape to the systemic circulation. Two days after the injection of Nanotax® the concentration of paclitaxel in peritoneal fluid was 450–2900 fold higher than in plasma. The study was completed in 2013 and it is not clear wether a Phase II trial will be initiated.

The second Phase I study evaluated the maximally tolerated dose, adverse effects and the pharmacokinetics of IP administered Abraxane®, an albumin-bound paclitaxel nanoformulation that was approved in 2005 for the treatment of metastatic breast cancer (Cristea et al., 2015). The study on 27 patients concluded that IP administered Abraxane has pharmacologic advantage over IV administered Abraxane and the inter- and intra-patient variability in drug uptake is low. The study completion date was 16th of January 2018. Presumably the final results will be published in the near future (Clinical Trial no. NCT00825201).

There have been no clinical trials on affinity targeted NPs for PC.

Table 1. IP-administered nanoparticles in PC clinical trials.

Intervention	Formulation	Condition	Phase / no. of patients	Outcome	Reference
Nanotax®	Nanoparticulate Paclitaxel; 600–700 nm; rod-shaped	Solid tumors confined to the peritoneal cavity	Phase I / 21 patients	IP administration results in higher and prolonged Paclitaxel levels with minimal systemic exposure compared to IV administration.	(Williamson et al., 2015)
Abraxane®	Albumin-bound Paclitaxel; 130 nm	Advanced peritoneal malignancy	Phase I / 27 patients	Higher peritoneal exposure compared to plasma.	(Cristea et al., 2015)

2.5. Tumor homing peptides

Vascular heterogeneity can be explored in unbiased manner by *in vivo* screening of phage libraries that display random peptide sequences (Hoffman et al., 2003, Pasqualini, Ruoslahti, 1996). The process of tumor homing peptide biopanning consists of intravenous administration of phage library into a tumor-bearing mouse, rescuing the phage from the malignant tissue, and repeating the process several times to derive a phage pool that selectively homes to the tumor. When peptide phage libraries are injected into the circulation, tumor-specific molecules on endothelial cells are primarily targeted. Phage display has yielded numerous peptides specific for many different conditions as summarized in Table 2. Importantly, this approach has yielded a variety of homing peptides specific for tumor vasculature and tumor cells (Arap, Pasqualini & Ruoslahti, 1998, Laakkonen et al., 2002, Ruoslahti, 2004, Fan et al., 2007, Zhang et al., 2006).Tumor homing peptides can be used for precision guided delivery of coupled drugs and contrast agents to tumor blood vessels to improve tumor detection and increase therapeutic index (Liu et al., 2017a). Coupled to tumor-homing peptides, different anti-cancer drugs show an enhanced anti-tumor effect (Arap, Pasqualini & Ruoslahti, 1998, Ellerby et al., 1999, Chen et al., 2001, Curnis et al., 2000, Karmali et al., 2009).

Table 2. Examples of homing peptides and their *in vivo* homing specificity. Adopted from Teesalu et al., 2012.

Peptide Sequence	*In vivo* homing specificity	Reference
1. AKRGARSTA (linTT1) **2. CKRGARSTC (TT1)**	Peritoneal tumors (p32/NRP-1) Breast tumors (p32/NRP-1) Breast tumors (p32/NRP-1)	(Hunt et al., 2017, Sharma et al., 2017, Paasonen et al., 2016)
3. CSPGAKVRC (UNO)	Tumor macrophages (CD206)	(Scodeller et al., 2017)
4. RPARSGRSAGGSVA (uCendR)	Breast tumors (NRP-1)	(Braun et al., 2016)
5. CAQK	Brain injury	(Mann et al., 2016)
6. CKRDLSRRC (IP3)	Peritoneal tumors	(Ikemoto et al., 2017)
7. CDAGRKQKC (DAG)	Alzheimer´s disease	(Mann et al., 2017)
8. CRNGRGPDC (iNGR)	Breast tumors (CD13/NRP-1)	(Alberici et al., 2013)
9. CGKRK	Breast tumors (p32) Glioblastoma (p32) Squamous cell carcinoma (p32)	(Agemy et al., 2013, Agemy et al., 2011, Hoffman et al., 2003)
10. RPARPAR	Prototypic CendR peptide (NRP-1)	(Teesalu et al., 2009)
11. CRGDKGPDC (iRGD)	Different tumors; prototypic tissue penetrating peptide ($\alpha v\beta_{3/5}$ intergrins; NRP-1)	(Sugahara et al., 2009)
12. CAGALCY	Brain	(Fan et al., 2007)
13. CREKA	Angiogenic vessels (fibrin clots)	(Simberg et al., 2007)
14. CARSKNKDC (CAR) **15. CRKDKC (CRK)**	Wound Wound	(Jarvinen, Ruoslahti, 2007)
16. CRAKSKVAC	Pan-endothelial homer	(Zhang et al., 2006)
17. CREAGRKAC **18. CAGRRSAYC**	Prostate carcinoma lymphatics Prostate carcinoma premalignant lymphatics	(Zhang et al., 2006)
19. CLSDGKRKC	Lymphatics in C8161 melanoma	(Zhang et al., 2006)
20. CNRRTKAGC (LyP-2)	K14HPV16 dysplastic skin lesions	(Zhang et al., 2006)
21. CGLIIQKNEC (CLT1) **22. CNAGESSKNC (CLT2)**	Blood clot Blood clot	(Pilch et al., 2006)

Peptide Sequence	***In vivo* homing specificity**	**Reference**
23. CRPPR	Heart	(Zhang, Hoffman & Ruoslahti, 2005)
24. CGNKRTRGC (LyP-1)	Tumor lymphatics, tumor macrophages and tumor cells in hypoxic areas (p32/ NRP-1)	(Laakkonen et al., 2002)
25. CSRPRRSEC	Dysplastic skin	(Hoffman et al., 2003)
26. gSMSIARL **27. gVSFLEYR**	Normal prostate Normal prostate	(Arap et al., 2002)
28. CTTHWGFTLC	Gelatinase A in angiogenic vessels	(Koivunen et al., 1999)
29. CNGRC	Angiogenic vessels (CD13)	(Arap, Pasqualini & Ruoslahti, 1998)
30. CRRETAWAC	$\alpha5\beta1$ integrins	(Koivunen, Wang & Ruoslahti, 1994)

2.5.1. Tumor penetrating peptides and the C-end Rule

The power of *in vivo* phage screening is illustrated by the recent discovery of peptides with unique tumor-penetrating properties. These tumor penetrating peptides (TPP) activate an endocytic transport pathway related to, but distinct from macropinocytosis by engaging a complex process that involves binding to a primary, tumor-specific receptor, a proteolytic cleavage, and binding to a second receptor, neuropilin-1 (NRP-1). The NRP-1 binding activates the transport pathway (Pang et al., 2014, Teesalu et al., 2009). NRP-1 has a binding pocket on its b1 domain that is capable of interacting with C-terminal peptides with consensus sequence R/KXXR/K (x-random amino acid). Such R/KXXR/K-containing peptides are due to strict requirement for C-terminal exposure termed C-end rule (Teesalu et al., 2009, Sugahara et al., 2009). The CendR receptor, NRP-1, is a pleiotrophic cell surface receptor with essential roles in angiogenesis, regulation of vascular permeability and development of the nervous system (Geretti, Klagsbrun, 2007, Gagnon et al., 2000). VEGF-A165 and some other ligands of NRP-1 possess a C-terminal CendR sequence that interacts with the b1 domain of NRP-1 and causes cellular internalization and vascular leakage (Becker et al., 2005). CendR peptides have similar effects, particularly when made multivalent through coupling to a molecular scaffold or a particle (Teesalu et al., 2009). NRP-1 is widely expressed in normal tissues and overexpressed in variety of tumors and tumor cell lines, including gliomas (Hu et al., 2007). Interestingly, overexpression of NRP-1 is correlated with poor prognosis of human glioma (Osada et al., 2004), and peptide-based interference of the transmembrane domain of NRP-1 appears to inhibit growth of orthotopic C6 glioma model by exerting anti-migratory, anti-angiogenic and anti-proliferation activity (Nasarre et al., 2010).

The trans-tissue CendR pathway mediates the exit of payloads from the blood vessels and their transport through extravascular tumor tissue. The CendR technology provides a solution to a major problem in tumor therapy, poor penetration of drugs into tumors. The tumor-penetrating CendR peptides can take a payload deep into tumor tissue in mice and into human tumors *ex vivo*. Tumor-penetration is seen even when the tumor is highly desmoplastic, as typically seen in the case of pancreatic tumors (Sugahara et al., 2009). These peptides specifically increase the accumulation in tumors of a variety of anticancer therapeutics, such as chemotherapeutic agents, antibodies and, particularly relevant to this application, therapeutic nanoparticles (NPs). Remarkably, the payload to be targeted does not have to be coupled to the peptide; the peptide activates a bulk transport system that sweeps along any compound present in the blood (“by-stander effect”). Treatment studies in mice show improved anti-tumor efficacy and less damage to normal tissues (Sugahara et al., 2009, Sugahara et al., 2010, Agemy et al., 2011, Alberici et al., 2013, Gu et al., 2013, Akashi et al., 2014, Wang et al., 2014, Schmithals et al., 2015, Zhang et al., 2015, Liu et al., 2013b, Liu et al., 2017b). In addition to a systemic route, the tumor penetrating peptides access tumors and induce the bystander effect by

direct contact with tumor tissue, including *ex vivo* tumor tissue "dipping" assay (Sugahara et al., 2009, Sugahara et al., 2010). The nature and regulation of the CendR pathway is partially understood; it is an endocytic transcytosis pathway that is regulated by availability of nutrients to a tumor and triggered through peptide binding to NRP-1 (Pang et al., 2014).

2.5.1.1. iRGD

The prototypic TPP iRGD (CRGDK/RGPD/EC) homes to angiogenic αv and β3/5 integrins expressed on plasma membrane of endothelial, fibroblast and malignant cells in tumors (Ruoslahti, 2012). After recruitment to integrins iRGD is proteolytically cleaved to expose C-terminally CRGDK CendR motif, and the truncated peptide loses most of its integrin-binding capacity and gains affinity for neuropilin-1 (NRP-1) (Figure 2). The proteolytic activation of the CendR sequence requires the integrin binding; the CendR motif is only minimally activated in an analogue (CRGEKGPDC) that lacks affinity for integrins (Sugahara et al., 2009). The likely reason is that the proteolytic processing step can only occur in close proximity to the cell surface. The αv integrin requirement makes iRGD activation specific to angiogenic tumor vessels. The tumor-penetrating properties of iRGD are in striking contrast to those of other RGD peptides that bind to αv integrins with affinities like that of iRGD but lack a CendR motif. Figure 3 depicts a multistep binding and penetration mechanism of iRGD peptide.

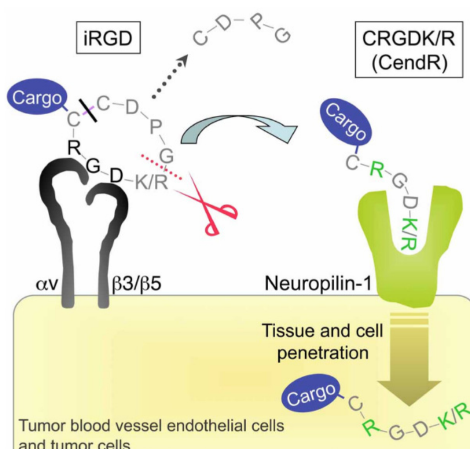

Figure 3. TPP multistep binding and penetration mechanism. iRGD peptide is recruited to integrins expressed on endothelial cells and other cells in tumors. After recruitment to integrins, iRGD is proteolytically cleaved to expose C-terminally CRGDK CendR motif, and the truncated peptide loses most of its integrin-binding capacity and gains affinity for neuropilin-1 (NRP-1). Binding to NRP-1 mediates penetration to cells and tissues. Adopted from Sugahara et al., 2009.

iRGD can be used to increase vascular and tissue permeability in a tumor-specific and neuropilin-1-dependent manner to enhance the penetration of chemically conjugated and co-administered anticancer drugs, imaging agents, and nanoparticles (Sugahara et al., 2009, Sugahara et al., 2010). iRGD is clinically developed for co-administration-based delivery of drugs and imaging agents to pancreatic cancer by a biotechnology company in the US, DrugCendR Inc. (www.drugcendr.com).

2.5.1.2. TT1

Another recently identified TPP, TT1 (cyclic TT1: CKRGARSTC; linear TT1: AKRGARSTA), is highly efficient in delivery of molecular and nanoparticle payloads to breast cancer lesions (Paasonen et al., 2016, Sharma et al., 2017, Simon-Gracia et al., 2018). The primary homing receptor for TT1 is p32, a mitochondrial protein aberrantly expressed on the cell surface of activated malignant and stromal cells in solid tumors (Fogal et al., 2008). TT1 peptide is after binding to p32 proteolytically cleaved to expose C-terminally the RGAR peptide that interacts with tissue penetration receptor NRP-1 (Sugahara et al., 2009, Teesalu et al., 2009, Sharma et al., 2017).

Cell surface p32 became relevant for systemic affinity targeting as the receptor for LyP-1 peptide that targets lymphatic vessels and macrophages (Laakkonen et al., 2002, Fogal et al., 2008). LyP-1 is widely used as an affinity module for tumor delivery of imaging agents, drugs, and nanoparticles to solid tumors (Luo et al., 2010, Yan et al., 2012, Roth et al., 2012, Miao et al., 2014, Timur et al., 2017, Teo et al., 2018). Success of LyP-1-based affinity targeting has inspired development of alternative targeting ligands to p32, including other homing peptides, antibodies, and low molecular weight compounds (Agemy et al., 2011, Paasonen et al., 2016, Kim et al., 2016, Yenugonda et al., 2017). P32 is prominently expressed in clinical samples and in mouse models of glioblastoma, and there is a significant correlation between high p32 expression and decreased survival of glioblastoma patients (Fogal et al., 2015). LyP-1 and several p32 targeting ligands possess an intrinsic antitumor activity (Laakkonen et al., 2004); in addition, genetic knockdown of p32 has been demonstrated to limit cell proliferation *in vitro* and tumor growth *in vivo* (Fogal et al., 2015). It is likely that alignment of intrinsic anticancer activity of LinTT1 with nanoparticle payload drugs that act in synergy can be used to potentiate the therapeutic efficacy of p32-directed nanosystems.

SUMMARY OF THE LITERATURE

Gastrointestinal and gynecological malignancies often disseminate in the peritoneal cavity. The condition is known as peritoneal carcinomatosis (PC) and it may cause complications such as bowel obstruction and the formation of ascites. PC results from the dissemination of the primary tumor or seeding after surgical intervention and is a cause for incurability of intra-abdominal cancers. In the treatment of peritoneal tumor lesions, intraperitoneal chemotherapy can be used to improve delivery of drugs into peritoneal tumors by providing direct contact and higher local concentration. Intraperitoneal chemotherapy was first introduced as a palliative tool to alleviate the symptoms arising from the formation of ascites in certain cancers. Later the approach was used in combination with cytoreductive surgery to kill the remaining micrometastasis that were impossible to remove during surgery.

IP chemotherapy is an attractive strategy to improve the outcome of PC. During the last decades, substantial amount of work has been put into improving the therapeutic outcome of PC by applying different therapeutic approaches that maximize selectivity and limit side effects. Multiple studies suggest that a delivery of chemotherapeutic drugs via IP route is a promising method and offers a direct pharmacokinetic advantage over intravenous (IV) administration. IP administration of drugs results in higher local concentrations and longer half-life of the drug in the peritoneal cavity thus improved outcome of the chemotherapy is achieved. Nanoparticles in the context of direct targeting of IP tumors are actively being evaluated in preclinical studies due to their potential of increasing the retention time in the IP cavity and to target drugs specifically to the tumor site compared to the conventional drugs. A few nanoparticle formulations of chemotherapeutic drugs have reached human trials, but there are no approved drug formulations for the specific use in the IP cavity.

Novel strategies such as development of precision nanomedicines to specifically target cancerous lesions and development of drugs/nanoparticles with extended residence time in the IP cavity may help to increase efficacy of IP chemotherapy.

3. AIMS OF THE STUDY

The goal of the research presented in this dissertation was the development of TPP-NP platform for IP delivery of anticancer drugs and imaging agents. A panel of complementary strategies were used to study the homing and anti-cancer efficacy of the peptide-guided therapeutic NPs on cultured malignant cells and *in vivo,* after IP administration into tumor bearing mice.

Specific aims:

1. To establish the specificity profiles for TPP-NP under cell-free conditions and on cultured IP tumor cell lines;
2. To determine the effect of TPP functionalization on the cellular uptake and cytotoxic activity of the NPs loaded with imaging agents and cytotoxic payloads;
3. To determine the effect of TPP functionalization on the IP tumor tropism and biodistribution of the IP administered NPs;
4. To evaluate the therapeutic efficacy of IP treatment of experimental PC using TPP-guided cytotoxic NPs.

4. MATERIALS AND METHODS

The methods used in the research presented in this thesis are described in detail in the respective publications. This section provides brief summary of the methods used in the studies.

4.1. Cell culture experiments and animal studies

We used five different cancer cell lines that originate from human or mouse tissues (Table 3). MKN-45P cells were isolated from parental MKN-45 cells (Koga et al., 2011). SKOV-3 and CT-26 cell lines were purchased from ATCC (SKOV-3 ATCC HBT-7; CT-26 ATCC CLR-2638). PPC-1 cancer cells were from the Ruoslahti laboratory at Sanford-Burnham-Prebys Medical Discovery Institute (SBPMDI), and M21 cells were a gift from David Cheresh at University of California San Diego (UCSD). The cells were cultivated in DMEM (Lonza, Belgium) containing 100 IU/mL of penicillin, streptomycin, and 10% of heat-inactivated fetal bovine serum (GE Healthcare, UK) in 5% CO_2 atmosphere.

For animal experimentation athymic nude mice were purchased from Harlan and Balb/c mice were purchased from Charles River. Animal experimentation protocols were approved by Estonian Ministry of Agriculture, Committee of Animal Experimentation (Project #42).

Table 3. Cell-lines used in the *in vitro* and *in vivo* studies

Cell-line name		Surface receptor		Application	Publication in thesis
		NRP-1	p32		
MKN-45P	Human gastric carcinoma	+	+	*In vitro /In vivo* Nude mice	I–IV
CT-26	Mouse colon carcinoma	+	+	*In vitro /In vivo* Balb/c mice	I–IV
SKOV-3	Human ovarian carcinoma	+	+	*In vitro /In vivo* Nude mice	I; III
PPC-1	Human prostate adenocarcinoma	+	+	*In vitro*	II
M21	Human melanoma	-	+	*In vitro*	II

4.2. Peptides and targeted nanoparticles

Peptides used in this thesis (Table 4) were synthesized using Fmoc/t-Bu chemistry on a microwave assisted automated peptide synthesizer (Liberty, CEM Corporation, NC, USA). Peptides were purified by HPLC using 0.1% TFA in acetonitrile-water mixtures to 90% – 95% purity and validated by Q-TOF mass spectral analysis. Fluorescent peptides were synthesized by using 5(6)-carboxyfluorescein (FAM) with 6-aminohexanoic acid spacer attached to the N-terminus of the peptide.

Three different nanoparticle (NP) platforms are used in this thesis (Fig. 4). All have recently been reported to facilitate nanoscale delivery of payloads to tumors (Sharma et al., 2017, Simon-Gracia et al., 2016a, Toome et al., 2017), but not been evaluated as delivery vehicles for IP tumor targeting. In publication I, we utilized for IP cancer targeting and imaging dextran-coated iron oxide nanoworms (NW) – theranostic NPs that serve as a carrier for anticancer compounds and possess intrinsic T2 contrast in MRI. In publications II and III we evaluated nontargeted and targeted pH-sensitive PS (PS), nanocarriers that can be loaded with hydrophobic chemotherapeutics, for treatment for IP tumors. In publication IV we used AgNP platform to show the homing of hyaluronic acid (HA) binding peptide to the IP tumors.

Table 4. TPP and NP platforms used in this thesis.

Peptide	Amino acid sequence	Receptor (homing specificity)	Nanoparticle platform	Publication in thesis
LinTT1 (KLAKLAK)*	AKRGARSTA $_D$(KLAKLAK)$_2$	p32	NW	I
iRGD	CRGDKGPDC	αv integrins; NRP-1	PS; NW	I–II
RPAR / R	GRPARPAR	NRP-1	PS; NW	I–II
IP3	CKRDLSRRC	HA	Ag-NP	IV

*chimeric with linTT1 peptide

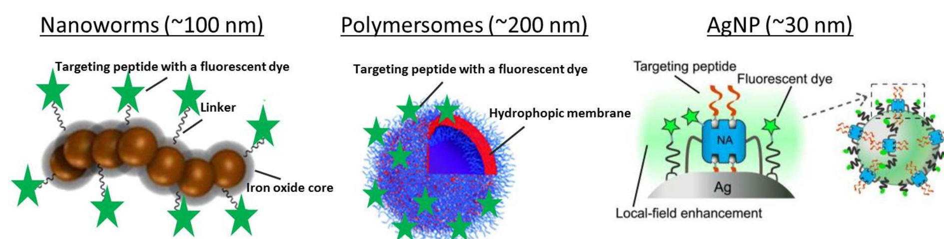

Figure 4. Nanoparticle platforms used in this thesis. Adapted from (Sharma et al., 2017, Simon-Gracia et al., 2016a, Braun et al., 2014).

4.2.1. TPP-NP preparation

NWs used in this thesis (publication I) were synthesized using a modified protocol adopted from methodology of preparing magnetic Iron oxide (IO) nanospheres (NS) based on the reaction of Fe (II) and Fe (III) salts in the presence of dextran (Palmacci S., Josephson L., 1993). To generate particles with a worm-like structure, higher concentrations of Fe-salts and higher MW dextran was used in the synthesis. In the last step of the synthesis, dextran coating of the iron cores was aminated to allow the attachment of N-hydroxysuccinimidyl (NHS) esters during the TPP coupling. Briefly, NHS-PEG-Maleimide linker was used to coat the aminated NW surface with malemide goups. Maleimides are electrophilic compounds that show high selectivity towards thiols present on cysteine residues of peptides and proteins. All the TPP used in this work were engineered to contain an extra cysteine residue for maleimide coupling.

PS used in publications II and III were prepared by self-assembly of amphiphilic copolymers in water. To label the PS with either 5(6)-carboxyfluorescein (FAM) or Rhodamine (Rho), the respective fluorophore was conjugated to the polymer during the synthesis. To encapsulate drugs (e.g. hydrophobic paclitaxel) inside the vesicles, organic solvents, such as $CHCl_3$ and MeOH, were used. The copolymer and the drug were mixed to form complexes. The complexes were dried under vacuum to remove the solvents and the final product-a polymer film, was dissolved in PBS. To functionalize the PS with TPPs, maleimide-cysteine reaction was used as for the NWs, except that the maleimide groups were incorporated into the copolymer during synthesis.

Ag-NPs used in publication IV were loaded with targeting peptides using neutravidin-biotin interaction. Neutravidin binds non-covalently with a very high affinity to biotin. Ag particles were prepared with neutravidin coating and the particles were coated with biotinylated IP3 peptide.

4.2.2. Characterization of nanoparticles

Throughout the current thesis, Dynamic Light Scattering (DLS) was used to assess the polydispersity and the average size of the nanoparticles. Transmission electron microscopy (TEM) was used to image the nanoparticles.

4.3. Bioactivity of TPP-NP *in vitro*

4.3.1. Binding of TPP-NP in a cell-free system (Publications I, II)

Recombinant hexahistidine–tagged p32 and NRP-1 b1b2 domain were bacterially expressed and purified as described (Paasonen et al., 2016, Teesalu et al., 2009). For cell-free binding assays, Ni-NTA magnetic agarose beads (Qiagen, Germany) in binding buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.05% NP40, 5 mM imidazole) were coated with p32 or b1b2 domain of the NRP-1 protein

(at 15 μg of protein/10 μL beads). Fluorescently labeled NWs or Rho-labeled PS were incubated with the protein coated beads in binding buffer containing 1% BSA at RT for 1 h. Incubation was followed by washes and elution with 400 mM imidazole containing binding buffer. The fluorescence of eluted samples, either at 490/520 nm with NW samples or at 526/555 nm with Rho-PS samples, was quantified using a fluorescence plate reader (FlexStation II, Molecular Devices, CA, USA).

4.3.2. Cellular binding and internalization of NPs

For flow cytometry experiments MKN-45P, CT-26, SKOV-3 cells in suspension were incubated with targeted or non-targeted NWs in complete cell culture medium for 1 h. The NW-containing solution was removed, cells were washed and analyzed by flow cytometry (Accuri, BD Biosciences, CA, USA). Anti-p32 antibody inhibition was done by pre-incubating the cells in suspension with 20 μg/mL of in-house rabbit polyclonal p32 antibody, followed by NW incubation for 1 h, washes and flow cytometry.

For fluorescence confocal imaging of FAM-labeled NWs and PS, MKN-45P, CT-26, SKOV-3, or PPC-1 cells (all at 5×10^4 cells/well) were seeded on glass coverslips in a 24-well plate. After 24 h, NWs (at 40 μg Fe/well) or PS (at 0.5 mg/mL) were added to the cells and incubated at 37 °C for 3 h (NW) or 1 h (PS). The cells were washed with PBS, fixed with 4 % of paraformaldehyde (PFA) in PBS pH 7.4, co-stained with DAPI and in-house rabbit anti-p32 or rabbit anti-NRP-1 antibody (Abcam, UK). Alexa 647-conjugated goat anti-rabbit IgG was used as a secondary antibody. The samples were imaged with fluorescence confocal microscopy (Zeiss LSM 510; Olympus FV1200MPE, Germany).

4.3.3. Subcellular localization studies

To study the subcellular localization of NWs, 5×10^4 MKN-45P cells were seeded on glass coverslips in a 24-well plate. On next day, the cells were incubated with NWs for 3 h. Subsequently, the cells were fixed and immunostained with rabbit anti-fluorescein antibody (cat. no. A889, Thermo Fisher Scientific, MA, USA) to detect FAM-labeled NWs and with mouse anti-cytochrome-C antibody (cat. no 89918, Thermo Fisher Scientific, MA, USA) to label mitochondria. The images were analyzed using the FV10-ASW 4.2 Viewer image software (Olympus, Germany).

To study cellular uptake of the PS samples a total of 5×10^4 PPC-1 cells were seeded in a 24-well plate with a coverslip. After 24 h PS loaded with Rho or with DOX were added to the cells at a concentration of 0.5 mg/mL and incubated for an hour. The cells were washed with PBS, fixed, stained with DAPI, and observed under the fluorescence confocal microscopy (Zeiss LSM 510).

4.3.4. Evaluation of nanoparticle cytotoxicity *in vitro*

4.3.4.1. MTT colorimetric assay

Cell viability was assessed by colorimetric assay based on reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by NAD(P)H-dependent cellular oxidoreductase to insoluble purple formazan. Briefly, cells were seeded in 96-well plates (10^4 MKN-45P or SKOV-3 cells, and 5×10^3 CT-26 cells) and grown in full medium at 37 °C. After 24 h different concentrations of NWs (3, 10, 30, 100, 300 μg/mL iron) were added to the wells.

After 6 h the medium was replaced with a fresh medium and after 18 h of incubation at 37 °C, the medium was aspirated and 10 μL of 5 mg/mL MTT reagent in PBS was added. The MTT reagent was removed in 2 h and the blue formazan crystals were dissolved in 100 μL of isopropanol and the absorbance was measured at 570 nm with a microplate reader (Tecan Austria, Austria). Alternatively, the cells were incubated for 24 h with the samples and the MTT assay was performed as described before.

4.3.4.2. xCELLigence® Real Time Cell Analysis (RTCA)

For real time cytotoxicity measurements, we used xCELLigence® RTCA DP instrument (Roche Diagnostics, GmbH, Mannheim, Germany and ACEA Biosciences, Inc. San Diego, CA, USA). Experiments were carried out using disposable 16-well xCELLigence® E-Plates with microelectrodes attached to the bottom of the wells for impedance measurements. To set the baseline, 50 μL of complete medium was added to the wells and background impedance was measured for each well. Subsequently, 50 μL of complete medium containing 10^5 cells was added to each well and E-plates were incubated in the RTCA DP device at 37 °C for 24 h. The impedance value was automatically collected in every 30 min and expressed as a cell index value (CI). Twenty-four hours after cell seeding, different compounds (PS, PS-PTX, RPAR-PS-PTX, iRGD-PS-PTX, and ABX) were added in triplicate at a final concentration of 100 nM of PTX. Complete medium alone was added to the control wells. CI was determined every 30 min over the following 25 h. All data were recorded and analysed using RTCA software version 1.2.1. CI-data from the experiments was normalized to the last data point prior to the addition of compounds. The data was expressed as percent viability relative to the untreated cells.

4.3.5. *Ex vivo* dipping assay on clinical tumor samples

Fresh surgical samples of peritoneal metastases of colon cancer patients were obtained under protocols approved by the Ethics Committee of the University of Tartu, Estonia (permit #243/T27).

The samples were immediately divided in explants of around 1 cm^3 and incubated with NWs (40 µg/mL Fe), PS (0.5 mg/mL) and AgNPs diluted in DMEM containing 1 % of BSA at 37 °C for 4 h. Next, the explants were washed 3 times with PBS, snap-frozen, cryosectioned at 10 µm, and immunostained using rabbit anti-fluorescein primary antibodies, followed by detection with Alexa-546 anti-rabbit secondary antibody (Invitrogen, Thermo Fisher Scientific, MA, USA). Alternatively, CF-555 dye labeled Ag-NP were used to visualize the tumor homing.

4.4. Biodistribution studies of TPP-NP In vivo

4.4.1. Experimental tumor mice

For *in vivo* homing studies nude mice were injected IP with 2×10^6 MKN-45P cells or 5×10^6 SKOV-3 cells. Balb/c mice received an IP injection of 2×10^6 CT-26 cells. Tumors were allowed to develop for 14 days in MKN-45P-injected mice and 4 weeks in SKOV-3-injected mice and 7 days in CT-26 injected mice. For dual tumors, nude mice were additionally inoculated with 10^6 MKN-45P or 0.5×10^6 CT-26 cells subcutaneously (SC) in the right flank.

4.4.2. Biodistribution studies of TPP-NP

To investigate the homing of NWs, the mice bearing MKN-45P, SKOV-3 or CT-26 IP tumors were injected IP or IV with FAM-labeled NWs (5 mg/kg Fe) and 5 h later the animals were perfused with 10 mL of PBS. After perfusion, the tumors and organs were excised, snap-frozen in liquid nitrogen, and stored at -80 °C for further analysis.

To test homing of PS, FAM-labeled PS were injected IP (0.5 mg in 500 µL of PBS) or IV (0.5 mg in 100 µL of PBS) and after 4 h the animals were perfused with 10 mL of PBS. Alternatively, CF-555 dye labeled Ag-NP were injected IP into MKN45-P tumor bearing mice and after 4h, animals were perfused with PBS.

After perfusion, the tumors and organs were excised, snap-frozen in liquid nitrogen, and stored at -80 °C for further analysis.

4.4.3. *Ex vivo* imaging

Targeted and non-targeted PS samples were injected into MKN-45P or CT-26 tumor bearing mice and after 4 h the tumors and organs were excised and washed with PBS for fluorescence visualization under Illumatool light source using the FAM filter set (Lightools Research, US), image acquisition using a digital camera, and fluorescence quantification using Image J software.

4.4.4. Magnetic resonance imaging (MRI)

Nude mice bearing MKN-45P IP tumors were injected IP with NWs coated with linTT1 peptide or FAM only (5 mg/kg Fe per injection). The mice were subjected to MRI before injection and 5 h after the NW injection. For each scan mice were anestesized with isoflurane (3.5% induction, 1.5–2.0% maintenance) in air/O_2 (2:1) and placed in a MR receiver mouse coil. Fast spin echo T2-weighted iron sensitive MRI scans were acquired using 9.4 T MR system (Bruker, USA) at TR/TE=1.8 s/23 ms and slice thickness of 0.5 mm.

4.4.5. *In vivo* evaluation of bystander activity

MKN-45P tumor mice were sequentially injected with 5 mg/kg of NWs and 0.3 mg Texas red-conjugated 70-kDa lysine-fixable dextran (Molecular Probes, MA, USA) in PBS into the abdominal cavity (total volume of injection: 1 mL). After 90 min, the mice were terminated, and the tissues were excised and processed for immunofluorescence.

4.5. Immunofluorescence and microscopic imaging

For immunofluorescence staining of tissues, 10 µm cryosections were equilibrated at RT, fixed in PFA for 15 min, permeabilized using PBS containing 0.2% Triton-X for 10 min, and blocked in PBS containing 0.05 % Tween-20, 5% FBS, 5% BSA, and 5 % goat serum (GE Healthcare, UK) for 1 h. The sections were immunostained with rabbit anti-fluorescein (cat. no. A889, Thermo Fisher Scientific, MA, USA), rat anti-mouse CD31, biotin rat anti-mouse CD11b (cat. no. 553370; 557395, BD Biosciences, CA, USA), rat anti-mouse LYVE-1 (cat. no. 14044382, eBioscience, CA, USA), rabbit polyclonal anti-Ki67 (cat. no. NB500-170, Novusbio, UK), and rabbit anti-Cleaved Caspase-3 (Asp 175), (cat. no. 966, Cell Signaling Technology, Inc., MA, USA), rabbit anti-NRP-1 (Abcam, UK) or rabbit anti-αv-integrin (Millipore, US) as primary antibodies. Alexa 488-conjugated goat anti-rabbit IgG, Alexa 647-conjugated goat anti-rat IgG, Alexa 546-conjugated goat anti-rabbit IgG (all Invitrogen, Thermo Fisher Scientific, MA, USA) and streptavidin Dylight® 550 (Thermo Fisher Scientific, MA, USA) were used as secondary antibodies. Nuclei were counterstained with 10 µg/ml DAPI. The stained tissue sections were examined by fluorescence confocal microscopy using Olympus FV1200MPE or Zeiss LSM 510 instrument, and the images were processed and analyzed using the FV10-ASW 4.2 Viewer image software (Olympus, Germany) or ZEN lite 2012 image software and ImageJ freeware.

4.6. Experimental tumor therapy

To test the *in vivo* efficacy of linTT1 proapoptotic NW, IP MKN-45P tumors were induced in nude mice by IP injection of 1.5×10^6 MKN-45P cells in 500 µL of PBS. Five days after tumor induction, the mice were randomized into 5 groups (8 mice in each group). Starting on day 5, mice were IP injected every other day with linTT1-NW, $_D$(KLAKLAK)$_2$-NW, linTT1- $_D$(KLAKLAK)$_2$-NWs, or non-targeted NWs at a dose 5 mg/kg Fe per injection, or with 500 µl of PBS. Body weight was monitored daily and the study was terminated when the body weight of the first animal in the study decreased by 20 % compared to the start of the treatment. The mice were perfused with 10 mL of PBS and larger tumors in the IP cavity were weighed and metastatic nodules smaller than 2 mm in diameter were counted.

To investigate the *in vivo* efficacy of targeted and non-targeted PS the athymic nude mice were injected IP with 2×10^6 MKN-45P cells. Three days after the cell injection, the mice were randomized in 4 groups (8 animals in each group). The mice were treated every other day with IP injections of 0.5 mL of ABX, PS-PTX, iRGD-PS-PTX, at the same PTX dose (cumulative dose: 7 mg/kg), or with PBS. 18 days after tumor induction the mice were perfused with 10 mL of PBS and the tumors and organs were excised. To estimate the tumor burden, the total weight of large (tumors bigger than 5 mm in diameter) and medium peritoneal tumors (tumors bigger than 2 mm and smaller than 5 mm of diameter) together with the small metastatic peritoneal nodules (tumor nodules smaller than 2 mm of diameter) was determined.

4.7. Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA) with Bonferroni comparison tests was performed using GraphPad Prism Software (Graphpad, CA, USA). ANOVA and Fisher LSD was performed with Statistica 8 software (StatSoft, OK, USA).

5. RESULTS

5.1. TPP-NP show receptor-dependent specificity and cytotoxicity *in vitro* (Publications I–III)

5.1.1. TPP-NP selectively bind to their target proteins

To study suitability of RPARPAR and TT1 homing peptides for affinity targeting of NPs, we first evaluated the interaction of peptide guided NPs with their cognate receptors (NRP-1 and p32, respectively) in a cell-free system. PS functionalized with RPARPAR (RPAR-PS) peptide readily bound to immobilized recombinant b1b2 domain of NRP-1 (Fig. 5A). The RPAR-PS showed only a background binding to recombinant p32 and untargeted PS did not bind to either protein.

In the case of NWs (Fig. 5B), we observed that fluorescein FAM-labeled linTT1-NWs readily bound to immobilized p32, whereas untargeted FAM-NWs, or FAM-NWs functionalized with a control RPARPAR peptide, showed only background binding.

These data show that homing peptides coated on PS or NWs are available for receptor interactions and that the tropism of nanoparticles can be specifically modulated by functionalization with TPPs.

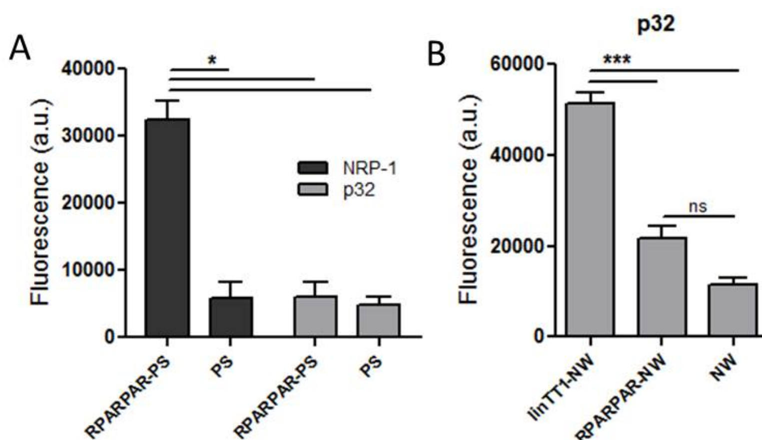

Figure 5. Binding of peptide-NPs to purified receptor proteins. (A) Binding of RPARPAR-PS or PS labeled with rhodamine to recombinant NRP-1 (b1b2 NRP-1) or to p32. For this assay, 0.5 mg/mL of PS samples were incubated with the recombinant proteins for 1h. Y-axis represents the bound PS fluorescence in arbitrary units (A.U.). N=3 (B) Binding of FAM-labeled linTT1-NWs, RPARPAR-NWs or control FAM-NWs to recombinant p32. Thirty µg/mL of NWs were incubated with immobilized his-tagged p32 for 1 h, followed by washes to remove unbound NWs and quantification of bound FAM-NWs by spectrometry. Y-axis represents the bound NW fluorescence in arbitrary units (a.u.). N = 3; statistical analysis by one-way ANOVA; error bars, mean + SEM, * $p < 0.05$; ***, $p < 0.001$.

5.1.2. TPP-NP bind to cultured PC cells in receptor-dependent manner

Since the target receptors for homing peptides used in this study, p32 and NRP-1, are predominantly upregulated on the surface of proliferating malignant cells, we established the relevance of p32 for linTT1-NW targeting and NRP-1 for RPAR-PS targeting. We determined whether on cultured PC cells plasma membrane p32 can be targeted with linTT1-NWs and cell surface NRP-1 can be targeted with RPAR-PS and iRGD-PS. To study interaction of linTT1-NW with p32-positive cells, we used flow cytometry analysis on non-permeabilized cells. The LinTT1-NWs, not FAM-NW, were found associated with the p32-positive cells after 1 h incubation. To further verify if the LinTT1-NW binding was p32 dependent, we pre-incubated the cells prior to incubating with NW samples with a function-blocking anti-p32 antibody. The blocking of p32 reduced LinTT1-NW binding up to 90 % (Fig. 6A, B). Confocal microscopy of cultured cells 3 h after addition of NWs revealed colocalization of linTT1-NW with p32 on the surface of the MKN-45P, CT-26 and SKOV-3 cells, whereas untargeted NW showed minimal binding (Fig. 6C). After 1 h of incubation, RPAR-PS showed a high uptake in both cell lines, whereas the control FAM-PS showed a low baseline uptake in both cell lines (Fig. 6D).

These experiments demonstrated that p32 and NRP-1 are expressed on the cell surface of cultured PC cell lines and that NP coating with linTT1, iRGD, or RPAR peptides can be used for robust and specific targeted delivery of compounds and nanoparticles to PC cells.

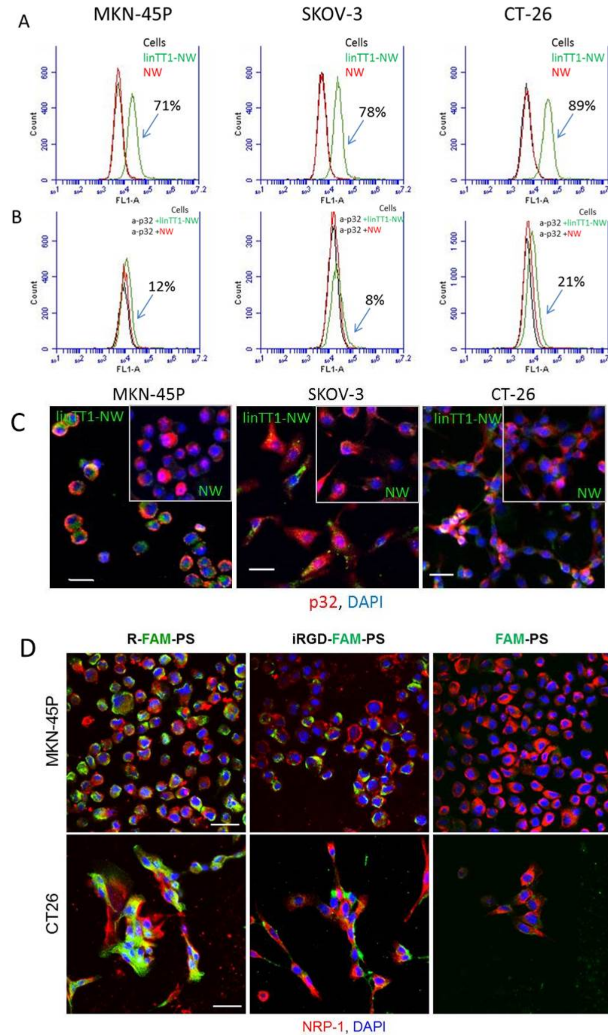

Figure 6. TPP-NP bind to cultured peritoneal carcinomatosis cells in a receptor dependent manner. (A) Flow cytometry of MKN-45P, SKOV-3 and CT-26 cells incubated with linTT1-NWs and control FAM-labeled NWs. Cells in suspension were incubated with NWs (at 30 µg/mL Fe) for 1h, followed by washes, and flow cytometry analysis. Green line: cells incubated with linTT1-NWs; red line: cells incubated with NWs; black line: cells without NW incubation. (B) Anti-p32 antibody inhibition of NW binding to MKN-45P, SKOV-3 and CT-26 cells. Suspended cells were pre-incubated with 20 µg/mL of p32 antibody, followed by NW incubation for 1h, washes and flow cytometry. The labeling color scheme is the same as in A. (C) Fluorescence confocal imaging of cultured adherent MKN-45P, CT-26 and SKOV-3 cells incubated with linTT1-NWs or non-targeted NWs for 3 h. Green: NWs; Red: p32; blue: DAPI. Scale bars: 30 µm. (D) Fluorescence confocal imaging of MKN-45P and CT-26 cells incubated with 0.5 mg/mL of RPAR-PS (R-PS), iRGD-PS or FAM-PS for 1 h. The cells were stained with DAPI and anti-NRP-1 antibody. Green: PS; red: NRP-1; blue: DAPI. Scale bars: 20 µm.

5.1.3. Internalized linTT1-NW are routed to mitochondria and have a cytotoxic effect on cultured IP tumor cells

In activated cells, p32 is present at the cells surface and in the mitochondria and by cycling between those two locations, could take payloads with it (Agemy et al., 2013). Thus, mitochondrial localization of linTT1 can be used to improve the cytotoxic activity of pro-apoptotic peptide, $_D$(KLAKLAK)$_2$, that exerts its effect by destabilization of mitochondrial membranes (Ellerby et al., 1999). We hypothesized that linTT1 peptide may be well suited for mitochondrial targeting of $_D$(KLAKLAK)$_2$ effector module in the p32-positive PC cells. First, confocal imaging of MKN-45P cells demonstrated that after 3 h of incubation intra-cellular linTT1-NWs colocalized with cytochrome C, a mitochondrial marker (Fig. 7A). Next, we studied the effect of NWs coated with linTT1-$_D$(KLAKLAK)$_2$ chimeric peptide on viability of cultured MKN-45P, CT-26, and SKOV-3 cells and found that linTT1-$_D$(KLAKLAK)$_2$-NWs reduced the viability of all three PC cell lines, with treatment at the highest concentration, 300 ug Fe/mL (~50 nmol/mL peptide) causing death of about 40 % cells (Fig. 7B-D). In contrast, control NWs coated with either peptide alone did not have an effect. These observations indicate that the linTT1-NW uptake pathway in PC cells is compatible with *in vitro* delivery of mitochondrially-acting proapoptotic peptide to induce cell death.

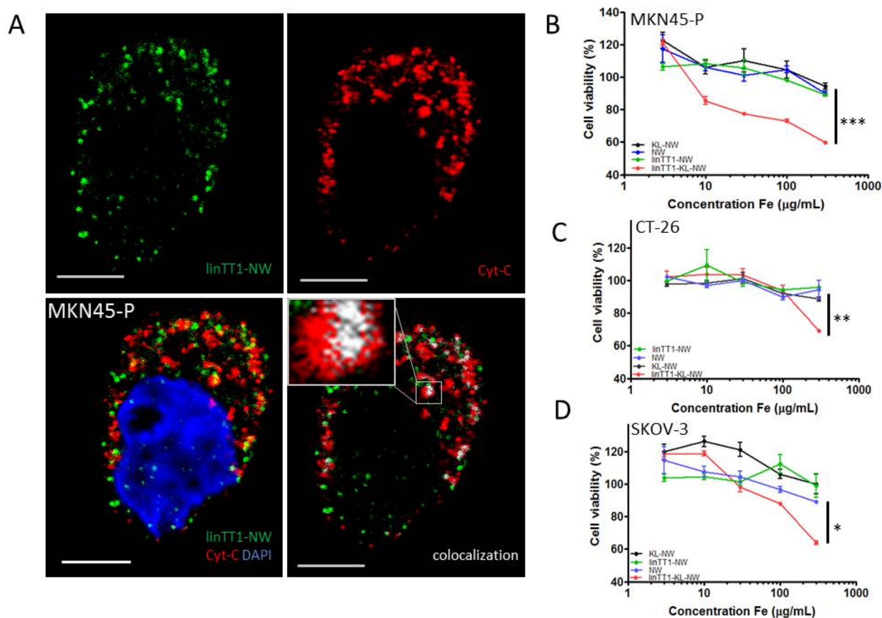

Figure 7. LinTT1-NWs are routed to mitochondria and potentiate the activity of a proapoptotic peptide payload. (A) Internalized linTT1-NWs colocalize with a mitochondrial marker, cytochrome C. MKN-45P cells were incubated with linTT1-NWs for 3 h, washed, and fixed for immunofluorescence staining. The cells were incubated with rabbit anti-FITC primary antibody to detect the NWs and with mouse anti-cytochrome C to label mitochondria, followed by incubation with anti-mouse Alexa Fluor-546 and anti-rabbit Alexa Fluor 647 secondary antibodies; nuclei stained with DAPI. linTT1-NW: green; cytochrome C (Cyt-C): red; DAPI: blue; colocalization of FAM and Cyt-C signal: white. Scale bar: 5 µm. (B-D) Treatment with linTT1-NW coupled to the proapoptotic peptide $_{D}$(KLAKLAK)$_2$, decreases viability of tumor cells. MKN-45P, CT-26 and SKOV-3 cells were incubated with the indicated NWs over a range of iron concentrations (3, 10, 30, 100, 300 µg/mL), and cell viability was assessed after 6 h incubation by a colorimetric assay based on reducing the tetrazolium dye MTT. KL: $_{D}$(KLAKLAK)$_2$. Statistical analysis was performed by ANOVA. n=3; error bars indicate ±SEM; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

5.1.4. TPP-PS release their cytotoxic cargo in the cytoplasm to exert time dependent cytotoxicity on target cells

Following cellular uptake via endosomal internalization pathway, PS cargo is released into the cytoplasm, possibly through endosomal membrane disruption by proton sponge effect (Massignani et al., 2009). We loaded RPAR-PS with fluorescent cargoes [Rhodamine B octadecyl ester (Rho) and Doxorubicin (DOX)] to study the *in vitro* internalization in PPC-1 cells and cytoplasmic release of cargo from peptide -guided PS. After 1-h incubation of PPC-1 cells with RPAR-FAM-PS loaded with Rho, a widespread Rho fluorescence was observed in the cytoplasm (Fig. 8A). Interestingly, already 1 h after incubation with the cells, the FAM-RPAR-polymer and Rho cargo exhibited clearly different intracellular distribution patterns (arrow in Fig. 8A). After 1 h incubation of PPC-1 cells with the DOX-loaded RPAR-PS, the intrinsic red fluorescence of DOX was observed in the nuclei of the cells (arrow in Fig. 8B). In contrast, only a weak DOX signal was seen in PPC-1 cells incubated in the presence of DOX-loaded untargeted PS (Fig. 8B). These data suggest that cellular uptake of TPP-PS is followed by disassembly of PS and cytoplasmic release of the payloads.

Next, we determined the cytotoxicity profile of TPP-PS loaded with cytotoxic drug, paclitaxel (PTX). As a reference, we used Abraxane (ABX), a colloidal suspension of paclitaxel and human serum albumin that is clinically approved for the treatment of several types of solid tumors (breast, lung, pancreatic, and gastric carcinoma), and is in advanced clinical trials for the treatment of colorectal cancer. We studied the viability of NRP-1-positive PPC-1 and NRP-1-negative M21 cells using x*CELL*igence RTCA DP technology – a real-time label-free technique to assess cellular proliferation, migration and invasion of cultured cells. We exposed cells to different compounds all at 100 nM PTX. In PPC-1 cells, PTX-PS targeted with RPAR and iRGD peptides were significantly more toxic than untargeted PTX-PS, or ABX (Fig. 8C). Approximately 50% of PPC-1 cells treated with RPAR-targeted or iRGD-targeted PTX-PS remained viable after 24 h of incubation, whereas about 80% of cells treated with untargeted PTX-PS were viable, and ABX had only a negligible effect (Fig. 8C). When observing NRP-1 negative M21 cells, the viability was not significantly affected by 24-h exposure to PTX-loaded PS targeted with iRGD or RPAR (Fig. 8C). Empty PS did not affect cell viability at any time point tested. These experiments show that PTX-PS targeted with TPP specifically decrease the viability of cultured NRP-1 expressing cells.

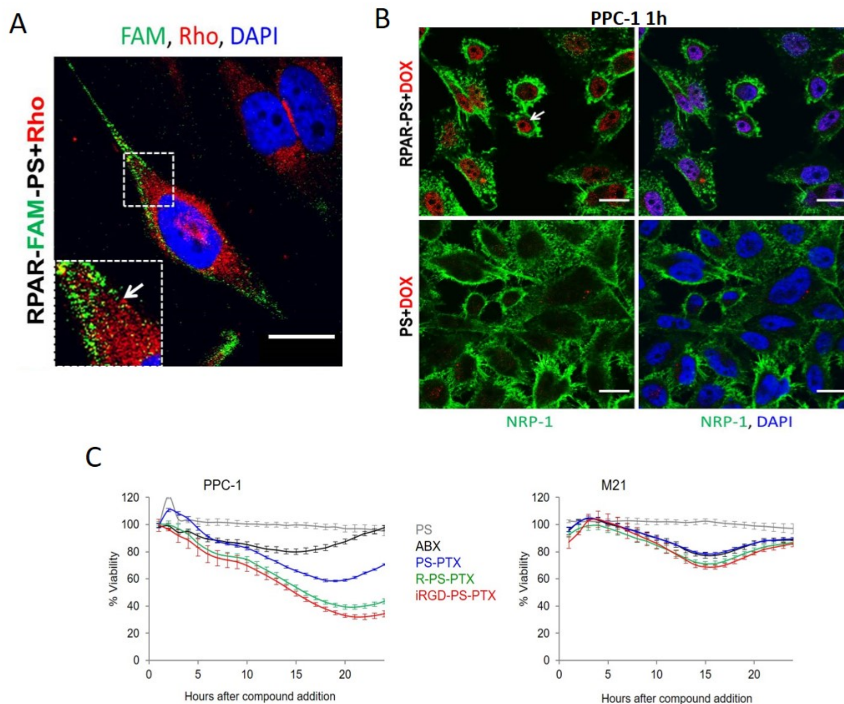

Figure 8. Peptide-guided PS release their cargo in the cytoplasm of target cells and potentiate the activity of an anticancer payload. (A) Fluorescence confocal imaging of PPC-1 cells incubated with 0.5 mg/mL of RPAR-FAM-PS loaded with Rho for 1 h. The cells were counterstained with DAPI. Green: PS; red: Rho; blue: DAPI. Scale bar: 20 µm. Representative fields from multiple areas of cultured cells from three independent experiments are shown. (B) Fluorescence confocal imaging of PPC-1 cells incubated with 0.5 mg/mL of RPAR-PS or PS loaded with doxorubicin (DOX) for 1 h. The cells were stained with DAPI. Green: NRP-1; red: DOX; blue: DAPI. Scale bars: 20 µm. Representative fields from multiple areas of cultured cells from three independent experiments are shown. (C) Growth rate dynamics of cultured PPC-1 and M21 cells after addition of the RPAR, iRGD, or untargeted PS loaded with PTX, and ABX at 100 µM PTX concentration, measured using the xCELLigence® real-time cell analyzer that allows continuous quantitative monitoring of attached cells. 100 % viability corresponds to untreated cells. N=3. Error bars: mean ±SEM.

5.2. TPP-NP home selectively to tumor lesions *ex vivo* and *in vivo* (Publications I–IV)

5.2.1. TPP-NP home to clinical tumor explants *ex vivo*

To evaluate the translational potential of TPP-NP we tested the binding of NW, PS and AgNP coupled with different tumor homing peptides on fresh surgical explants of peritoneal metastasis of human colon cancer. The *ex vivo* tumor dipping assay results showed TPP-NP binding in human colon cancer tissues, whereas the control NP only weakly labeled the surface of the tumors (Fig. 9). These findings serve as an initial starting point towards the translation of the TPP-NP platforms into clinical setting.

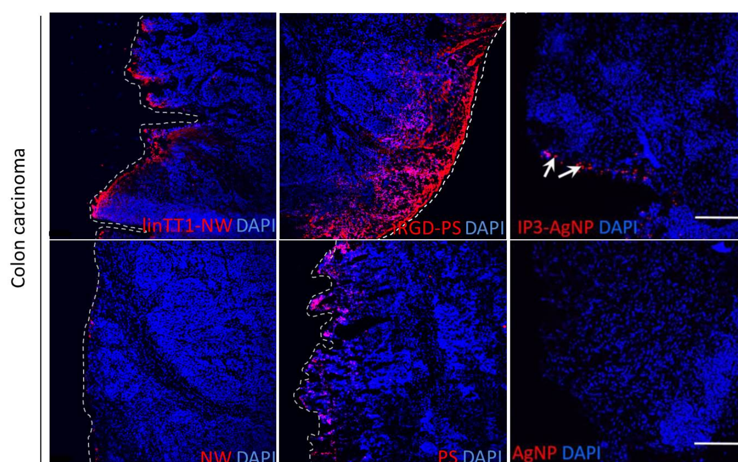

Figure 9. Confocal fluorescence microscopy imaging of TPP-NW binding to human colon cancer explants. Fresh surgical samples of colon cancer were incubated with targeted NPs or untargeted NPs for 4 h at 37 °C, followed by sectioning and staining with anti-FITC (rabbit) and Alexa Fluor 546 (anti-rabbit) secondary antibody. Alternatively, CF-555 dye labeled Ag-NP were used to visualize the homing of the IP3-labeled AgNP. Arrows point to IP3-AgNP bound to the edge of the tumor. Red: NPs; blue: DAPI. Scale bars: 200 μm.

5.2.2. Intraperitoneally-injected linTT1-NW have improved tumor selectivity over systemically-injected NWs

We next studied the biodistribution and accumulation of linTT1-NW in tumor tissue and control organs by comparing two administration routes: systemic IV injection and locoregional IP injection. For that we used MKN-45P peritoneal tumors in mice. Five h after IP injection of linTT1-NW, we observed wide-spread signal in the tumor tissue and low background in the control organs (Fig. 10B). In contrast, tumor signal in mice injected with systemic linTT1-NW was accompanied by a strong non-specific background in control organs,

particularly in the liver (Fig. 10A, B). Quantitative imaging of the mice dosed IP or IV with linTT1-NWs showed that the FAM signal in the tumors was almost twice as high after IP injection, whereas the signal in control tissues was more than 3-fold lower after IP administration (Fig. 10C) further supporting the superiority of IP administration route for peptide NWs for PC targeting.

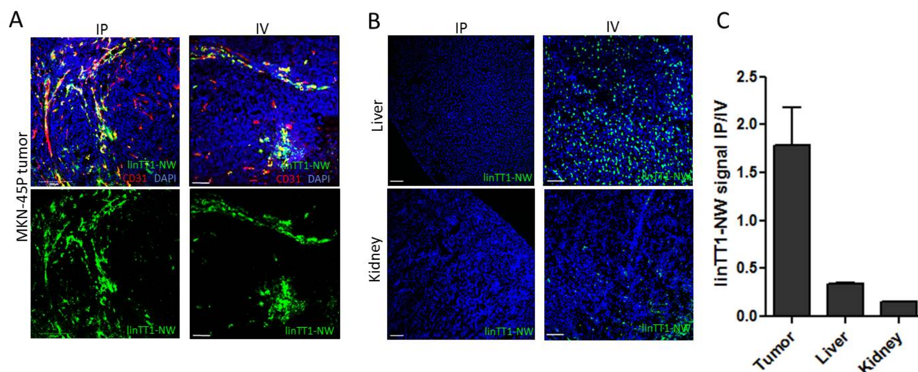

Figure 10. Intraperitoneal linTT1-NWs have improved tumor selectivity over systemically administered NWs. (A) Representative fluorescence confocal images of linTT1-NW at 5 h after IP (first column) or IV injection (second column) demonstrate tumor homing for both routes of administration. Cryosections of tumor tissues were stained with a CD31 antibody to visualize the blood vessels. Green: NWs; Red: CD31, Blue: DAPI. Representative fields from multiple sections (n ≥3) prepared from at least 3 tumors are shown. Scale bars: 100 µm. (B) Biodistribution of linTT1-NWs injected IP or IV in non-target organs (liver and kidney) in mice bearing MKN-45P tumors. NWs were injected at a dose of 5 mg/kg, and tissues were collected after 5 h. Blue, DAPI; green: FAM. Scale bars: 100 µm. (C) Quantification of green fluorescence in tumor, liver and kidney after IP or IV injection of linTT1-NWs. Fluorescence signal intensity was quantified by ImageJ freeware and normalized for tissue area. Representative fields from multiple sections from tumors in 3 mice are shown.

5.2.3. LinTT1-NW home to peritoneal tumor lesions *in vivo*

Immunophenotyping of cells positive for linTT1-NWs with a panel of cell type specific marker antibodies demonstrated that in MKN-45P tumor model linTT1-NWs co-localized with CD31-positive blood vessels (Fig. 11A, B). Outside tumor blood vessels, a partial overlap was seen with lymphatic vessels and macrophages (Fig. 11). Similar pattern was observed in SKOV-3 ovarian tumor tissue where linTT1-NW co-localized with blood vessels and macrophages (Fig. 11), suggesting that a combination of direct penetration from the IP cavity and indirect accumulation via systemic circulation drives tumor accumulation of the linTT1-NWs. In the case of CT-26 tumors, we observed near-complete colocalization of linTT1-NWs with CD11b-positive macrophages (Fig. 11) suggesting that in this model particle accumulation is primarily direct, not vascular-mediated.

The distinct localization pattern in different types of tumor tissue can be supported by previous data that cell surface p32 is expressed besides tumor cells also on other activated cells such as vascular and lymphatic endothelial cells and tumor-associated macrophages, especially in hypoxic and nutrient-deprived areas (Fogal et al., 2008, Agemy et al., 2013) and that p32 expression level and subcellular localization has also been demonstrated to be affected by the tumor type and -stage.

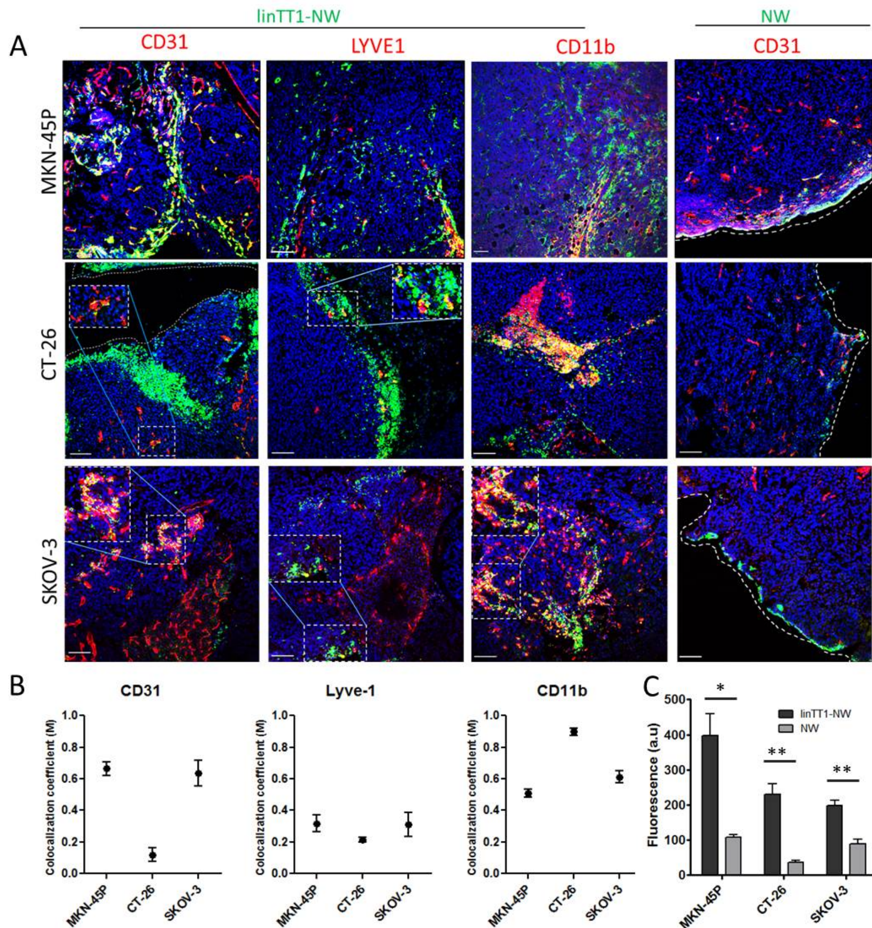

Figure 11. Characterization of LinTT1-NW target cell populations in different models of peritoneal carcinomatosis. (A) Confocal imaging of tumor sections. Mice bearing MKN-45P (upper panel), CT-26 (middle panel) and SKOV-3 (lower panel) tumors were IP injected with linTT1-NWs (5mg iron/kg). Tissues were collected after 5 h circulation, and cryosections of tumor tissue were stained with antibodies against CD31 (blood vessels), LYVE-1 (lymphatic vessels) and CD11b (macrophages). Green: NWs; Red: CD31, LYVE-1 or CD11b. Blue: DAPI. Scale bars: 100 µm. (B) Colocalization analysis of linTT1-NWs with CD31, LYVE-1 and CD11b in MKN-45P, CT-26 and

SKOV-3 tumor models based on Manders (M) coefficient (Manders, Verbeek & Aten, 1993) 0= no colocalization; 1= complete colocalization. Analysis was performed by ImageJ software. Error bars, mean ±SEM (C) Quantification of green fluorescence intensity in the confocal images of tissue sections prepared from MKN-45P, CT-26 and SKOV-3 tumors. Representative fields from multiple sections of tumors from 3 mice per group are shown. Analysis by ImageJ, N ≥ 3 mice; Statistical analysis: Student's t-test; error bars: mean ± SEM; $**p < 0.01$, $*p < 0.05$.

5.2.4. LinTT1-NWs as a tumor-seeking contrast agent

Iron oxide is a well-known imaging agent that produces hypointense areas in T2-weighted MR images (McAteer et al., 2007). To investigate the potential of linTT1-NWs for PC imaging we performed MR imaging experiments. Nude mice bearing MKN-45P IP tumors were injected with linTT1-coated or control NW. The tumor areas in mice injected with TT1-NW showed hypointense regions, whereas untargeted NW produced no detectable signal decrease under the same imaging conditions (Fig. 12A and B). Post- MRI *ex vivo* imaging of tumors and control organs showed selective accumulation of fluorescently labeled TT1-NW in tumor tissue (Fig. 12C).

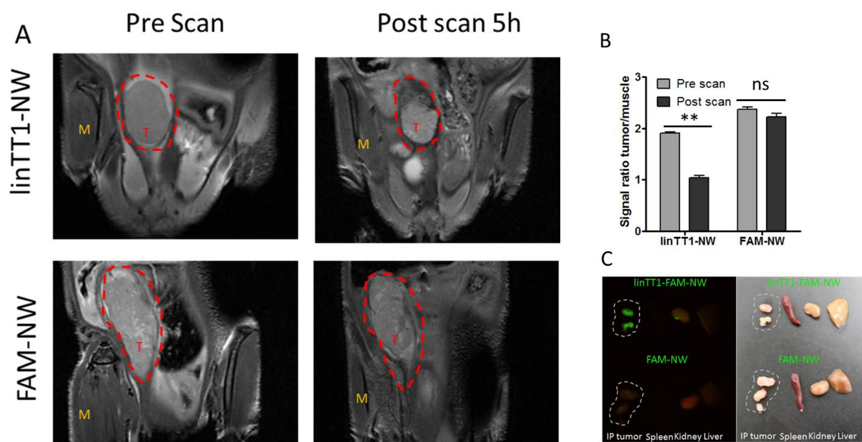

Figure 12. PC imaging with linTT1-NWs. (A) T2-weighted magnetic resonance images of mice bearing IP MKN-45P tumors. The mice were injected intraperitoneally with linTT1-coated or control FAM-coated NWs (5 mg/ kg of iron). (A) Coronal images of the tumors were acquired using 9.4 T Bruker MR system (TR/TE=1.8 s/23 ms; slice thickness 0.5 mm) before NW injection (pre-scan) and 5 h after NW injection (post scan). T, tumor; M, muscle (B) linTT1-NWs produced significant hypointensities in the tumor tissue whereas control NWs gave no signal. The signal intensity was normalized to the adjacent muscle tissue. Five images per time point were analyzed. Statistical analysis: Student's t-test; error bars: mean + SEM; $** p < 0.01$. (C) (C) After MR imaging, tumors and control tissues were excised and fluorescent signal was imaged *ex vivo* by Illumatool (Lightools Research, CA).

5.2.5. LinTT1-NW trigger bystander effect after IP injection

LinTT1 belongs to the family of CendR peptides and a relevant feature of these peptides is to induce a bystander effect, an increased accumulation of coadministered compounds. For example, the prototypic CendR peptide iRGD increases the accumulation of coadministered payloads in tumor tissue (Schmithals et al., 2015, Deng et al., 2017). To determine whether linTT1 coupled to NW could trigger a trans-tissue transport pathway for coadministered payloads (Sugahara et al., 2010), we administered via IP injection linTT1 peptide coupled to NW (or iRGD-NWs as a positive control) simultaneously with 70 kDa fluorescently-labeled dextran, collected the tissues 90 min later, and studied the biodistribution of the dextran in tissue sections. We observed a significant increase in dextran accumulation in the tumor with linTT1-NWs and iRGD-NWs, whereas only a minimal signal was present when dextran was injected alone (Fig. 13).

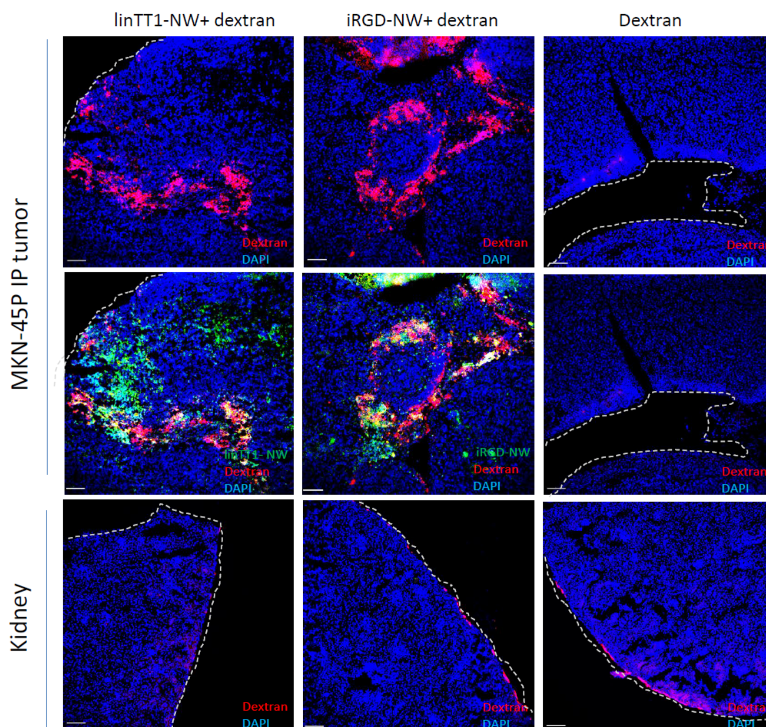

Figure 13. IP linTT1-NWs increase MKN-45P tumor accumulation of coadministered 70 kDa dextran. Mice bearing disseminated MKN-45P tumors were injected IP with the indicated NW formulations (5 mg/kg Fe) and 0.3 mg of 70 kD dextran in 1 ml PBS. After 90 min, the mice were perfused, and tissues processed for confocal imaging. Red: dextran, green: TPP-NP.

5.2.6. Conjugation of TPP to the surface of PS improves tumor accumulation after IP injection

To determine the targeting effect of iRGD or RPAR-PS in a mouse model of PC, we studied the homing of IP-administered PS to peritoneal and subcutaneous MKN-45P and CT-26 tumors in mice. Macroscopic imaging of tissues after 4 h of IP injection demonstrated that FAM-labeled targeted PS accumulated in both MKN-45P and CT-26 peritoneal tumors, but not in control organs, whereas untargeted FAM-PS gave a weaker tumor signal (Fig. 14A and B). In both tumor models, iRGD-PS group showed highest fluorescence in IP tumors and only background signal was detected in the control organs (Fig. 14C and D).

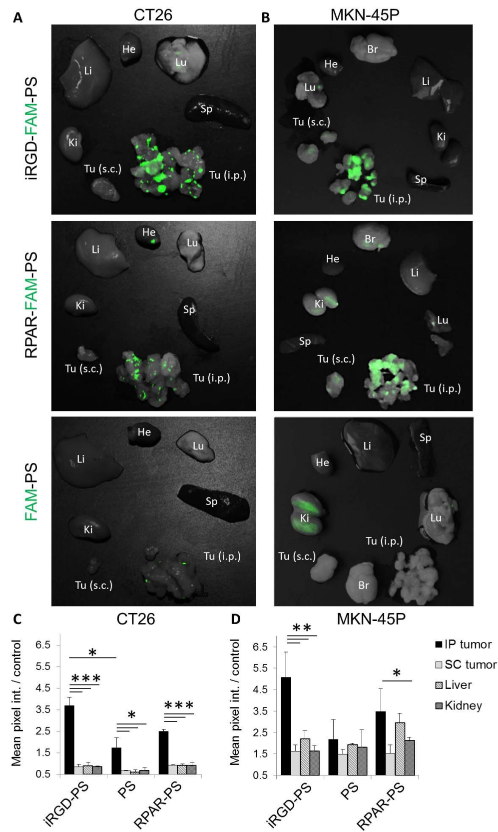

Figure 14. *In vivo* biodistribution of IP-administered PS. (A) Mice bearing dual IP and SC MKN-45P or CT-26 tumors were IP injected with 0.5 mg of FAM-labeled iRGD-PS, RPAR-PS, or untargeted PS, and after 4 h the tumors and organs of interest were excised and fluorescent signal was imaged by Illumatool (Lightools Research, CA). Representative compound fluorescent and bright-field images from three independent experiments are shown. He, heart; Lu, lung; Sp, pleen; Ki, kidney; Li, liver; Br, brain, Tu, tumor. (B) Quantification of the fluorescent signal in tumors and control organs by the ImageJ software. $N \geq 3$ mice; statistical analysis was performed by one-way ANOVA; error bars, mean + SEM; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

5.2.7. IP-administered TPP-PS home to peritoneal and subcutaneous tumors

To obtain information on the distribution of the FAM-labeled PS, we immunostained the tissue sections with anti-FAM antibody. In peritoneal MKN-45P tumors, iRGD-PS accumulation was seen in the tumor periphery and deep within the tumor mass (Fig. 15B, arrows), partially co-localizing with αv-integrins and NRP-1 (Fig. 15A). In IP CT-26 tumors iRGD-PS accumulated predominantly in the tumor periphery (Fig. 15C and D). In SC MKN-45P tumors, iRGD-PS were found scattered in the tumor parenchyma, partially overlapping with CD31-positive blood vessels (Fig. 15B). In both models, untargeted PS only weakly labeled the surface of the peritoneal tumors (Fig. 15B and D, arrowhead). The extent iRGD-PS accumulation was lower in the SC CT-26 than in MKN-45P tumors (Fig. 15B and D). This result correlated with the expression of NRP-1, which was lower in the CT-26 tumors (Fig. 15A and C). These data indicate that iRGD-functionalization improves the accumulation of PS in IP and, to some extent, in SC tumors.

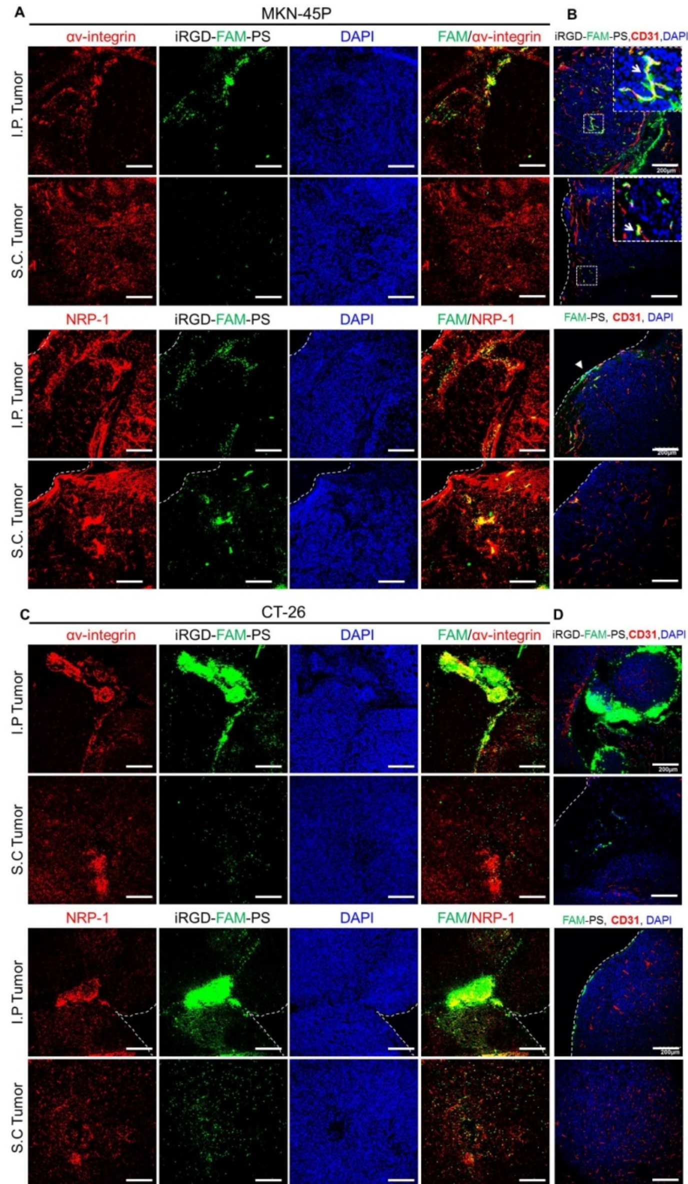

Figure 15. Confocal imaging of PS in tumor tissue. Co-localization of FAM-labeled iRGD-PS with αv-integrins and NRP-1. (A-D) Fluorescence confocal images of tissue sections prepared from IP and SC MKN-45P and CT-26 tumors collected 4 h after IP injection of PS. iRGD-FAM-PS and control FAM-PS (0.5 mg) were IP-injected and the tumors were excised 4 h later. (A, C) Tissue sections were stained for αv-integrins, or NRP-1 (red) (B, D) Tissue sections were stained for blood vessels: CD31 (red) and the nuclei were counterstained with DAPI (blue). The green fluorescence corresponds to PS, which were labeled with FAM. Scale bar: 200 μm. Representative images of three independent experiments are shown. Arrows point to PS co-localizing with blood vessels; arrowheads point to PS in tumor periphery.

5.2.8. IP-administered IP3 peptide-conjugated AgNPs home peritoneal gastric and colon carcinomas (Publication IV)

IP3 peptide was discovered using a T7 peptide phage library that was IP injected into a MKN-45P tumor bearing mice. The library consisted of cyclic peptides with seven random amino acids; CX7C and with a diversity of 10^8. The biopanning combined *ex vivo* and *in vivo* selection rounds. Subsequently, the peptide-encoding portion of the phage genome was subjected to High Throughput Sequencing (HTS) with Ion Torrent and analyzed with bioinformatics tools for peptide identification. During analysis critical parameter for peptide selection was high tumor-to-kidney ratio and based on the results IP3 was selected for individual evaluation. IP3 peptide contains a hyaluronic acid (HA) binding motif and targets tumor extracellular matrix and macrophage rich regions in tumors.

We further assessed whether IP3 has potential as a targeting ligand for NPs, biotinylated IP3 and control peptides were coated on ~30 nm silver NPs (AgNPs). Peptide-functionalized AgNPs were injected IP into mice bearing MKN-45P and CT-26 tumors. Confocal images revealed a robust accumulation of IP3-AgNPs in the outer rim of both MKN-45P and CT-26 tumors. In addition to the peripheral accumulation, IP3-AgNPs penetrated deeper in the tumor (arrows in Fig. 16A, B). In contrast to IP3-AgNPs, only low, background levels of the control AgNPs (biotin AgNA555) were observed at the edge of both MKN-45P and CT-26 tumors (arrowheads in Fig. 16C, D).

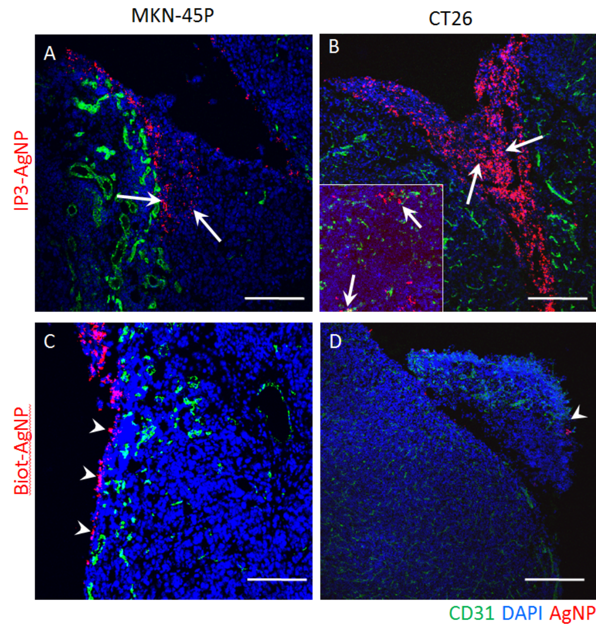

Figure 16. IP-administered IP3 peptide-conjugated AgNPs home to peritoneal gastric and colon carcinomas. Confocal imaging of MKN-45P and CT-26 tumors after 4 h of injection with AgNPs (red). The cryosections were immunostained anti-CD31 (green) and the nuclei were counterstained with DAPI (blue). (A, B) Tumor homing and penetration of IP3-AgNPs. (A) IP3-AgNPs located away from the edge of the tumor to some degree while being in close proximity to blood vessels or without association with blood vessels (arrows in Figure 4A). (B) IP3-AgNPs were found associated with blood vessels and accumulated in the avascular regions. Arrows point to IP3-AgNPs in the avascular region. Inset in B: IP3-AgNPs overlapped with blood vessels inside the tumor (arrows). (C, D) Imaging of control nanoparticles. The control AgNPs located on the edge of the tumor for both MKN-45P (C) and CT-26 tumors (D) (arrows). (A-D): Red, AgNPs; green, CD31; blue, DAPI. Scale bar: 200 µm; inset in B: 100 µm.

5.3. TPP functionalization enhances therapeutic efficacy of NPs (Publications I–III)

5.3.1. TPP functionalization enhances therapeutic efficacy of PS-PTX and proapoptotic NW in mouse models of PC

Based on preferential accumulation of TPP-NP *in vitro* and having established selective tumor targeting *in vivo*, we evaluated the therapeutic efficacy of PTX-loaded iRGD-PS and pro-apoptotic linTT1-NW in a mouse model of PC.

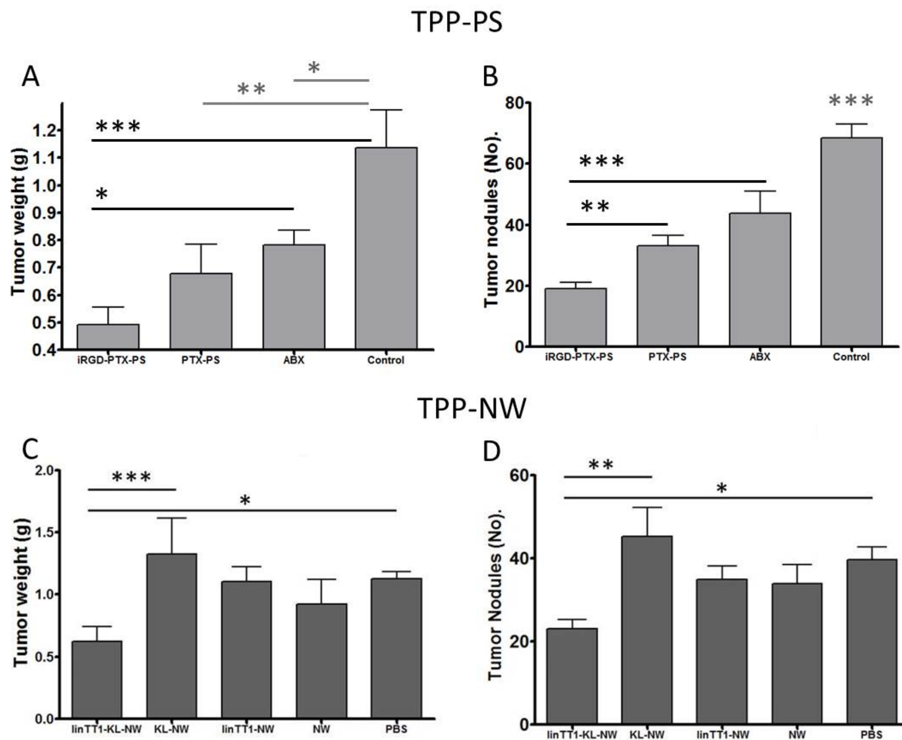

Figure 17. Experimental therapy of mice bearing peritoneal MKN-45P tumors. (A, B) Experimental treatment results from TPP-PS study. Mice bearing disseminated peritoneal MKN-45P tumors were injected IP every other day during two weeks with indicated formulations (cumulative dose of the treatment: 7 mg PTX/kg). The tumor weight (weight of large peritoneal tumors combined with small peritoneal tumor nodules) and number of peritoneal tumor nodules after treatment are shown. (C, D) Experimental treatment results from TPP-NW study. Mice bearing disseminated IP MKN-45P tumors were injected IP every other day during two weeks with the indicated NW formulations (5 mg/kg Fe). (B) Total tumor weight and number of peritoneal tumor nodules after treatment are shown. KL: $_{D}$(KLAKLAK)$_2$ apoptotic peptide coupled to NWs. N=8 mice in each group. Statistical analysis was performed by one-way ANOVA; error bars, mean +SEM; *** $p < 0.001$. **$p < 0.01$, *$p < 0.05$.

First, we tested the efficacy of targeted PTX-loaded iRGD-PS in mice bearing MKN-45P disseminated IP tumors. Mice were treated with iRGD-PS-PTX, untargeted PS-PTX, or ABX, all at the cumulative PTX dose of 7 mg/kg. Among the formulations used, iRGD-PS-PTX gave the strongest antitumor response. The total weight of all the detectable peritoneal tumors treated with iRGD-PS-PTX was significantly lower than those in the ABX and untreated groups (Fig. 17A, B). Importantly, treatment with iRGD-PS-PTX also reduced the number of peritoneal MKN-45P tumor nodules significantly more effectively than the other treatments (Fig. 17A, B).

Since the MKN-45P tumor model in nude mice is well established and studied in our lab, we evaluated the therapeutic effect of targeted pro-apoptotic linTT1-NW in MKN-45P tumor bearing mice as well. The treatment with linTT1-$_D$(KLAKLAK)$_2$-NWs resulted in a significant decrease in tumor burden (weight of peritoneal malignant tissue) and in significant reduction in the number of tumor nodules (Fig. 17C and D). The control NW treatments did not differ from the vehicle control.

The biggest challenge in the treatment of PC is local dissemination and microscopic metastasis. Though visible tumor and metastatic nodules can be surgically removed, microscopic metastasis remains, producing recurrence of the tumor. In both treatment studies, TPP-PS treated, and TPP-NW treated, we detected significantly lower number of tumor nodules in the peptide targeted NP group compared to other treatment groups (Fig. 17B, D) These data suggest that functionalizing nanoparticles with either iRGD or linTT1 peptide can have a significant reducing effect and antitumor activity on metastatic spread in PC.

6. DISCUSSION

6.1. Significance

The development of novel strategies to treat peritoneal malignancies is a high priority as there is no effective cure. Intraperitoneal (IP) chemotherapy is increasingly used to treat peritoneally disseminated tumors, however the methodology is complex and the effect on antitumor efficacy is modest. The field is rapidly advancing by numerous preclinical and clinical studies. For example, compared to free chemotherapeutic drugs, IP-administered therapeutic nanoformulations have superior pharmacokinetic and biodistribution profiles (Dakwar et al., 2017) and at least two paclitaxel-containing nanoformulations (Nanotax® and Abraxane®) are currently under evaluation for PC in clinical trials (Williamson et al., 2015, Cristea et al., 2015).

The work presented in this thesis explored the preclinical application of IP administration route for TPP-guided therapeutic nanoparticles to peritoneally disseminated tumors. Our studies show that precision-guided locoregional delivery can be used to improve efficacy of different classes of NPs loaded with anticancer compounds.

The discovery of novel approaches to treat IP tumors is translationally relevant. In our studies we used established clinically relevant tumor models and validated the tumor selectivity of the peptide coated particles using fresh clinical explants from PC patients. Follow up clinical studies will determine the applicability of the TPP-mediated affinity targeting for IP chemotherapy.

6.2. Main findings

Our study was designed to preclinically evaluate the effect of TPP-mediated affinity targeting on tumor accumulation and antitumor efficacy of IP-administered NPs. We found that TPPs increase selectivity of NPs towards PC lesions and that this selectivity translates for therapeutic NPs in improved efficacy. Our studies also show that increased residence time in the IP cavity was achieved for IP-administered NP which enables potentiated direct penetration (as opposed to circulation-mediated delivery) of the particles from the IP space.

6.2.1. LinTT1 functionalization increases tumor selectivity of IP injected NPs

The p32 protein is a well-validated target molecule for systemic treatment of tumors (Sharma et al., 2017, Agemy et al., 2011, Karmali et al., 2009), but prior to our studies it was not evaluated for IP affinity targeting of PC. We show that a number of cell lines representing PC tumors express cell surface p32 at levels

relevant for peptide-based tumor affinity targeting. The binding of linTT1-coated NPs to surface p32 of these cells resulted in cellular internalization into p32-expressing PC cell lines *in vitro* and in tumor accumulation *in vivo*. Importantly, the internalized NPs were found associated with the mitochondria, as has been shown previously for a related p32-targeted peptide (Agemy et al., 2010).

Coating of NWs with linTT1 peptide potentiated their tumor selectivity and anti-tumor activity upon IP administration in p32-dependent manner. In several mouse models of PC and fresh clinical tumor explants linTT1-NWs showed efficient accumulation that was not present in the case of untargeted NWs. These observations warrant follow-up pre-clinical and clinical studies to validate the system for the IP treatment of PC.

6.2.2. iRGD peptide conjugation potentiates IP tumor delivery of PTX-PS

Our study was the first to show that PS are effective in IP drug delivery. We demonstrated that PS loaded with cytotoxic drug Paclitaxel exhibit an intrinsic selectivity towards IP tumor lesions by efficiently delivering payloads into tumor cells in mouse models of PC *in vitro* and *in vivo*. Importantly, we show in a mouse model of PC that Paclitaxel-PS have a superior efficacy at a very low drug concentration. The PS target peritoneal tumors through a combination of direct local penetration and indirect homing via the circulation. This enhanced tissue penetration can be attributed to PS being flexible and able to pass through pores much smaller than their size (Pegoraro et al., 2014). After demonstrating the efficacy of the non-targeted PS in PC we show that by affinity targeting of IP administered PS-PTX with TPPs RPARPAR and iRGD that their tumor selectivity and antitumor activity can be increased. As the experiments with non-targeted PS show a combination of direct and vascular-mediated tumor accumulation then by functionalizing the PS surface with iRGD the tumor accumulation can be further increased. An explanation can be that whereas the iRGD-PS actively penetrated the tumors through a CendR motif-driven tissue transcytosis process, the untargeted PS accumulated passively in the periphery of the tumors. We determined in an experimental treatment study that PTX-loaded PS have better antitumor efficacy than PTX or Abraxane, and that conjugation of iRGD to PTX-PS renders them even more effective against PC.

6.2.3. TPP as ligands for efficient targeting of PC

Tumor penetrating peptides used in this thesis, linTT1 and iRGD, are both characterized by the presence of cryptic R/KXXR/K C-end Rule (CendR) motif (iRGD: CRGDKGPDC, linTT1: AKRGARSTA). To be activated, this motif requires proteolytic cleavage to activate NRP-1 binding to trigger the CendR

cell- and tissue penetration pathway (Teesalu, Sugahara & Ruoslahti, 2013). For initial tumor recruitment iRGD and linTT1 use different receptors: LinTT1 binds to cell surface p32 – a mitochondrial protein aberrantly displayed on the surface of activated tumor cells and cells in tumor stroma (macrophages, lymphatic endothelial cells, endothelial cells) (Paasonen et al., 2016) and iRGD recognizes integrins upregulated on angiogenic endothelial cells and tumor cells (Sugahara et al., 2009). Therefore, linTT1 and iRGD have different targeting specificities in the tumor environment. Since tumors can be heterogenous in their molecular patterns, the peptides can be used as personalized targeting ligands based on specific molecular profile of an individual tumor or used in combination for synergistic targeting of the PC.

6.2.4. Hyaluronan targeting peptide as a targeting ligand in PC

Hyaluronic acid (HA) is a glycosaminoglycan component of the extracellular matrix and distributed widely in epithelial, connective and neural tissues. In peritoneal space, HA is present in mesothelial surface, and is upregulated in peritoneal tumors of gastrointestinal (Ikemoto et al., 2017) and ovarian origin, where it is known to facilitate the peritoneal dissemination (Ween, Oehler & Ricciardelli, 2011). The IP3 peptide contains a hyaluronan-binding motif and was found to target IP tumors. After IP injection of AgNP coated with IP3 peptide as a targeting ligand, we saw tumor specific accumulation of IP3-AgNP in peritoneal tumors of gastric and colon origin. HA is an abundant target in solid tumors and may provide a high capacity target for affinity targeting of solid tumors. These data suggest that IP3 peptide has the potential to guide drugs, nanoparticles and imaging agents to extracellular matrix of peritoneal tumors.

6.2.5. TPP-NPs accumulate in avascular tumor nodules and are effective against micrometastasis

Clinically the biggest challenge in PC is the peritoneal seeding of tumor. Avascular tumor nodules are largely inaccessible for systemic chemodrugs and locally-administered anticancer drugs might not exhibit sufficiently long retention time in the IP cavity to penetrate and effectively destroy the remaining tumor cells. Our experimental treatment study using TPP-NP on a gastric cancer model showed a significant decrease in the number of tumor nodules found in the peritoneal cavity thus pointing to effective therapeutic effect on metastatic spread in PC.

6.3. Future directions

It has been a century since Paul Ehrlich introduced the concept of “magic bullet” – an entity capable of recognizing a specific target to provide a therapeutic action at the desired site (Ehrlich, 1908). Modern version of the magic bullet engages different carefully fitted components: 1. A potent drug payload; 2. A high-capacity nanocarrier; 3. A targeting moiety capable of directing the drug-loaded carriers to the target site. This concept is a basis of the design of novel smart anticancer therapies (Fornaguera, Garcia-Celma, 2017) and is actively explored for locoregional targeting of intraperitoneal tumors (Dakwar et al., 2017, Van Oudheusden et al., 2015).

Personalized medicine applies precision treatments for patient or patient groups by considering their genetic and phenotypic factors for optimal therapeutic response (Zhang et al., 2012). Image-guided personalized therapies can provide critical information on the specific biomarkers /molecular profiles and allow design/application of nanosystems based on the presence of affinity ligand target molecules in a specific patient cohort (Man, Lammers & T M de Rosales, 2018). IP tumors can be extremely heterogenous and it is clear that no single treatment can provide a successful cure for all. The versatility at the nanoparticle drugs and imaging agents enables a variety of specifically tuned treatment modalities.

Currently marketed nanoproducts represent the first generation nanoparticles. They lack targeting molecules and mainly rely on the EPR effect to passively reach the tumor site (e.g Doxil) (Hare et al., 2017, Shi et al., 2017). The second generation of nanosystems is defined by having a targeting ligand on their surface (e.g a monoclonal antibody) and they are yet to be clinically approved. At the moment four polymeric NP loaded with a chemotherapeutic drug and coated with HER2 (human epidermal growth factor); EGFR (epidermal growth factor); PSMA (prostate specific membrane antigen) and TfR (transferrin receptor) are being evaluated in clinical trials (Shi et al., 2017).

IP therapies of PC are particularly well suited for nanotherapies. The IP administered NPs increase in retention time of the drug in the tumor proximity and the IP administered nanocarriers are not subject to nonselective uptake in the organs of reticuloendothelial system. Our work showing that affinity targeted IP NP have improved tumor selectivity and penetration provide a solid rationale for follow up translational studies on improved PC nanotherapies. The goal is to support the translation of pre-clinical results to pharmaceutical industry and to commercialize the novel nanomedicinal drug products.

7. CONCLUSIONS

1. TPPs: LinTT1, iRGD and RPARPAR coated onto NPs are available for receptor interactions and therefore, can be used to selectively target NPs to p32 and NRP-1 expressing cultured cells. IP3 peptide allows targeting of the HA (Hyaluronic acid) in tumor extracellular matrix.
2. Internalized linTT1-NWs are routed to mitochondria and have a cytotoxic effect on different IP tumor cell lines. TPP-PS release their cargo in the cytoplasm and show cytotoxicity on malignant cells.
3. IP injected TPP-NPs have improved IP tumor selectivity over IV injected TPP-NPs. After IP injection TPP-NPs are specifically taken up and accumulate in peritoneal tumor lesions using both direct penetration and systemic circulation. Tumor accumulation of the NP can be improved by targeting with TPPs where TPP-NP homed to and penetrated through peritoneal tumors, whereas untargeted NP accumulated only in the tumor periphery.
4. Our data show that TPP functionalization enhances therapeutic efficacy of PS-PTX and proapoptotic NW in mouse models of PC as significantly lower number of metastatic nodules were detected in the treatment group compared to the control groups.

8. SUMMARY IN ESTONIAN

Intraperitoneaalsete kasvajate sihtmärgistatud ravi kasutades peptiididega suunatud nanoosakesi

Seedetrakti ja günekoloogiliste pahaloomuliste kasvajate puhul on kasvajarakkude levik kõhuõõnes ehk peritoneaalne kartsinomatoos (PK) üks sagedasemaid ilminguid. PK haigete keskmine elulemus on 4 kuud. PK ravivõimalused on piiratud, kuna süsteemne keemiaravi on madala efektiivsusega ning patsiendile manustatavat ravimidoosi piiravad kõrvalnähud kõhuõõnevälistes kudedes. Võrreldes intravenoossete ravimitega saavutavad otse kõhuõõnde manustatud vähiravimid kasvajakoes kõrgema kontsentratsiooni ning on oluliselt efektiivsemad. Sellegipoolest põhjustavad intraperitoneaalselt manustatud tsütotoksilised ravimid kõrvaltoimeid kõhuõõne normaalsetes kudedes.

Üheks võimaluseks ravimite ja kontrastainete efektiivsemaks muutmiseks ja kõrvalnähtude vähendamiseks on nende laadimine nanoosakestesse. Nanoosakeste abil on võimalik parandada ravimite lahustuvust, koeselektiivsust ja vabanemist sihtmärkkoes. Vähiravimite ja nanoosakeste koeselektiivsuse ja efektiivsuse parandamiseks saab neid suunata keemiliselt konjugeeritud afiinsusligandidega (nt. antikehad, peptiidid, aptameerid). Meie uurimisgrupp kasutab sellel eesmärgil vähiselektiivseid peptiide, näiteks iRGD vähkipenetreerivat peptiidi (TPP). Pärast seondumist rakupinna integriinidega läbib iRGD proteolüütilise lõikamise, mis aktiveerib seondumise teise vähirakkudel üleekspresseeritud valgu, NRP-1'ga ja käivitab rakuinternalisatsiooni raja. TT1 vähkipenetreeriva peptiidi retseptor on vähirakkude pinnal ekspresseeruv valk p32, mis normaalsetes rakkudes paikneb mitokondrites. TT1 peptiid kinnitub kasvajarakkude pinnal olevale p32'le ning käivitab seejärel NRP-1'st sõltuva rakkusisenemise protsessi.

Käesolev prekliiniline töö keskendub kõhuõõne vähkkasvajate (maovähk, soolevähk ja munasarjavähk) uute kuvamis- ja ravimeetodite väljatöötamisele kasutades erinevate koostisega nanoosakesi (nanoravimid) ning suunavaid vähiselektiivseid peptiide. Töös uuriti polümeeridel ja hõbedal põhinevate ning raudoksiidi sisaldavate nanoosakeste selektiivsust kasvajakoe suhtes peale kõhuõõnde süstimist. Katses kasutati erinevatel kõhuõõne kasvajarakkudel põhinevaid hiire loommudeleid.

Uurimistöö eesmärgid

1. Uurida TPP-NP spetsiifilisust sihtmärkvalkude suhtes rakuvabas keskkonnas ja rakukultuuris.
2. Hinnata TPP-ga kaetud tsütotoksliste nanoosakeste rakku sisenemise võimet ja tsütotoksilisuse efekti.
3. Määrata intraperitoneaalselt manustatud TPP-ga suunatud nanoosakeste biodistributsioon kõhuõõne kasvajatega hiirtes.
4. Hinnata TPP-ga suunatud tsütotoksiliste nanoosakeste terapeutilist efektiivsust kõhuõõne kasvajatega hiirtes.

Materjal ja Metoodika

Uurimistöös oli kasutusel kokku 5 erinevat kasvaja rakuliini, mis pärinevad kas inimeselt või hiirelt. Loomkatseteks kasutati atüümseid nude hiiri või Balb/c hiiri ning kõik loomkatseid olid kooskõlastatud Eesti Põllumajandusministeeriumi vastava komisjoni poolt ning loomkatsetele oli väljastatud luba numbriga 42. Värskete inimese soolevähi proovide saamine ja kasutamine oli kinnitatud Tartu Ülikooli Eetikakomisjoni poolt (luba numbriga 243/T27). Töös kasutati erinevaid tuumorispetsiifilisi peptiide (iRGD, LinTT1, RPARPAR, IP3), mis olid konjugeeritud nanoosakeste pinnale. Nanoosakestest kasutati polümersoome ning raud- ja hõbeosakesi. Nanoosakeste sünteesil kasutati erinevaid varem publitseeritud meetodeid; raudoksiidi osakeste sünteesil kasutati Fe-soolasid ja kõrge molekulaarmassiga dekstraani, et saavutada usjalik-kuju ning polümersoomid saadi amfifiilsete kopolümeeride ja vee vastastikkusel toimel. Peptiidide lisamine nanoosakeste pinnale toimus kas NHS-PEG-maleimiid-linkerit kasutades või läbi neutravidiin-biotiin interaktsiooni. Nanoosakeste iseloomustamiseks kasutati DLS tehnoloogiat ja transmissioonielektronmikroskoopiat. Selektiivsuse hindamisel rakuvabas süsteemis kasutati Ni-NTA magneetilisi agaroosiosakesi millele seondati rekombinantsed p32 ja NRP-1 valgud. Fluoresentsmärgisega nanoosakeste seondumine valkudele tehti kindlaks kasutades flouresentsplaadilugejat. *In vitro* seondumiskatsed vähirakkudele viidi läbi kasutades voolutsütomeetriat ja konfokaalmikroskoopiat kasutades relevantseid primaarseid ja sekundaarseid antikehi. Tsütotoksilisuse hindamiseks kasutati MTT kolorimeetrilist meetodit ja xCELLigence® tehnoloogiat. *In vivo* koedistributsiooni katseteks indutseeriti hiirtes kõhuõõne kasvajad süstides kasvajarakud otse kõhuõõnde ning hiljem teostati kasvajatest ja kontroll-organitest valmistatud koelõikudel immuunohistokeemilised värvingud ja visualiseerimine kasutades konfokaalmikroskoopiat. Lisaks visualiseeriti raudoksiidi seondumist kasvajakoega *in vivo* MRT abil. Ekperimentaalteraapia hindamaks tsütotoksiliste nanoosakeste ja kullerpeptiidide efektiivsust viidi läbi mao-või soolevähiga hiiremudelitel süstides nanoosakesi otse kõhuõõnde ning katse lõpus hinnati kasvajakoe kaalu ning kasvajanoodulite arvu võrreldes kontroll-osakestega süstitud hiirtega.

Uurimistöö peamised tulemused ja järeldused

1. LinTT1 ja RPARPAR peptiididega kaetud nanoosakesed seonduvad nende peptiidide teadaolevate retseptoritega (vastavalt p32 ja NRP-1) ning seda selektiivsust retseptori suhtes saab kasutada nanoosakeste suunamisel p32 ja NRP-1 ekspresseerivate rakkude pinnale. IP3 peptiid seondub hüaluroonhappega *in vitro*.
2. Rakku sisenenud linTT1-NW suunatakse mitokondritesse ning nad avaldavad tsütotoksililist toimet erinevatel IP kasvajarakkudel. TPP-PS´st vabanevad lastmolekulid tsütoplasmas ning avaldub tsütotoksline toime IP kasvajarakkudel.

3. TPP'ga konjugeerimine aitab kaasa nano-osakeste paremale akumulatsioonile kasvajakoes ja rakku sisenemisele. IP süstitud TPP-NP on võrreldes IV süstitud osakestega vähikoe suhtes selektiivsemad. IP manustatud TPP-NP akumuleeruvad kasvajakoes nii otsese seondumise teel kasvajarakkudele kui ka kaudselt, vereringe kaudu.
4. Polümersoomidel ja raudoksiidil põhinevate nanoosakeste suunamine TPP-ga võimendab osakeste terapeutilist efektiivsust.

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PUBLICATIONS

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