Teaser Loco-regional cancer drug therapies have been advanced to increase drug concentrations in tumors while minimizing systemic toxicity. We review benefits and limitations of current approaches and discuss a rapidly reversible hydrophobization of drugs for solid tumor treatment.

Loco-regional cancer drug therapy: present approaches and rapidly reversible hydrophobization (RRH) of therapeutic agents as the future direction☆

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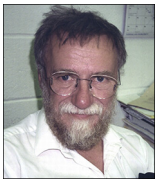
Insufficient drug uptake by solid tumors remains the major problem for systemic chemotherapy. Many studies have demonstrated anticancer drug effects to be dose-dependent, although dose-escalation studies have resulted in limited survival benefit with increased systemic toxicities. One solution to this has been the idea of loco-regional drug treatments, which offer dramatically higher drug concentrations in tumor tissues while minimizing systemic toxicity. Although loco-regional delivery has been most prominent in cancers of the liver, soft tissues and serosal peritoneal malignancies, survival benefits are very far from desirable. This review discusses the evolution of loco-regional treatments, the present approaches and offers rapidly reversible hydrophobization of drugs as the new future direction.

Background

During the past century many great ideas and methodologies have molded the present approach to cancer treatment, which saves lives and prolongs survival. At the same time, in spite of all the achievements, the outcome of cancer treatments, especially that of solid tumors, remains unsatisfactory. The aim of this review is to highlight the evolution of loco-regional treatment of solid tumors, and outline the latest and potential future methodologies. It has to be noted from the beginning that, although this review enumerates related events in medical history in seemingly time-ordered fashion, in reality, ideas, innovations and methodologies in oncology are often intertwined, and some apparent successions have been used only for a narrative purpose – there are several reviews highlighting a chronological history of oncology [1–7].

More than 30 years ago, the multimodality approach was introduced for the treatment of solid tumors, and it is still successfully used today. The basis of this approach is to remove tumor tissues surgically, and further employ other techniques to kill any remaining tumor cells physically (i.e. electrocoagulation, radiofrequency, microwave, high intensive focused ultrasound, laser and cryosurgery) [8–12]. Chemotherapy has become an irreplaceable tool to eliminate tumor tissues



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that are not visible or that could not be ablated by the above techniques. This approach, termed adjuvant therapy, is a standard methodology in cancer treatment [8–12].

It has long been known that the antitumor effect of chemotherapeutics is dose-dependent. Several strategies have been investigated to increase chemotherapeutic concentrations during treatment, including dose escalation and treatment intensification. Early studies on dose escalations have shown positive responses [13,14]; however, later investigations have concluded that benefits appeared marginal whereas toxicity increased [15–18]. Recent reports concluded that the dose-escalation regimen does not significantly extend overall survival in advanced cancer, as compared to standard dose [19], whereas associated hematologic and nonhematologic toxicities increased [20].

Because systemic anticancer chemotherapy targets all replicating cells, including those of normal tissues, limitations of all chemotherapy, and especially of the dose-escalating strategy, are obvious and have been known for a long time [20–24]. One apparent solution to the problem is to localize a high concentration of drug(s) to the region of the tumor. Such an approach, later termed loco-regional treatment, was pioneered and advanced in the 1950s by Klopp and co-workers using intra-arterial drug administration to tumor-affected regions [25–31]. Intra-arterial drug delivery has developed into an important therapy for a variety of solid tumors [32–40]. However, despite all the advances, the long-term outcomes of patients receiving intra-arterial therapy (drug alone, chemoembolization or drug-eluting beads) remain unsatisfactory [41]. One obstacle remains: systemic toxicity, because the drug inevitably enters the circulation following delivery.

An alternative logical approach in loco-regional chemotherapy was reported by Ryan and colleagues in 1957 (in a dog model) [42]. The new approach enabled the delivery of a much higher dose of a drug to tumors without systemic toxicity at all, using an oxygenated extracorporeal circuit connected to a heart–lung machine [42]. This technique was successfully implemented in the clinic within a year [43]. Significant tumor reduction was observed after extracorporeal perfusion of the extremities (i.e. melanoma and rhabdomyosarcoma), pelvis (i.e. sarcoma) and lung (i.e. epidermoid carcinoma) [43]. By 1962, 350 patients with different types of cancers had been treated, with positive responses seen in 50% [44]. By avoiding systemic and minimizing local toxicities this methodology has enabled drug levels in tumor tissues up to ten-times higher than with systemic treatments [45]. Extracorporeal circuits or isolated drug perfusion protocols have since been implemented by other groups for a variety of cancers of different localizations [46–48], and all have reported positive outcomes to a certain degree.

However, overall survival benefits with either direct arterial delivery or isolated drug perfusion protocols have appeared to be modest or negligible when they are viewed in the scale of achieved drug concentrations [46–52]. This lack of correlation between the local dose achieved and tumor response is frustrating and somewhat puzzling. We believe that this impediment could be better understood and approached by reviewing evolution and effects of loco-regional treatment in cancers for which direct application of the drug is attainable (i.e. for serosal surface malignancies). The following analysis of the loco-regional approach as a direct drug application to cancer tissue is based on an example of serosal peritoneal malignancies.

There are four major serosal cavities in the human body: the peritoneal, the pericardial and the two pleural cavities, which are lined by mesothelium over basal membrane [53]. Although the mesothelium has been known for a long time, for a review see [54], the significance of mesothelial cells in cancer was recognized only recently [55]. The peritoneal cavity is of specific interest for the current analysis because: (i) it is the dominant serosal cavity in respect to cancer; (ii) its entire surface is amendable for loco-regional treatment; and (iii) anatomical peculiarities allow extended manipulations on surface malignancies and visual control. Among other cancer types, peritoneal carcinomatosis and particularly ovarian cancer is analyzed in this review as the most studied peritoneal cancer. It has become evident that most recent achievements in the treatment of ovarian cancer are applicable to other peritoneal surface malignancies.

Ovarian cancer is a highly lethal disease, with only a minority of treated patients showing good survival rates [56,57], and remains the fourth-or-fifth most common cause of cancer death among women and the main lethality from gynecologic malignancies [56–59]. Although early-stage ovarian carcinoma allows curative surgery, unfortunately patients are usually diagnosed when the disease has already spread beyond the ovaries. The asymptomatic beginning of the disease [60] and the early peritoneal dissemination of cancerous cells [61,62] reflect the transcoelomic origin of ovarian surface epithelial malignancy [63,64]. Even with progression, peritoneal dissemination often results in indistinct and nonalerting mass effect symptoms [65].

The specifics of the circulation of the intraperitoneal fluid and the peristalsis predetermine the pattern of peritoneal dissemination [66]. After this stage of the disease is established, known as a peritoneal disseminated ovarian cancer, pathoanatomical conditions usually do not allow an *en bloc* resection of the tumor [67,68]. Whole-abdominal radiation therapy for the disseminated tumors was considered noneffective and severe toxicity was reported [69]. Systemic chemotherapy, as initial or adjuvant treatment, is effective only temporarily and, ultimately, most patients will die from disease recurrence [70–74].

Early reports had shown positive responses using the dose-escalation approach [13,14]; however, more-recent results have concluded that it appears not to be the solution to improving patient outcome [15,17]. Möbus *et al.* used a dose-intensification approach to treat ovarian cancer with the support of marrow recovery (by the infusion of autologous blood stem cells), but this high-dose chemotherapy did not appear to be superior to conventional-dose chemotherapy [75]. As a result some experts strongly oppose systemic dose-escalation therapy for ovarian cancer. For example, Ozols wrote in his Editorial: 'The randomized trial comparing high-dose sequential chemotherapy with standard intravenous chemotherapy by Möbus *et al.* in this issue of the *Journal of Clinical Oncology* is the last nail in the coffin of this once important concept' [76]. This sobering opinion was confirmed by a recent randomized Phase II clinical trial evaluating increasing the dose intensity of cisplatin for the treatment of disseminated ovarian cancer [77].

More than 35 years ago, cytoreductive or debulking surgery was introduced [78,79], and this procedure still remains the main tool in the treatment of disseminated peritoneal ovarian cancer [80–82]. However, in spite of repeated debulking and second-look

laparotomy, further combined with multi-agent chemotherapies, the five-year survival rate is still low [83,84].

Exploiting the fact that all peritoneal tumors by default are exposed to the peritoneal cavity, loco-regional intraperitoneal chemotherapy was introduced for ovarian cancer [85]. Although the exact technical term for application of chemotherapeutics into the peritoneum to treat peritoneal cancer is 'intracavitary chemotherapy', in this review we use the term 'loca-regional chemotherapy', because prominent specialists and founders of intracavitary chemotherapy used terms 'intracavitary chemotherapy', 'loca-regional treatment' and 'loca- regional therapies' interchangeably, for example see [86,87]. In its essence, intracavitary chemotherapy is loca-regional, as opposed to systemic.

It was reasoned that this approach, although limited by the depth of penetration of the drug into the tumor (free-surface diffusion), could facilitate very high drug doses at peritoneal tumors while avoiding systemic toxicity [88–91]. However, treatment outcomes of therapies for disseminated ovarian cancer (as well as for others peritoneal surface malignancies) remained unchanged until two methodologies merged: complete elimination of all tangible peritoneal tumors (by means of dissection, electrocoagulation and laser evaporation) followed immediately by perioperative intraperitoneal chemotherapy aimed to kill and remove all remaining cancer cells. This innovative methodology and its progress should be credited to Dr Paul H. Sugarbaker and colleagues; their persistent exploration and educational efforts [49,66,92–117] have resulted in worldwide acceptance and successful application of the technique known as 'Sugarbaker's protocol' [118] for the treatment of peritoneal malignancies [119–125]. Sugarbaker's protocol emphasizes the importance of combination of two procedures: aggressive management of all visible tumors, including peritoneal resection, and perioperative intraperitoneal hyperthermic chemotherapy, as mandatory factors to prolong survival. Beneficially and surprisingly, an extent of peritoneal resection did not affect systemic drug levels [126]. Oddly enough, each approach, taken separately, historically was not sufficient to change overall survival significantly; and, furthermore, as a single modality each approach by itself faces obvious theoretical limitations.

Theoretical limitation no. 1: complete elimination of all tumor tissues by ablation

It is known that even the earliest studied stages of the ovarian surface epithelial cancer (as well as other peritoneal surface malignancies) are associated with exfoliation or shedding of malignant cells into the peritoneal cavity, leading to widespread dissemination [127,128]. Spreading cancer cells attach to the peritoneal surface and grow into tumor nodules [64,129]. Secondary tumors also spread cancer cells into the peritoneum by continued exfoliation, generating another wave of peritoneal micrometastases, thus maintaining the vicious cycle of the disease. Therefore, we have to conclude that the total number of undetectable single cancer cells and clusters is always much greater than the number of visible tumors, and none of the ablative procedures alone would be able to eliminate cancer cells, even with the most thorough and aggressive protocols [92,130–133].

Theoretical limitation no. 2: efficient killing of cancer cells via loco-regional drug application, as the result of drug penetration into the tumor based on free-surface diffusion (facilitated by perioperative intraperitoneal chemotherapy)

It became evident during the past decades that an increased interstitial fluid pressure in solid tumors is one of the main obstacles in solid cancer therapy [134–144]. In normal organs, the interstitial fluid pressure is always lower than the intravascular pressure, facilitating molecular exchange between blood and tissue compartments. In all studied solid tumors interstitial fluid pressure is increased, forming a barrier to transcapillary transport of drugs [135,137,140,143,144]. Increased interstitial fluid pressure is also a characteristic of peritoneal malignancies [111,145–147] and, by default, should interfere with free-surface diffusion of drugs. Closed perfusion procedures with elevated intra-abdominal pressure [146,148] could overcome tumor interstitial pressure by convection-driven drug penetration [146], although accompanied by side-effects [149,150]. Therefore, an open peritoneal drug application as the means to kill all cancer cells in peritoneal tumors via free-surface diffusion should face the same obstacles [135,137,140,143,144]. The current knowledge on tumor pathophysiology suggests that, as long as cancer tissue exists as intact nodules (even small), high tumor interstitial fluid pressure would interfere with drug penetration even with increased local concentration. However, when Sugarbaker and co-workers combined these two techniques they synergistically enhance each other: the aggressive cytoreduction protocol with peritonectomy removes all visible tumors (or breaks tumor nodal integrity) and, hence, removes tumor high interstitial fluid pressure, facilitating an effective interaction of chemotherapeutics with remaining single cells and cell clusters. Yet, one additional obstacle remains – efficient drug uptake into the cancer cells.

There are numerous research publications [87,124,151–160] and analyses [161–167] on the rationale, mechanisms, experimental techniques and clinical benefits of the combined application of hyperthermia and chemotherapeutics, especially emphasizing the benefit of hyperthermic intraoperative intraperitoneal chemotherapy (HIPEC) for surface peritoneal malignancies. In this treatment model, assuming that a complete cytoreduction is achieved, there are only two interacting components: the plasma membrane of the cancer cells and the drug. In theory, we can take out of the equation all active membrane transporters and apply Fick's law of diffusion, which states that diffusion linearly depends on concentration [168]. In experiments with cell membranes it was shown that membrane permeability increases with temperature [169]. Therefore, the question as to why does HIPEC work in patients with complete cytoreduction [170,171] is not a reasonable one in our opinion. We suggest that another question should be asked: why does not Sugarbaker's protocol provide a better outcome for all patients? We have earlier proposed that the main obstacle preventing local drug extraction by the tumor cells is the hydrophilic nature of the drug formulations themselves, and suggested a new approach for loco-regional drug treatment of cancer [172].

New approach: rapidly reversible hydrophobization of drugs for first-pass drug extraction

The idea of increasing the hydrophobicity of a drug is not new. Lipophilization of ionic drugs, without modification of their

chemical structures, for transport through hydrophobic cell membranes, was considered as an ideal approach long ago [173]. In fact, drug hydrophobization utilizing relatively stable modifications such as esters and amides has previously been shown to increase drug interactions with cellular membranes and has correlated with improved cellular uptake and lowered IC_{50} values [174–178]. However, concerns involving compound aggregation and embolization (following drug administration), as well as the sequestering of the drug in the cell membrane [179], have limited advances in this area.

To limit the possibility of drug aggregation, or losing the compound owing to membrane sequestration, we developed an approach to drug hydrophobization in which the drug is linked to a hydrophobic moiety by highly labile chemical linkages [termed rapidly reversible hydrophobization (RRH)] [172,180,181] to form a prodrug that is mixed with an aqueous solution before delivery. Hydrophobization drastically enhances cell-membrane association of the prodrug and, consequently, drug uptake, and the rapid lability protects nontargeted tissues from exposure to the highly active agent. Because the attachment linkages for the hydrophobic moieties are rapidly hydrolyzed following administration, the hydrophobic prodrug that is not extracted during a first-pass exposure to tumor tissues rapidly reverts to the less-membrane-permeable parent drug. This effect enables selective targeting of tumor and organs via loco-regional treatments. Reversible hydrophobization also greatly expands the number of compounds that could be effective drug candidates, by separating the delivery function from the active portion of the molecule. This is ideologically the same concept as targeted drug conjugates and liposomal and polymeric drug delivery systems. The conceptual basis of this approach was previously outlined [172] and is presented in Fig. 1.

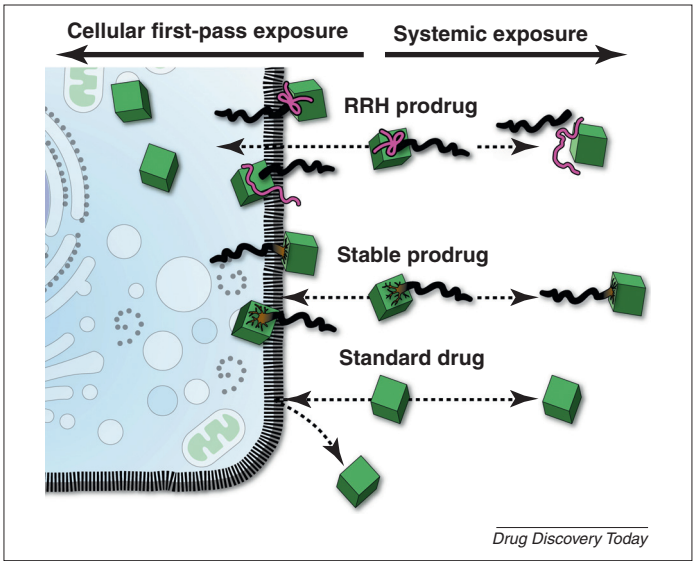

FIGURE 1

Conceptual schematic of the cellular delivery of a rapidly reversible hydrophobization (RRH) prodrug, a stable prodrug and a standard drug in the context of first-pass extraction. The RRH prodrug consists of the drug (green cube) which is linked to a hydrophobic moiety (black tail) by highly labile chemical linkages (pink tied or untied ribbon). The RRH prodrug provides high levels of membrane attachment and internalization but upon a loss a hydrophobic moiety it is expelled from the membrane to either the cytoplasm or outside the cell. The RRH prodrug that was not extracted by cells reverts to the less-membrane-active drug form. The hydrophobically modified prodrug with a stable linkage (brown root) provides high levels of membrane attachment, followed by internalization by endocytosis. The drug itself, although available to other cells and tissues systemically, has little cellular uptake potential.

Reporter drug and prodrug

To demonstrate the idea and utility of RRH prodrugs, we looked for a reporter system that would possess the following: (i) a membrane-impermeable drug; (ii) a site suitable for modification to the prodrug; and (iii) facile intracellular detection of the drug inside cells. A suitable system that has been long understood in biomedical research is propidium iodide (PI). PI is a well characterized membrane, impermeable DNA intercalator and is routinely used to detect cells with compromised membranes. Chemically, PI possesses two amino groups at the three and eight positions of the phenanthridinium ring system that are available for modification. Additionally, PI exhibits a 20–30-fold enhanced fluorescence upon intercalation into DNA, facilitating easy detection of targeted cells. However, every potential drug that possesses modification sites could be derivatized to a RRH prodrug. For example, melphalan, a long known DNA alkylation chemotherapeutic, was similarly modified at the primary amine of the amino acid [172]. Modification of the parent drug was conducted with either an alkyl chlorosilane to form a silazane or a derivatized maleic acid to form a maliamic acid (Fig. 2) [172,180,181]. Both prodrug modification types are very reversible, with half-lives in the order of 10–20 s (depending on pH environment) when mixed with aqueous buffers [172]. We believe this rapid reversal to the parent drug is a crucial strategy for effective first-pass extraction and minimization of systemic exposure to the highly membrane-permeable prodrug.

In vitro and *in vivo* studies on RRH prodrugs

To deliver the highly hydrolytically unstable prodrugs, we relied on a system for formulating a concentrated solution of the prodrug in an organic solvent (for example DMSO) that rapidly mixed with an aqueous buffer immediately before local administration. Rapid and homogeneous mixing of the prodrug solution with isotonic glucose was crucial to avoid membrane damage by the DMSO. To achieve efficient mixing, a small passive mixing chamber with colliding flows was constructed, and the mixed solution was immediately applied to a target tissue. The principles of passive mixing and the design of the mixing chamber were previously reported [172,180,181] and details are given in the supplementary material (Figs S1 and S2 in Supplementary material online). Using programmed syringe pumps, the prodrug solutions (concentrated in DMSO) were mixed and diluted at least tenfold with isotonic glucose while it was delivered to tissues. No effect of the DMSO was observed in any experiment when compared to Isotonic Glucose (ITG) alone (no DMSO) controls.

Results of *in vitro* experiments

To determine if RRH prodrug modification led to increased cellular uptake, RRH-PI uptake was investigated in a number of cell lines: [B16 (murine melanoma), Hepa 1-6 (mouse hepatoma cells), SKOV-3 (human ovarian carcinoma), OVCAR-3 (human ovarian carcinoma), Jurkat (human T-lymphocyte), HEK 293 (human embryonic kidney epithelial cells) and MC38 (mouse colon

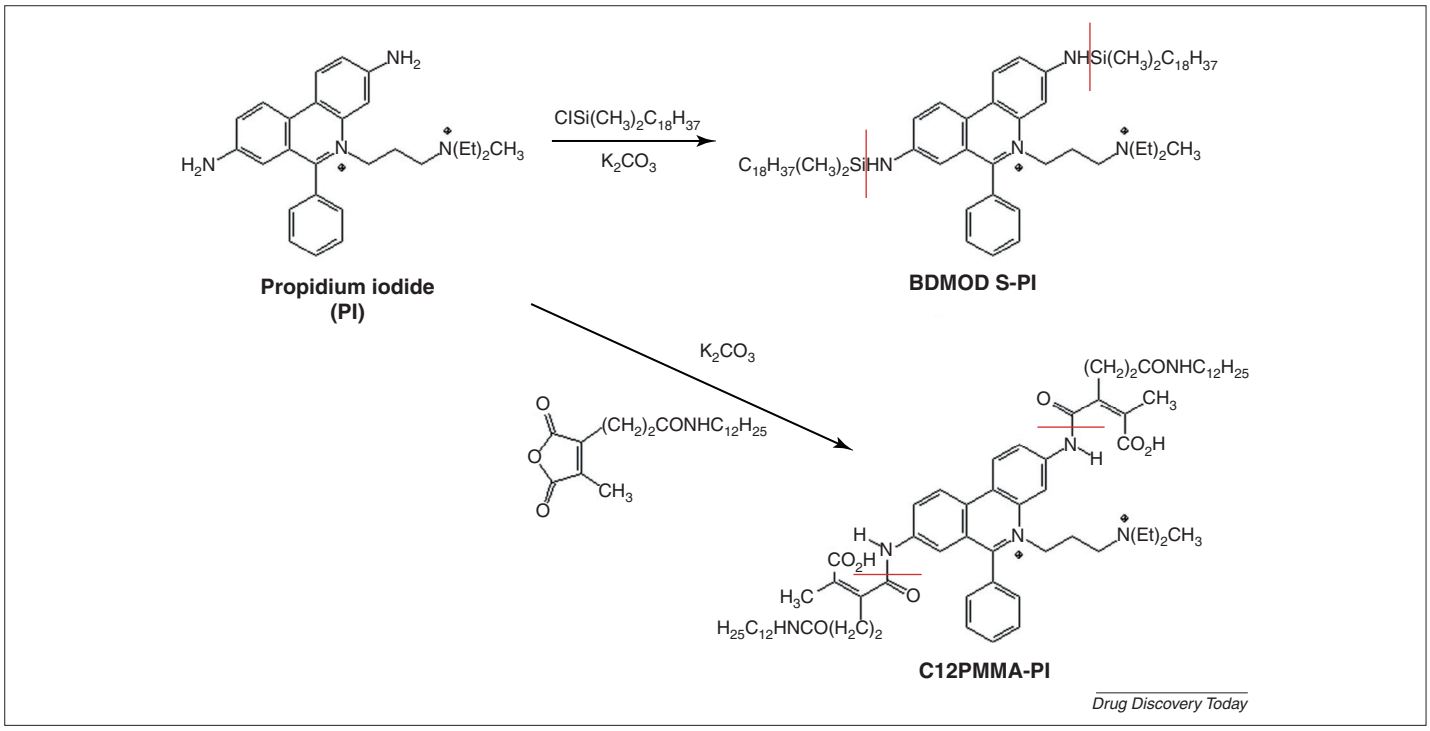

FIGURE 2

Hydrophobic modification of propidium iodide (PI) with either an alkyl chlorosilane to form a silazane or a derivatized maleic acid to form a maliamic acid. Red lines indicate sites of reversible linkage.

carcinoma cells)]. Both reversible modifications of PI behaved similarly in the cellular uptake studies. In all cases, RRH-PI was readily observed in nuclei of nearly 100% of cells within ~2 min following treatment. The cells appeared to be morphologically normal, with no signs of damage following treatment. When RRH-PI was hydrolyzed for 5 min before treatment, only a small portion of cells showed PI-positive nuclei (similar to treatment with PI alone). Treatment with RRH-PI was equally effective on suspended cells (Jurkat). Quantitative determination by flow cytometry indicated that >99% of the cells treated with RRH-PI were positive for PI uptake compared with only about 9% for PI-treated cells when Jurkat cells were suspended in ITG and passed through the mixing chamber [172].

RRH prodrugs were tested for cytotoxic antiproliferative activity in a number of proliferating cell lines. In the case of melphalan, treatment with the parent drug resulted in ~80% cell viability at the highest drug concentration tested (1458 μM) in Hepa 1-6 and SKOV-3. In MC38 cells, the IC_{50} was determined to be 1176 μM. By contrast, an IC_{50} antiproliferative activity of RRH-melphalan was achieved at dramatically lower concentrations (330, 384 and 304 μM, respectively) [172].

Targeting normal mouse tissues

Utilizing the same micro mixing chamber (Figs S1 and S2 in Supplementary material online) and three-syringe pumps (Fig. S3 in Supplementary material online), *in vivo* experiments using loco-regional delivery were conducted and previously reported [172,180,181]. To visualize cell targeting, tissues were snap-frozen in O.C.T. compound, cryosectioned and stained for confocal microscopy [actin (green, Alexa 488); cell nuclei (blue, ToPro-3)]. Additionally, unstained frozen sections were examined by fluorescent microscopy in a rhodamine and a fluorescein isothiocyanate (FITC) channel. The red PI nuclear fluorescence was strong enough for detection of PI-labeled cells in the FITC channel and under a long-pass FITC emission filter (505 plus), although the red fluorescence was a bleed-through fluorescence that represented only a small fraction of the real signal from PI intercalation. This technique was advantageous as very fast examination, whereas the green autofluorescence (observable in the FITC channel) was used to outline general tissue morphology. For histopathology analysis, formalin fixation was followed by routine processing and paraffin embedding. Light microscopy of hematoxylin–eosin-stained paraffin sections was performed for all experiments.

As detailed earlier, a variety of delivery techniques have been developed to deliver drugs in a local setting to normal tissues [172,180,181]. Using RRH prodrugs, we demonstrated that most liver cells (i.e. hepatocytes and vascular cells) were targeted with a single bolus injection to the portal vein (with occluded blood flow) (Fig. S4 in Supplementary material online). A single bolus injection of RRH-PI into the hepatic artery of normal mice (with preserved portal blood flow) targeted all endothelial and most smooth muscle cells of the hepatic artery, biliary plexus and gall bladder arteries, as well as epithelial cells close to arterial trees (Figs S5 and S6 in Supplementary material online). A single bolus injection of RRH-PI into the common bile duct resulted in significant targeting of all biliary epithelial cells and neighboring hepatocytes (Fig. S7 in Supplementary material online). A single bolus injection into the urinary bladder and ureter resulted in targeting of the all-accessible urothelium (transitional epithelium), including that of the renal pelvis and big distal collecting tubules (Figs S8–S10 in Supplementary material online). A single bolus injection into the right internal carotid artery of a normal mouse resulted in strong

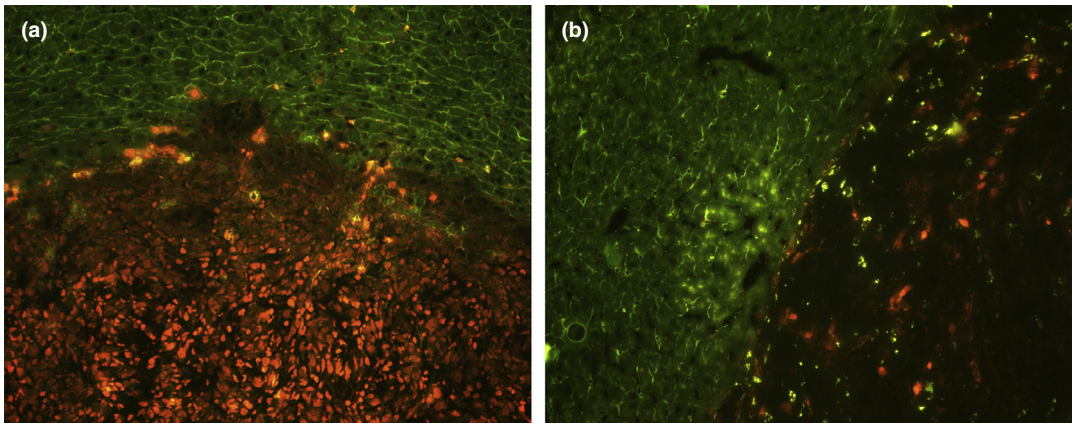

FIGURE 3

Mouse MC38 colon carcinoma cells (10^4) were inoculated into liver via the ileo–colic vein of C57BL mice. Twenty-five days post-inoculation either rapidly reversible hydrophobized (RRH)-propidium iodide (PI) **(a)** or unmodified (hydrolyzed) PI **(b)** was administrated via the hepatic artery while portal flow was preserved. Unstained frozen sections, fluorescent microscopy, fluorescein isothiocyanate (FITC) excitation with long-pass FITC emission filter (505 plus). (a) Practically all tumor cells are labeled after RRH-PI administration; (b) only a small number of scattered cells (probably apoptotic) are labeled in a tumor after unmodified PI administration and none in preserved parenchyma. Note, the red PI nuclear fluorescence was strong enough for detection of PI-labeled cells in the FITC channel using a long-pass FITC emission filter (505 plus), although the red fluorescence was a bleed-through fluorescence that represented only a small fraction of the real signal from PI intercalation. Axioplan-2 microscope, magnification x400.

targeting of the brain vascular cells and neurons and glial cells in right brain hemisphere and in weak targeting in left hemisphere (Fig. S11 in Supplementary material online). Some animals were allowed to recover after administration of the RRH-PI, and were sacrificed 24–30 hours later. In these cases, the RRH-PI-targeted tissues still showed strong nuclear PI-labeling, for example in the hepatic artery (Fig. S12 in Supplementary material online).

Topical administration of RRH-PI resulted in strong nuclear labeling of cells to which the prodrug was directly applied. For example, topical application of RRH-PI to the cornea, to the skin or into the lumen of the intestine resulted in strong nuclear PI-labeling of the cornea epithelium (Fig. S15 in Supplementary material online), the epidermis or the enterocytes, respectively. Targeting with RRH-BDMODS-PI and RRH-C12PMAA-PI was equally effective for normal and malignant tissues, therefore a particular form of RRH-PI is not always detailed.

Loca-regional cancer targeting with RRH prodrugs in mouse tumor models

We thought that two mouse cancer models were of particular interest for testing the RRH prodrug approach for loco-regional drug therapy: liver tumors and disseminated ovarian cancer. Both malignancies, as previously mentioned in this review, have been intensively studied and approached in the clinic with loco-regional drug therapy. Both malignancies possess anatomical specifics that are very appealing for loco-regional treatment. However, survival benefits, even with the most advanced loco-regional drug applications, remain very far from desirable [39,50,119–125, 182–188].

RRH prodrug testing in mouse models of liver neoplasms

Loco-regional drug treatment of liver tumors via the hepatic artery route relies on the well-established fact that liver tumors (primary and secondary) are exclusively supplied by the hepatic artery, whereas normal liver parenchyma is supplied mostly by portal flow [40,187,189]. We therefore tested RRH prodrugs in mouse models of liver neoplasms, to determine if the concept behind the RRH prodrug would enable increased drug uptake in the tumor.

Four different mouse syngeneic liver tumor models were developed during the course of this work: colon carcinoma (MC38 colon carcinoma cells/C57BL), hepatocellular carcinoma (Hepa1-6/C5BL), melanoma (B16 melanoma cells/C57BL) and neuroblastoma (NXS2 neuroblastoma cells/A/J) [172,180,181]. Surgical aspects of hepatic artery access and infusion in mouse models of liver neoplasms were previously reported [172,180,181] and are also detailed in the supplementary material (Fig. S14 in Supplementary material online: hepatic artery access and infusion in mouse models). In general, the methods of hepatic artery access were similar to the procedures used clinically [187].

Following hepatic artery injections, we observed an intensive targeting of all cells in tumors by RRH prodrug (Fig. 3a), in a dramatic contrast to only a small number of targeted cells by hydrolyzed prodrug (Fig. 3b), which were probably apoptotic cells. These tumor-targeting patterns were observed in all animals treated with RRH-PI or with native PI and hydrolyzed prodrug.

Another important characteristic of RRH prodrug delivery via the hepatic artery was that, although all tumors were heavily targeted, only a few cells in the liver parenchyma were targeted. As expected, cells in all hepatic arteries were heavily targeted with RRH-PI. Targeted hepatocytes and sinusoidal cells were scattered in the vicinity of tumors and portal tracts, and by nonmorphometric approximation constitute ~1% of parenchymal volume. This approximation was a result of fluorescent microscopic examination of numerous frozen liver sections from tumor-bearing animals after the RRH prodrug delivery via the hepatic artery. We acquired six consecutive overlapping fields, consisting of parenchyma and a tumor, and combined them in one image

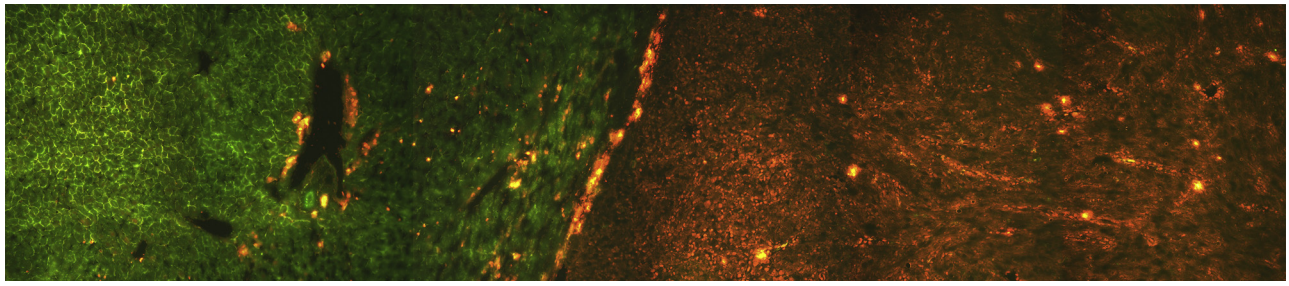

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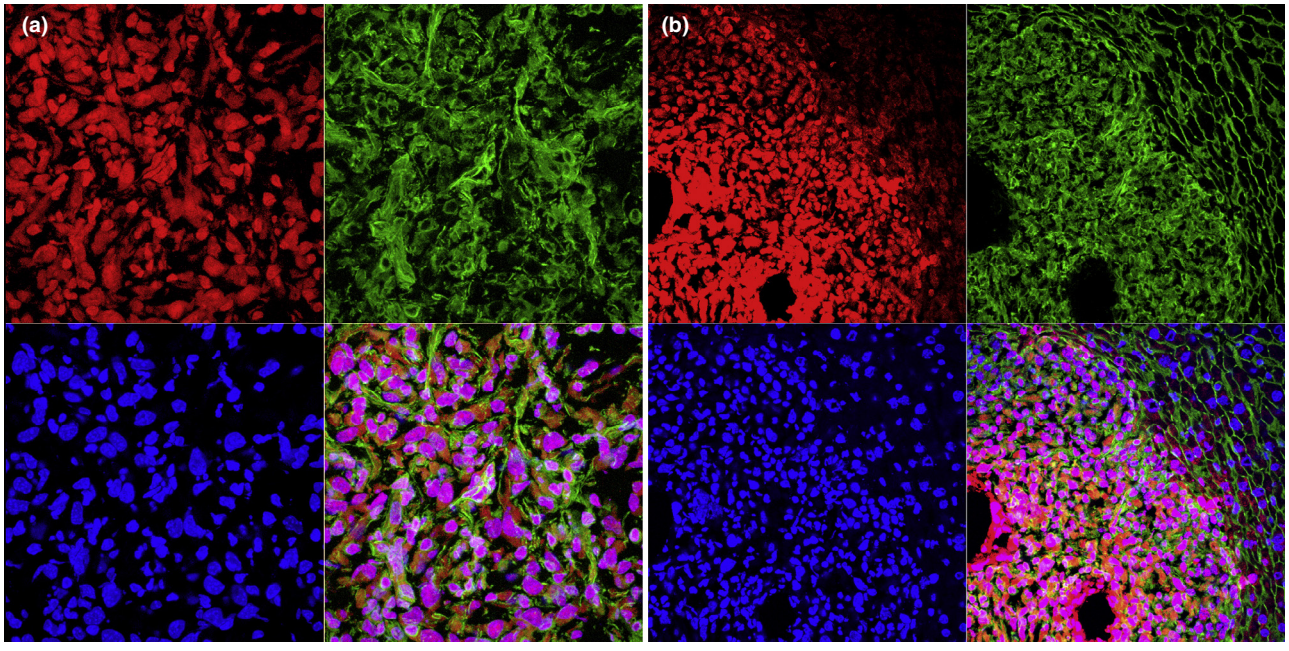
FIGURE 4

Liver tumors were established by inoculation of MC38 cells (10^4, syngeneic colonic carcinoma) into the ileo–colic vein of C57Bl mice. Three weeks later, C12PMMA-PI [0.16 mmol of rapidly reversible hydrophobized (RRH)-propidium iodide (PI) in 20 μl of DMSO mixed with 200 μl of isotonic glucose, mixing chamber] was infused via the hepatic artery, portal flow was preserved. Unstained frozen sections were air-dried cover-slipped and examined using 488 nm illumination and long-pass fluorescein isothiocyanate (FITC) emission filter (505 plus). Using Axioplan-2 microscope, under magnification 200× images, six consecutive overlapping fields were collected and combined in one image. Note, extremely high targeting of all tumor cells and hepatic arteries, whereas only a few cells in the parenchyma are targeted.

(Fig. 4). Figure 4 shows: (i) the dramatic drop in targeting between liver tumor and parenchyma; (ii) very strong targeting of all tumor vasculature and liver hepatic artery that appeared in portal triads. We hypothesize that such a dramatic drop in targeting is a result of fast RRH-PI hydrolysis and specifics of the blood drainage from the mouse liver tumors. In mouse tumors, blood drained from tumor capillaries to the superficial venous network of the metastases and further to hepatic veins [190]. In this scenario, any remaining active RRH-PI drained from hepatic arteries to sinusoids would be immediately extracted by membranes of erythrocytes, which constitute the paramount membrane compound in vasculature, but lacking nuclei.

Most animals were sacrificed 5–10 min after the restoration of hepatic artery blood flow and reperfusion of liver tumors, which was monitored and confirmed by surgical microscopy. Several animals were allowed to recover following surgery and sacrificed at 4–5 or 24 hours post-procedure. Results at the later time points mirrored the results obtained at the early time points. Treatment



Drug Discovery Today

FIGURE 5

Mouse liver colonic carcinoma tumors were established as described before. Three weeks later, C12PMMA-PI [0.16 mmol of rapidly reversible hydrophobized (RRH)-propidium iodide (PI) in 20 μl of DMSO mixed with 200 μl of isotonic glucose, mixing chamber] was infused via the hepatic artery, portal flow was preserved. Frozen sections were stained with Phalloidin Alexa 488 (actin – green) and ToPro-3 (DNA – blue). Red channel showed fluorescence of DNA-intercalated PI. Sections were examined under laser scanning microscope (LSM 510) confocal microscopy. **(a)** This image represents the center of the tumor, where literally all cells, including all the hepatocellular carcinoma (HCC) and all cells of tumor microenvironment, are strongly labeled. All DNA ToPro-3 fluorescence is co-localized with PI fluorescence, resulting in the bright pink color. By contrast, there is a dim red signal, which is probably hydrolyzed PI that was retained in the tumor matrix, magnification 630×. **(b)** The same fluorescent staining, image taken from tumor–parenchyma interface. All tumor cells were targeted; the most targeted cells are probably around the venous network of the tumor [190]. Some bordering hepatocytes are targeted as well, magnification 400×.

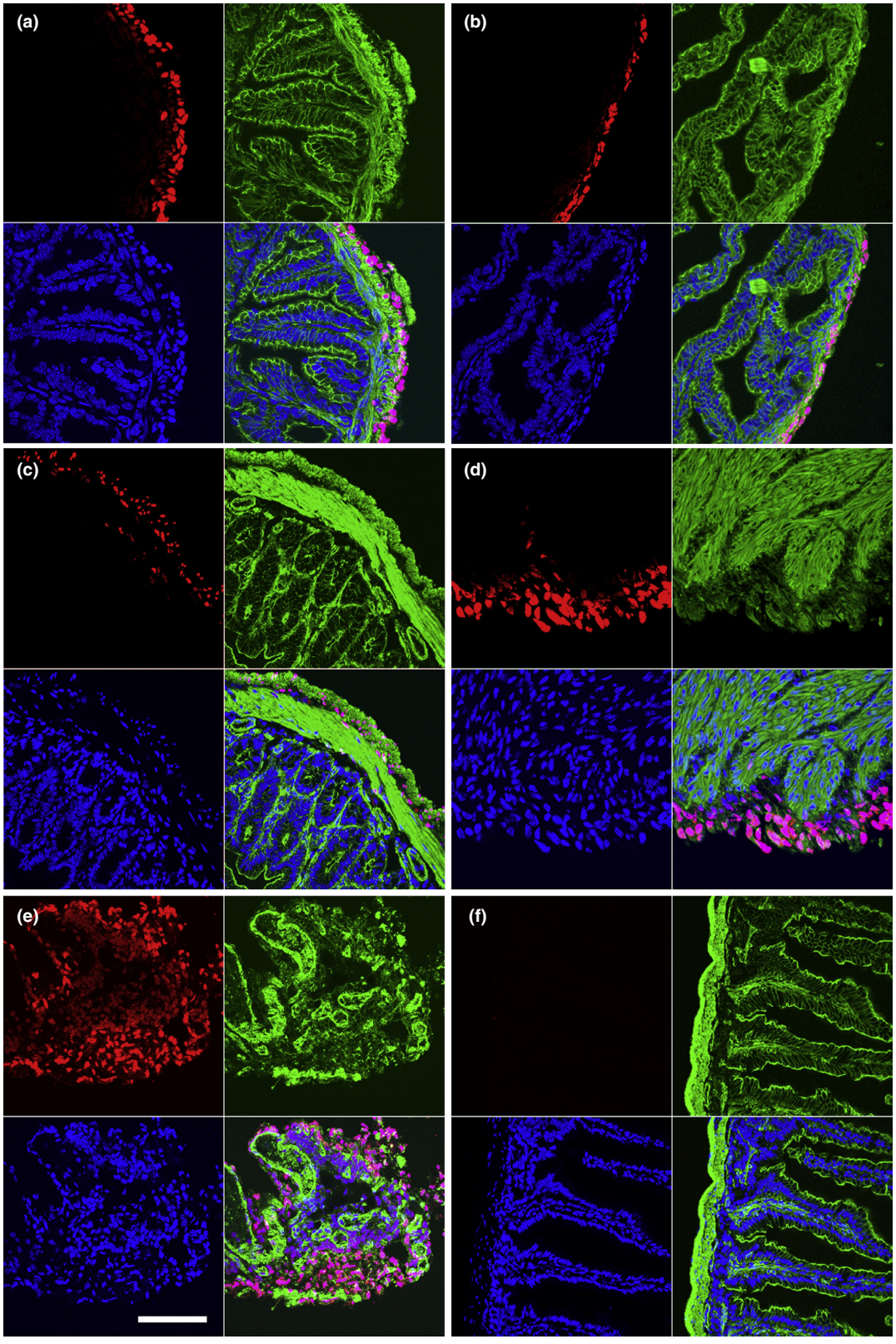


FIGURE 6

Targeting of peritoneal organs and tissues in normal ICR mice. **(a–e)** Intraperitoneal application rapidly reversible hydrophobization (RRH)-propidium iodide (PI) to normal peritoneal tissues. (a) Fallopian tube (BDMODS-PI); (b) fallopian tube (C12PMMA-PI); (c) jejunum (C12PMMA-PI); (d) uterus (C12PMMA-PI); (e) milky spot in visceral mesentery (C12PMMA-PI); **(f)** application of hydrolyzed C12PMMA-PI on jejunum. Red channel – fluorescence of DNA-intercalated PI; green – actin stain with Phalloidin Alexa 488; blue – nuclear stain with ToPro-3. Frozen sections, laser scanning microscope (LSM 510) confocal microscopy, bar = 100 μm. Note that cells situated deeper in the tissues (beyond serosal layer) were labeled at a much lower intensity or not at all. Application of hydrolyzed RRH-PIs resulted in extremely little if any drug uptake (f).

of other liver tumors (i.e. hepatocellular carcinoma, melanoma and neuroblastoma) showed similar high levels of tumor targeting while leaving the normal parenchyma free from drug uptake.

In parallel to fluorescent microscopy of unstained sections, we performed a confocal microscopy of the same liver samples. Figure 5a shows that practically all cells in the tumor (cancer cells and cells of the tumor microenvironment) were heavily labeled after RRH-PI delivery via the hepatic artery, providing a sufficient delivery of RRH drug to the tumor. The sufficient delivery means that an effective tumor perfusion was confirmed under a surgical microscope. To get access to a hepatic artery (or celiac artery), a mouse liver was kept toward a diaphragm with gauze soaked in saline. In this position hepatic lobes occasionally were slightly twisted, impeding the lobe vascular supply at the hepatic hilum. However, because unmodified PI and RRH-PI solution have a purple color, a sufficient RRH drug delivery to liver tumor was easily monitored by a completely changed color of tumors that was visible under a surgical microscope. Liver tumors in twisted lobes changed color only in some areas, or did not change color at all, and were not analyzed. Figure 5b shows that practically all cells in

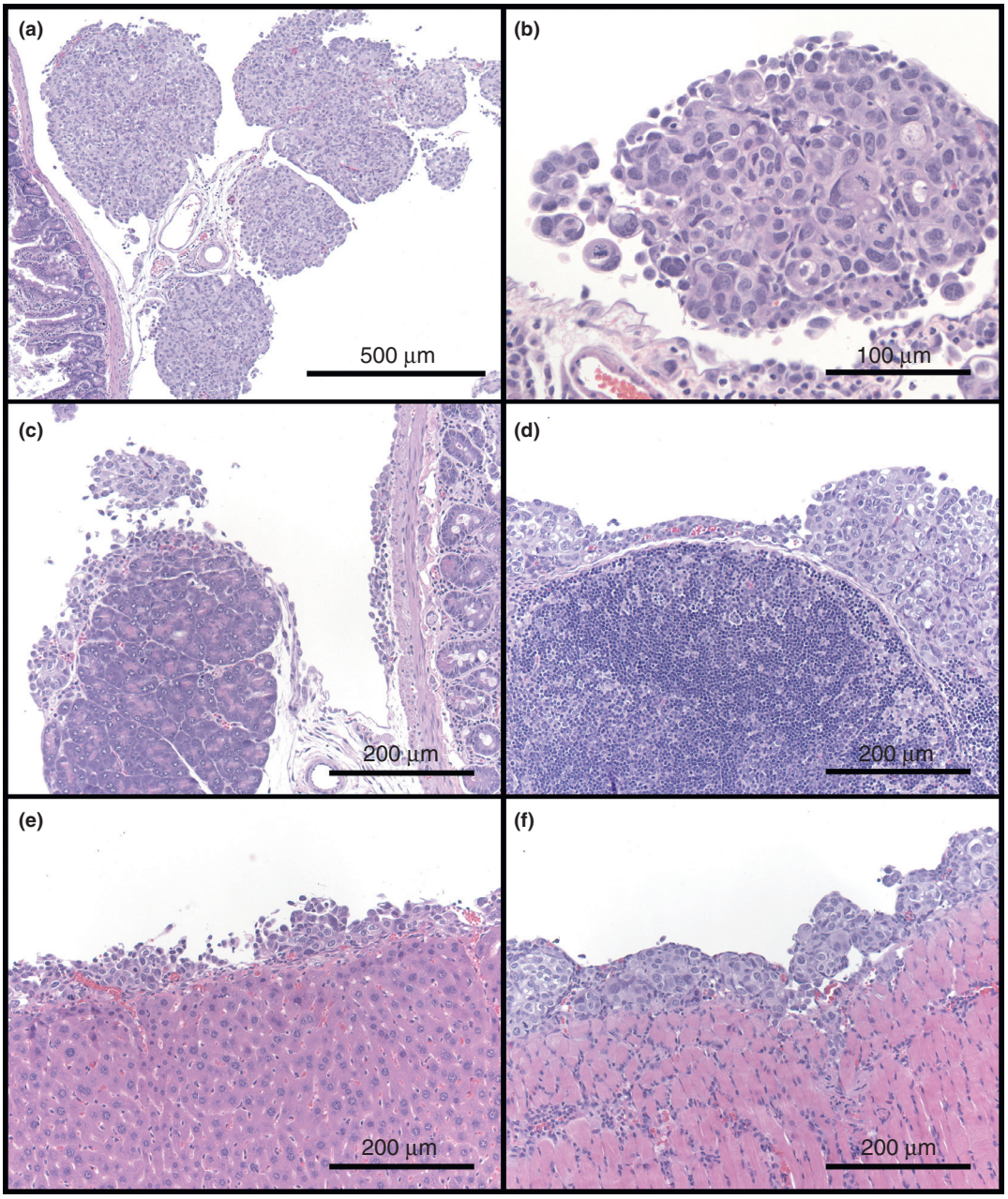


FIGURE 7

Pathological features of mouse model of disseminated peritoneal ovarian cancer, 5 weeks after Nude-*Foxn1*^{nu} mice inoculation with human SK-OV-3 cancer cells. **(a)** Micro tumor growth on duodenal mesentery. **(b)** Defoliating cells from mesentery tumor. **(c)** Loose tumor cell growth on duodenal wall and pancreas. **(d)** Tumor cell growth on mesenteric lymph node. **(e)** Tumor cell growth on abdominal surfaces of liver. **(f)** Tumor cell growth on diaphragm with invasion, paraffin sections, hematoxylin and eosin (H&E) stain.

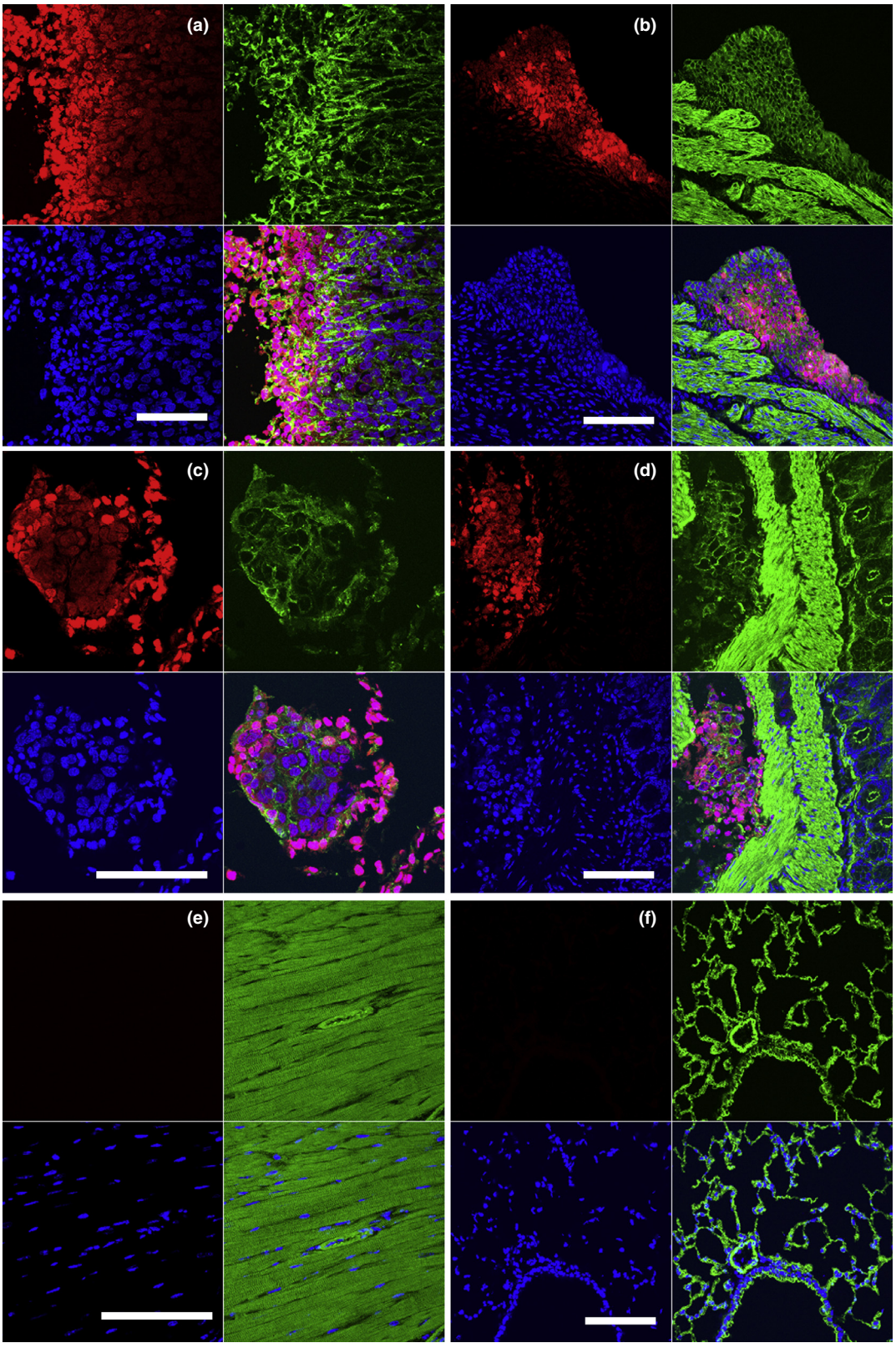


FIGURE 8

First-pass targeting of peritoneal disseminated ovarian cancer in mouse with rapidly reversible hydrophobized (RRH)-propidium iodide (PI). **(a)** Targeting of multiple cell layers in big ovarian tumor; **(b)** targeting of tumor tissue growing on colon wall; **(c)** targeting of mesenteric micrometastasis; **(d)** targeting of tumor cell cluster growing on and invading large bowel; **(e,f)** heart and lung tissues of animal receive RRH-PI intraperitoneal perfusion. None of heart or lung tissues showed any PI-labeling. Red channel – fluorescence of DNA-intercalated PI; green – actin stain with Phalloidin Alexa 488; blue – nuclear stain with ToPro-3. Frozen sections, laser scanning microscope (LSM 510) confocal microscopy, bar = 100 μm. Note, the RRH-PI had targeted all of the single tumor cells, cell clusters and microtumors

the tumor (cancer cells and cells of tumor microenvironment) were heavily labeled after RRH-PI delivery via the hepatic artery, including tumor cells at the tumor–parenchymal interface. In addition, some liver cells neighboring the tumor were also labeled. In this image the most-targeted cells are probably a part of the venous network of the tumor [190].

RRH prodrug testing in a mouse model of peritoneal disseminated ovarian cancer

Before experiments with the ovarian cancer mouse model, we conducted studies to investigate RRH-PI targeting of peritoneal organs and tissues in normal mice (ICR, female). The method of the RRH-PI prodrug application and the results were detailed in previous publications [180,181] presented at the McArdle Symposium on Cancer [191] (see also supplementary material online).

Briefly, the abdominal cavity was opened and the RRH-PI prodrug was directly applied to peritoneal organs using methods described above (i.e. mixing chamber and syringe pumps). Alternatively, RRH-PI prodrug was injected intraperitoneally using methods described above (i.e. mixing chamber and syringe pumps). After 10 min to 1 hour the abdominal organs were harvested and analyzed. The application of RRH-PI resulted in strong PI-targeting of all cells exposed to the peritoneal cavity (Fig. 6a–e). The cells situated deeper in the tissues (i.e. beyond the serosal layer) were labeled at a much lower intensity or not at all. Application of hydrolyzed RRH-PIs resulted in extremely little if any drug uptake (Fig. 6f).

Intraperitoneal perfusion with RRH-PI in a mouse model of disseminated ovarian cancer

To test for RRH prodrug uptake in a peritoneal cancer, we established a mouse model of disseminated ovarian cancer. Briefly, Athymic Nude-*Foxn1*^{nu} mice (female, 4–5 weeks) were injected intraperitoneally with human ovarian adenocarcinoma cells (SK-OV-3 cells, 2×10^6 cells in 1 ml PBS). The animals were treated with RRH-PI after 2–3 weeks post-inoculation, or at the time of the first manifestation of ascites (4–5 weeks post-inoculation). Following tissue fixation (10% NBF, *en bloc*), microscopic examination on the mice treated 2–3 weeks post-inoculation showed that multiple microtumors (i.e. 1 mm and smaller) were present throughout the peritoneal cavity, most notable on the mesentery. At 4–5 weeks after SK-OV-3 cell inoculation the maximum tumor size increased (5–7 mm); however the bulk of ovarian cancer mass was still present as microtumors (0.1–1.0 mm; Fig. 7). The striking feature of this ovarian cancer model was that at 5 weeks post-inoculation most peritoneal surfaces were affected by growing cancer cells, coating the visceral and parietal peritoneum (e.g. liver, pancreas and diaphragm; Fig. 6a–f). All tumors showed defoliation of cancerous cells. Thus, the pathological analysis indicated strong similarities in peritoneal perpetuation and dissemination between the SK-OV-3 mouse model and human ovarian cancer.

At 4–5 weeks after SK-OV-3 cell inoculation all of the animals developed ascites. At this time point an intraperitoneal perfusion with RRH-PI prodrug, followed by drug solution aspiration, was conducted on tumor-bearing mice under isoflurane anesthesia (Fig. S15 in Supplementary material online). The mice were allowed to recover from anesthesia, monitored for 3–5 hours and then sacrificed. Additional animals were monitored for 24 hours to insure tolerability of the procedure (i.e. no adverse effect of treatment noted).

Microscopic examination indicated the RRH-PI had targeted all of the single tumor cells, cell clusters and microtumors (0.1–1.0 mm) within the peritoneal cavity. This targeting of the spreading tumor cells and microtumors was total in regard to abundance and significant in regard to intensity (Fig. 8). Larger tumors were targeted to a depth ~500 μm (i.e. 25–30 cell layers) within 5–7 min following exposure to the prodrug (half-life of RRH-PI is ~10–20 s). Interestingly, deeper cell layers of relatively compact tumors were also targeted (Fig. 8a). Considering fast RRH-PI hydrolysis, this depth of cancer cell targeting in tumors could be due to membrane-to-membrane transfer of the hydrophobic prodrug rather than diffusion [192,193]. As expected, the adjacent mesenteric cells also showed RRH-PI uptake and staining, as did the outer layer of cells in all of the normal tissues exposed to the peritoneal cavity (and the RRH-PI solution). However, the tumor tissues appeared to be much more susceptible to RRH-PI uptake, showing greater tissue penetration and more intense PI-positive staining as compared with nonmalignant tissues beyond the serosal layer. Remote tissues such as heart and lung did not show any RRH-PI targeting (Fig. 8e,f).

Concluding remarks and some thoughts on clinical applications of RRH drug modification

In this review we outline the evolution of the loco-regional drug therapy for solid cancer and recent developments in this field. Loco-regional drug therapy, as a part of adjuvant treatments, has shown increased survival benefits for many types of cancer. We started our investigation of RRH prodrugs for regional-local treatment based upon the question of why, given the much greater drug exposure to tumor tissue using these techniques (in some cases 1000-times higher than with systemic treatment) [194], have the clinical effects not been better? We hypothesize that the drugs themselves showed poor tumor uptake even in the higher concentration environment. We (as others have) reason that hydrophobization of the drug enables greater cell uptake, and greater first-pass extraction of the prodrug. We further surmise that, by using a highly labile linkage, this prevents the highly membrane active prodrug form affecting normal tissues distal to the region of treatment. Over all, the effect would be to increase the tumor uptake for a given dose for a short time while exposing remote tissues to the less active parent drug, thereby effectively reducing systemic exposure of the drug, which is often the factor limiting drug dose.

Our experimental results have increased our belief that RRH prodrug modification could be an effective tool in the treatment of a multitude of cancers. Additionally, these modifications could open a new door in drug development. Traditional therapeutics show only a minimal degree of cellular uptake, and thereby

(0.1–1.0 mm) within the peritoneal cavity, as well as larger tumors at a depth ~500 μm (25–30 cell layers). Deeper cell layers of relatively compact tumors were also targeted (a). The adjacent mesenteric cells and the outer layer of cells in all normal tissues exposed to the peritoneal cavity also showed very high RRH-PI uptake. Nonmalignant tissues beyond the serosal layer showed very little, if any, RRH-PI uptake. Remote tissues such as heart and lungs did not show any RRH-PI targeting (e,f).

insufficient antitumor activity. However, this property helps to decrease systemic toxicity. Utilizing a RRH prodrug approach, drugs that show effectively little cellular uptake (and potentially little systemic toxicity) could be converted to active therapeutics for a short time in the tissue of interest (i.e. cancer). Our results using PI have shown that a long-known membrane-impermeable DNA intercalator can effectively target cells if hydrophobized and even have a therapeutic effect in a tumor model [172,180,181].

Using this approach, we demonstrated that RRH prodrugs were taken up by nearly all of the tumor cells in vascularized liver tumors. Similarly, the RRH prodrugs demonstrated surface penetration into ovarian tumors to a depth of at least 500 mμ (~25–30 cell layers) within seconds following RRH prodrug treatment. This is much more rapid than uptake seen with conventional therapies [195]. In other tumor types, conventional drug penetration in this range usually takes hours to achieve [196–198]. We further demonstrated that the effect was specific to the RRH modification because PI or hydrolyzed RRH prodrug showed little if any cellular uptake in ether treatment system. Although delivery to liver tumors and disseminated ovarian cancer were the only disease models in which we have tried our approach, the results in combination with targeting of normal tissues suggest that the potential applications could be broad. From studied animal models it became apparent that RRH methodology is capable of overcoming an increased interstitial fluid pressure in solid tumors, which is one of the main obstacles in solid cancer therapy [134–144].

Our results have shown that, when an artery was used as the route to deliver RRH-PI (hepatic or carotid), all arterial endothelial and smooth muscle cells showed a large amount of drug uptake. It is logical to suggest that arterial intimal hyperplasia, the main cause of failure in percutaneous coronary interventions and post-transplant coronary arteriosclerosis, should be the obvious target [199]. RRH drug technology could be used for intra-arterial luminal application to suppress proliferating neointimal cells in natural vessels, after bypass surgery, in stents or prosthetic vessels. Because intensive cell targeting along a route of vascular delivery is a property of RRH prodrug methodology, every vascular disease involved in active cellular transformation would be a potential target. If the hypothesis on the initiation of coronary atherosclerosis due to intimal cell proliferation in epicardial arterial *tunica intima* [200] is correct, reversal of coronary atherosclerosis before plaque formation is theoretically possible. Similarly to cases of angioplasty failure, the vascular target in coronary atherosclerosis is discrete, accessible and usually is no more than a few centimeters long. Angioplasty technology is such a rapidly evolving field that creation a vascular catheter with a mixing chamber in its tip is just an engineering matter, because catheters for isolated intravascular perfusion are long-understood in cardiovascular intervention (e.g. Schneider Europe, Bulach, Switzerland) and the technique aimed to inhibit neointimal formation in an isolated coronary segment with the double-balloon catheter was reported [201].

In a larger scope, every diseased organ or tissue compartment that has accessible vasculature could be amendable for application of local RRH drug therapy. It follows from all our observations that delivery of RRH drug via duct to exocrine organ should target all cells that are amendable to first-pass extraction. For example, delivery via the pancreatic duct should target all duct and exocrine cells but not pancreatic islets. Using devices similar to catheters for isolated intravascular perfusion [198], RRH drug delivery to prostate tissues via *Ductus ejaculatorii* is an achievable procedure.

Furthermore, our results on the targeting of all cell layers in the stratified epithelium certainly indicate that this methodology could be applicable for all surface malignancies, whether they appear in the dermis, airways or gastrointestinal tract or originate from urinary transitional epithelium. Again, mounting a mixing chamber on a tip of a cystoscope should not be difficult. The irrigation of the surface of the transitional epithelium with RRH prodrug followed by drug removal could be a simple procedural task.

When the RRH prodrug methodology is utilized with drug delivery to the tumor via a vascular route, it enables a precise calculation of the volume of the prodrug needed. This should significantly reduce the total dose of drug and significantly limit systemic toxicity. Similarly, in the case of surface applications to internal cavities (e.g. peritoneal cavity: bladder, ureter, kidney or pelvis) a prodrug mixture could be removed shortly after application, thereby preventing or significantly reducing systemic drug concentration. Although the RRH prodrug must be taken into solution with a small amount of an anhydrous solvent (e.g. DMSO), the limited amounts utilized in these studies did not cause any side-effect. Because we did not investigate how little DMSO was required for effective mixing, it is possible that in treatments requiring large volumes solvent doses could be a concern. However, the DMSO (or other nonpolar solvent) is not necessary if the prodrug solution is not being mixed with the blood. In cases when large surfaces need to be irrigated (e.g. peritoneum, bladder, ureter, kidney pelvis) or tissues of exocrine glands that drain secretion via ducts (e.g. exocrine pancreas, prostate) any nontoxic oil could be a carrier for the RRH drug. We suggest that the RRH prodrug behavior at an oil–cell interface would be similar to that in our experiments, but that the RRH prodrugs stability would be enhanced. Furthermore, RRH-drug–oil could be infused intraperitoneally during cystoscopy procedure. Because the RRH drug internalization into the cellular membrane would take place only at the oil–cell interface, free drug ingestion into the vasculature and lymphatic system would be limited. Injected patients could be active for some period of time, allowing natural circulation of administrated drug solution. This is very important for all peritoneal malignancies, because the route of peritoneal cancer dissemination is usually the same as peritoneal fluid circulation. After a predetermined time needed for treatment, the RRH prodrug in oil could be removed by cystoscopy under ultrasound control. Of course, long-term RRH prodrug effect on serosal membranes and normal urothelium should be further investigated, because all surface cells are targeted. However, we suggest that such adverse effects would be inversely proportional to rate of cell proliferation in treatment area. We suggest that RRH of drugs for first-pass drug extraction could significantly contribute to current achievements in the field of loco-regional cancer therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.drudis.2014.08.009.

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