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REVIEW

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Platelets for advanced drug delivery in cancer

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ABSTRACT

Introduction: Cancer-related drug expenses are rising with the increasing cancer incidence and cost may represent a severe challenge for drug access for patients with cancer. Consequently, strategies for increasing therapeutic efficacy of already available drugs may be essential for the future health-care system.

Areas covered: In this review, we have investigated the potential for the use of platelets as drug-delivery systems. We searched PubMed and Google Scholar to identify relevant papers written in English and published up to January 2023. Papers were included at the authors' discretion to reflect an overview of state of the art.

Expert opinion: It is known that cancer cells interact with platelets to gain functional advantages including immune evasion and metastasis development. This platelet-cancer interaction has been the inspiration for numerous platelet-based drug delivery systems using either drug-loaded or drug-bound platelets, or platelet membrane-containing hybrid vesicles combining platelet membranes with synthetic nanocarriers. Compared to treatment with free drug or synthetic drug vectors, these strategies may improve pharmacokinetics and selective cancer cell targeting. There are multiple studies showing improved therapeutic efficacy using animal models, however, no platelet-based drug delivery systems have been tested in humans, meaning the clinical relevance of this technology remains uncertain.

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Platelets; platelet extracellular vesicles; cancer; tumor microenvironment; drug delivery

1. Introduction

Cancer is a major public health burden, with global death rates parallel to cardiovascular disease [1]. However, the incidence of cancer is rapidly increasing, with a predicted doubling in 2070 from 2020 [2]. Although improved strategies for cancer prevention and the development of new methods for early detection are essential, more efficient medical treatments for advanced cancer are necessary to keep mortality rates low. However, as the pre-launch research and development costs for new anticancer drugs may reach several billion US dollars [3], it is unsurprising that the rising cost of cancer drugs leads to treatment abandonment due to affordability, especially in low-income countries [4]. Thus, a low-cost strategy to increase the efficiency of already available anticancer drugs is intriguing and necessary for future healthcare systems. In this review, we discuss the potential of platelets as a drug delivery system to serve this purpose.

2. Platelet biology

Platelets were first recognized as critical contributors to hemostasis in the late 19th century [5]. Later, that platelets exhibit many other functions, with essential roles in tissue regeneration, immunology, and cancer biology became apparent [6–11]. Platelets are anucleated membrane cell fragments derived from megakaryocytes (MKs). Thus, platelets are considerably smaller than other nucleated blood cells, typically with a diameter of 2–3 µm and a height of 350–800 nm [12]. MKs originate partly from a shared progenitor with erythroid cells; however, earlier restricted progenitors have also been identified [13–16]. MK development is primed by thrombopoietin (TPO) [17]. However, at least some TPO-independent platelet production exists, as double knockout of the *Tpo* gene and TPO-receptor gene *Mpl* does not entirely abrogate thrombopoiesis in mice, which can also be salvaged with different chemokines [18]. MKs usually reside close to blood vessels [19], and shear stress promotes MK maturation [20]. Under normal conditions, platelets are produced by membrane budding or proplatelet formation [21,22]. However, upon greater need, MK rupture induced by interleukin-1α may be an emergency mechanism to restore the platelet pool [22].

Although platelets are anucleate cell fragments with a rapid mRNA time decay [23], limited protein synthesis persists [24]. However, most platelet proteins are inherited by parental MKs, and a recent analysis of the platelet proteome identified over 5,000 unique proteins [25]. Some proteins are packed in granules, mainly alpha granules and dense granules, which are released upon platelet activation, yielding a complex releasate [26]. Furthermore, the composition of the platelet releasate differs depending on the activation stimuli [27,28]. Platelet alpha granules contain several hundred proteins, including growth factors and chemokines [29,30].

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Article highlights

- Platelets interact with cancer cells in the blood and the tumor microenvironment, which confers survival advantages in the cancer cells.
- Platelets can transfer molecules to cancer cells by shedding platelet extracellular vesicles.
- Platelets can be loaded or conjugated with multiple drugs, thereby slowing drug elimination and increasing selective targeting because of platelet-cancer interactions.
- Many of the advantages of platelet drug vectors can be transferred to synthetic nanocarriers by coating them with platelet membranes.
- Although multiple animal studies confirm the potential of platelet-based drug delivery, currently there are no human studies.

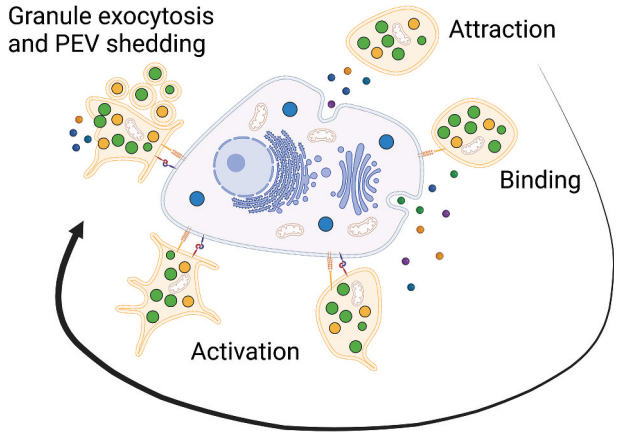

Figure 1. Platelet-cancer interactions. The tumor microenvironment attracts platelets followed by binding and activation of the platelets by cancer cells. Platelets may be activated through multiple pathways including secretion of soluble mediators or direct receptor stimulation yielding functional advantages for the cancer cells.

Conversely, dense granules primarily contain small coagulation-active substances such as ADP, serotonin, and calcium. However, mass spectrometry analysis has also revealed a minor selection of cell signaling proteins [31].

As discovered by Heijnen et al., platelets secrete different microvesicles up to 1 µm in diameter via plasma membrane shedding or multivesicular body and alpha granule exocytosis following thrombin receptor agonist peptide or thrombin stimulation [32]. Nowadays, the terms 'microvesicles' or 'microparticles' are generally replaced with 'extracellular vesicles' (EVs) and denominated small, medium, or large, depending on size [33]. Platelet extracellular vesicles (PEVs) are the most abundant EVs in plasma, with substantial contributions from other blood and endothelial cells [34,35]. Although PEVs are generally 'small,' with a diameter <200 nm depending on origin [36,37], larger PEVs exist and can contain large organelles such as mitochondria and proteasomes [38]. PEVs are shed by platelets upon storage or activation by different stimuli [36,37,39]. Regarding coagulation, PEV generation follows membrane ballooning, which both increase the total procoagulant surface area [40,41]. Different platelet activators yield quantitative and qualitative differences in the PEV composition, suggesting that formation and packing involve active rather than stochastic processes [42]. Consequently, PEVs generated in cancer may have specific qualities, as the proteome of PEVs generated by the co-incubation of platelets with the breast cancer cell line MDA-MB-231 differs from that of thrombin-generated PEVs [43].

3. The role of platelets in cancer

Patients with cancer have an increased risk of venous thromboembolism (VTE), which varies with cancer type and stage [44,45]. Additionally, an elevated platelet count is often observed and is generally indicative of poor prognosis [46–48]. However, the increased VTE risk in patients with cancer is not merely a result of elevated platelet counts, as there is no association between VTE and elevated platelet counts in the general population [49]. This phenomenona is surely multifactorial and likely involves a complex interplay between platelets and cancer cells [50]. It is known that the tumor microenvironment attracts platelets, as immunohistochemical investigations of tumor biopsies reveal that platelets can infiltrate tumors [51,52] (Figure 1). However, platelets are generally absent in normal tissues. Based on findings from murine knockout models, platelet tumor infiltration may be dependent on select G proteins [53], meaning extravasation is not a random event. In addition, platelet tumor infiltration leads to worse overall survival in patients with colorectal or pancreatic cancer [51,54,55]. Thus, one can surmise a critical role of platelet-cancer interactions in cancer biology.

3.1. Platelet-cancer interactions

Cancer cells activate platelets via multiple pathways. For instance, the high mobility group box 1 protein is released by cancer cells and activates platelets via Toll-like receptor 4 (TLR4) [56]. Multiple cell lines likely generate the strong platelet activator thrombin via different mechanisms, with varying dependency on tissue factor [57]. Cancer cells can also activate platelets directly via binding to receptors and adhesion molecules such as Clec-2, P-selectin, glycoprotein (GP) VI, integrin α6β1, and platelet FcγRIIa [58–63]. Furthermore, cancer cells secrete ADP, a weak platelet activator [64]. Treatment with the commonly used platelet inhibitor ticagrelor, which inhibits the ADP receptors P2Y12 and P2Y1, reduced tumor growth in ovarian cancer-bearing mice by 60% compared to aspirin and 75% compared to placebo [65]. Similar results have been obtained using ADP receptor inhibitors in melanoma, breast cancer, and pancreatic cancer models, underscoring the importance of platelet ADP stimulation by cancer cells [66,67].

There was early enthusiasm about the potential of aspirin treatment to break the platelet-cancer axis, and observational studies have shown a reduced risk of multiple cancer diseases and better cancer-specific survival in patients with colorectal cancer [68–71]. However, a recent systematic review including more randomized controlled trials (RCTs) for prophylactic aspirin against colorectal cancer did not confirm these previous conclusions [72]. Nonetheless, the authors reported high statistical heterogeneity due to variable study designs, including aspirin dosage and treatment duration. Multiple platelet

inhibitors are in clinical use, but the studies on the relationship between platelet inhibition and cancer risk or mortality generally includes only aspirin. Although some data on the effects of other platelet inhibitors, usually in combination with aspirin, are available, RCTs are lacking [73].

Platelets interact with circulating tumor cells (CTCs) not only by binding to single cells, but also by formation of larger aggregates and both mechanisms may confer functional advantages to cancer cells, including protection against mechanical forces and CTC arrest [74–76]. This interaction also facilitates epithelial-to-mesenchymal transition (EMT) through transforming growth factor (TGF)-β1 secretion [58,77]. In SK-OV-3 and OVCAR-3 ovarian cancer cells, inhibiting the TGF-β1 receptor reversed the effect of platelets on EMT, thereby reducing metastatic potential [78].

3.2. Platelets in cancer immunology

Cancer cells can evade natural killer (NK) and T cells by interacting with platelets (Figure 2). Platelets express MHC class 1 molecules (MHC-1), which exhibit time-dependent decay [79]; however, MHC-1 expression is upregulated upon activation [38]. Thus, platelet binding can lead to pseudo-expression in CTCs, disrupting recognition by NK cells, and directly impairing their cytotoxic function [80]. However, platelet binding may also exert MHC-1-independent inhibitory functions [81]. The use of platelet releasate weakened NK cytotoxicity against tumor cells by downregulating the NK group 2D (NKG2D) receptor expression, and this effect was abolished when neutralizing TGF-β1 [82]. Similar findings have been reported using PEVs co-cultured with NK cells, where activation receptors were downregulated in a TGF-β1-dependent manner [83]. Platelet binding may also cause the shedding of NKG2D receptor ligands on cancer cells, thus impairing NK cell-mediated lysis [84].

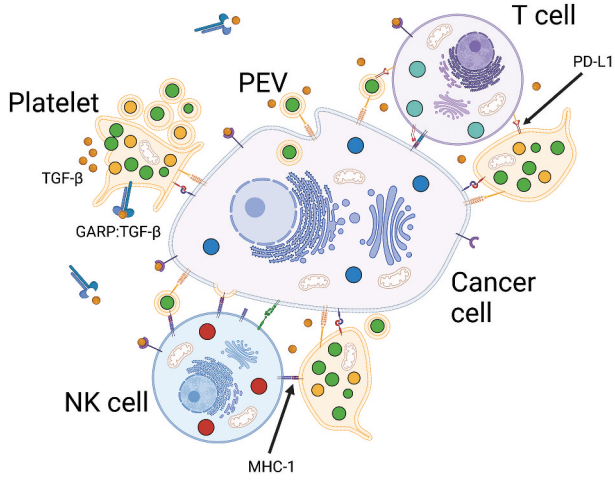

Figure 2. Platelet-assisted immune evasion. Platelet-cancer interactions assist cancer cells in evading NK and T cell cytotoxicity partially through TGF-ß-mediated mechanisms. In addition, binding of platelets also leads to pseudo-expression of MHC-1 and PD-L1, thus cancer cells may avoid recognition by NK cells through expression of platelet self-antigens and impair T cell cytotoxicity through checkpoint inhibition.

T cells activate platelets through the CD40-CD40L axis, which may increase T cell recruitment through C-C motif chemokine ligand 5 secretion [85]. Furthermore, in lung cancer, the frequency of platelet-T cell aggregates increases [86]. Although platelets can also activate cytotoxic T cells through antigen presentation [38,87], platelet-T cell interactions are likely detrimental in cancer because the surface-bound GARP-TGF-β complex on platelets is a crucial mediator of platelet-associated T cell suppression in murine cancer models [88,89]. In addition, similar to the pseudo-transfer of MHC-1, platelet binding may lead to the pseudo-expression of programmed death-ligand 1 (PD-L1), thus impairing anticancer T-cell activity through checkpoint inhibition [90]. Riesenberg et al. studied the potential interplay between platelets and the tumor microenvironment in heat shock protein gp96 knockout mice, which exhibit thrombocytopenia and severe platelet dysfunction [91]. SMAD3 phosphorylation was lower in tumor cells and tumor-infiltrating lymphocytes (TILs), indicating less stimulation by TGF-β. Moreover, the relative CD4+ or CD8+ TIL frequencies, PD-1 expression, and tumor necrosis factor (TNF)-α and interferon (INF)-γ production upon stimulation were higher. The tumor growth-inhibitory effect of platelet inhibition described in the previous sections was also confirmed and it was abrogated when combined with an antibody against CD8.

Bispecific T cell-recruiting antibodies are a novel modality of immunotherapy in which monoclonal antibodies (mAbs) connect cancer cells with T cells. Although only a few drugs have reached clinical use, this technology has shown promising therapeutic efficacy, and multiple drugs are under clinical investigation [92]. However, recent evidence indicates that activated platelets may reduce bispecific antibody-mediated CD4+ and CD8+ T cell reactivity, which is restored with TGF-ß blockade [93]. Platelets may also play a role in the resistance to conventional anti-PD-1-based immunotherapy. The combination of dual platelet inhibition and anti-PD-1 antibody was more effective than anti-PD-1 antibody treatment alone in a murine MC-38 colon cancer model [91]. Although the platelet-mediated anticancer effect of aspirin in humans is controversial, one prospective study showed that aspirin only reduced the risk of colorectal cancer with a low TIL count, suggesting a possible immune-mediated pro-tumor effect of platelets [94]. Thus, our knowledge of the interactions between platelets and cytotoxic NK and T cells supports new studies on platelet inhibition to overcome resistance to various immunotherapies.

4. Drug delivery systems

Drug delivery systems exist in many forms, including novel techniques using drug-loaded nanoparticles [95]. Liposomes are likely the best-known and most used of these platforms, and several drugs with liposomal formulations have been approved since the 1990s [96]. Liposomes are membrane-permeable lipid bilayer vesicles with aqueous cores that can be loaded with drugs to alter their pharmacokinetics and pharmacodynamics [96]. Other notable platforms include poly(lactic-co-glycolic acid) (PLGA) and protein-based nanoparticles. PLGA nanoparticles are manufactured from polymers

comprising lactic and glycolic acid in different ratios to obtain specific physicochemical properties [97]. Protein-based nanoparticles have huge potential as they can be manufactured with various functionalities and responsiveness depending on the protein composition [98]. However, their clinical use remains limited. Despite their early promise, nanopharmaceuticals have disadvantages, most notably cost and certain immunological phenomena [99]. Liposomes are cleared by macrophages in the reticuloendothelial system [100], and macrophage uptake of liposomes depends on their size and lipid composition [101]. Pegylation is used to alter the physicochemical properties of liposomes and increase their stability [102]. However, this leads to the risk of hypersensitivity reactions, possibly through interactions with anti-PEG IgM and complement activation [103].

Generally, drug delivery systems are used to optimize the pharmacokinetics and pharmacodynamics of a drug, thus improving its therapeutic efficacy and safety. However, promising preclinical data on liposome-formulated chemotherapeutics usually do not translate into substantially better therapeutic efficacy in clinical studies [104]. However, a liposomal formulation may be more beneficial than the free drug if it reduces the frequency or severity of adverse events. Doxorubicin (DOX) is an anthracycline frequently used to treat solid and hematological malignancies as a liposomal formulation and a free drug. Thus, as much clinical data are available, DOX is ideal for comparing toxicity. However, systematic reviews generally conclude that conventional and liposomal formulations have similar toxicity profiles. Although the risk of cardiotoxicity may be reduced in liposomal formulations, the risk of hand-foot syndrome increases [105–107].

One example of the cost issue in developing advanced drug delivery systems for existing drugs is CPX-351, now registered as a Vyxeos liposomal. CPX-351 is a relatively new liposome-encapsulated formulation of cytarabine and daunorubicin that maintains a favorable molar ratio of 5:1 in the plasma and bone marrow (BM) for a prolonged time [108]. In addition to its favorable pharmacokinetics, preclinical data suggest that it may selectively target leukemic cells over normal hematopoietic progenitors [109,110]. A phase III trial published in 2018 showed that CPX-351 markedly improved overall survival and remission rates compared with conventional treatment, with similar toxicity in older patients with newly diagnosed high-risk secondary acute myeloid leukemia [111]. Post hoc analysis also showed improved quality-adjusted time without symptoms of disease or toxicity (Q-TWiST) [112]. However, a 2021 analysis concluded that it was far from cost-effective at the current price, yielding an incremental cost of $115,000 per patient [113]. Thus, even if drug delivery platforms have been in clinical use for a long time, they still have several shortcomings, including the failure to increase therapeutic efficacy or to substantially reduce toxic side effects, at least in a cost-effective manner.

5. Platelets as a drug delivery system

Cancer cells interact with platelets to gain functional advantages. Thus, using platelets for drug delivery could provide a platform for selectively targeting cancer cells, at least to a greater degree than free drugs or existing drug delivery systems. Some apparent advantages of a platelet-based versus a synthetic drug delivery system are that platelet concentrates are readily available, well tolerated, and have relatively low direct production costs [114]. Although it would increase the hospital stay for patients with cancer, platelets for drug delivery can also potentially be harvested from the patients themselves, thus eliminating potential issues with alloimmunization and further minimizing the risk of transfusion reactions.

Platelets or PEVs as drug delivery systems also share the theoretical benefits of nanopharmaceuticals in terms of changes in pharmacokinetics, as the drugs are encapsulated and protected from degradation processes in the blood, phagocytosis by macrophages, and renal clearance. PEVs are also known to infiltrate tumors, lymphoid organs, BM, and inflamed tissue [38,115–117]. Platelets can transfer content to various malignant and normal cells via PEV shedding and subsequent internalization by the target cells [115,118–122]. Thus, PEVs shed from drug-loaded platelets may transfer molecules independent of specific transporter proteins, thereby circumventing limiting drug uptake, which is a suggested drug-resistance mechanism [123].

Strategies for using platelets as drug delivery systems can be summarized into three main categories: drug-loaded, drug-bound, and platelet-based hybrid vesicles (Figure 3).

5.1. Drug-loaded platelets

Several studies have used platelets as a delivery system by loading cytotoxic compounds and the most commonly used drug is DOX [124–129]. However, other chemotherapeutics have also been tested in use with platelet hybrid vesicles [130,131]. Because anthracyclines passively permeate lipid membranes, drug loading is uncomplicated [132]; however, human organic cation transporter 1 (OCT1; SLC22A1)-mediated transport has also been reported [133]. Administering DOX-loaded platelets increased drug uptake and apoptosis induction in Raji cells compared with free DOX [124]. Furthermore, toxicity in cardiomyocytes was reduced, which may have clinical relevance, as cardiomyopathy is a feared consequence of DOX treatment. Platelets have migratory capabilities [134], which were further enhanced by coating DOX-loaded platelets with polydopamine to increase tumor infiltration and drug transfer in MCF-7 breast cancer-bearing mice upon irradiation with near-infrared light [129]. One possible challenge of using platelets as drug vectors is their limited storage potential. Wu et al. used cryopreserved DOX-loaded platelets from clinical-grade platelet concentrates to study apoptosis induction in cell-lines from different solid cancers, creating an 'off-the-shelf product' [126]. Cryopreservation of DOX-loaded platelets did not markedly impair platelet function or the cytotoxic effect, which was generally in line with that of free DOX, although it was higher than that of liposomal DOX. Moreover, drug release, although partially a time-dependent and pH-sensitive process, was stimulated by tissue factor-expressing cancer-derived EVs.

Platelets loaded with the tyrosine kinase inhibitors sorafenib and lenvatinib led to increased drug uptake and necrosis in tumors in hepatocellular carcinoma-bearing rats compared

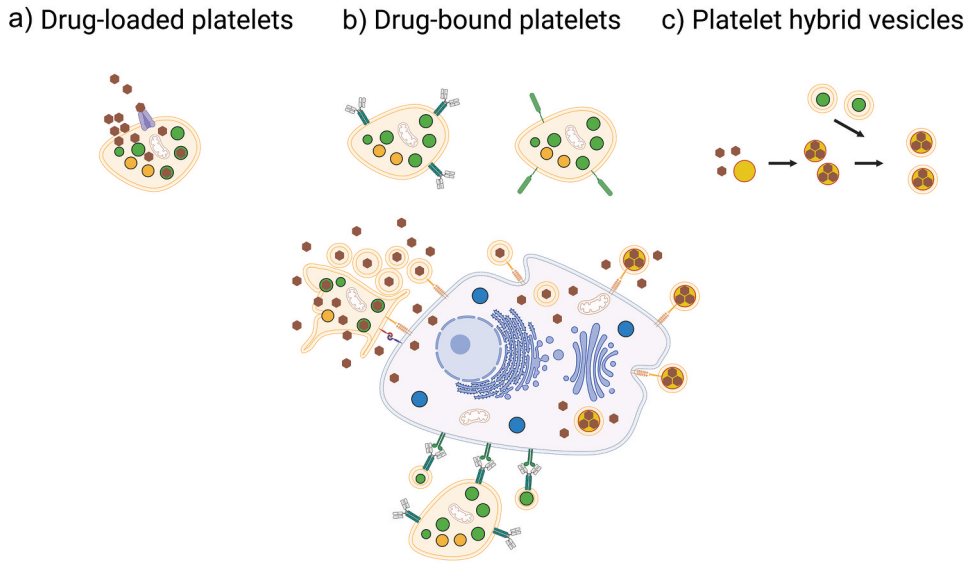

Figure 3. Platelets as drug-delivery systems. Platelets can be used as drug-delivery systems mainly through three different strategies. a) by drug-loading where platelets encapsulate the drug, then accumulate in the tumor microenvironment followed by activation and drug release. b) by drug-binding where large molecules such as antibodies or cytotoxic complexes are conjugated to the surface and kill cancer cells by cell-to-cell contact or by shedding of PEVs. c) by implementing platelet membranes in hybrid vesicles through fusing of PEVs with synthetic drug-loaded nanocarriers or other cell membranes to slow macrophage clearance and increase accumulation in the tumor microenvironment.

to treatment with free drugs [135]. Furthermore, drug release increased when the platelets were exposed to ADP, indicating the importance of platelet activation. The contribution of platelet activation to loaded drug release has also been corroborated in other studies [127,129]. Li et al. suggested in their study that the polydopamine-coated platelets could bind DOX through π–π stacking, which was released in weak acidic environments [129]. In addition, the transfer of drugs from DOX-loaded platelets decreased by inhibiting endocytosis in the target cancer cells. Thus, drugs are partially transferred from drug-loaded platelets by shedding PEVs, followed by internalization by cancer cells.

Photothermal therapy (PTT) is a treatment modality closely related to the better-known photodynamic therapy. In PTT, photothermal agents (PTAs) may be administered via intratumoral or intravenous routes before laser treatment, which induces heating, followed by apoptosis or necroptosis [136]. Thus, tumor-selective uptake is necessary to avoid excessive damage to the surrounding tissue, which currently limits its application outside preclinical research. Platelets were used as vectors for gold nanostar (AuNS)-directed PTT in a murine head and neck squamous cell carcinoma model [137]. Platelet cloaking protected the AuNSs against macrophage clearance, yielded better therapeutic efficacy, and led to less weight loss than treatment with uncoated AuNSs. Combining PTT with the loading of cytotoxic compounds may also be prudent. By loading platelets with the PTA IR-820 and DOX before laser irradiation, Zhang et al. obtained synergistic effects, as heat induction increased DOX release, thus improving anticancer efficacy in breast cancer-bearing mice [138].

5.2. Drug-bound platelets

Other studies have investigated platelets as drug delivery systems with antibody conjugates or binding of other large molecules. This may also be useful for increasing cancer cell targeting because low platelet binding to cancer cells may be a potential limitation of using platelets as drug vectors. Transferrin conjugation increased platelet adherence to myeloma cells with high transferrin receptor expression [139]. Furthermore, the anticancer effect of DOX-loaded platelets was optimized by conjugating anti-CD22 to the platelets, leading to more selective drug transfer and increased DOX uptake and apoptosis of CD22+ lymphoma cells [140]. Fan et al. conjugated platelets with a tumor microenvironment-responsive nanogel comprising the pore-forming peptide GALA and serine protease granzyme B (GrB) [141]. Platelets accumulated at the tumor site, where the GALA/GrB complex was cleaved under acidic conditions and internalized by cancer cells. GALA was essential for lysosomal escape by GrB for optimal apoptosis induction in a murine melanoma model. Yap et al. used single-chain antibodies against activated GP IIb/IIIa linked to the microtubule inhibitor monomethyl auristatin E (MMAE) [142]. Thus, the activated platelets were selectively targeted for drug delivery *in vivo*, and following tumor infiltration, MMAE was released via linker cleavage by cathepsin B. This system effectively treated primary breast cancer tumors and metastasis in murine models and showed excellent selectivity with no drug uptake in the BM or spleen. Li et al. engineered TNF-related apoptosis-inducing ligand (TRAIL)-expressing platelets by lentiviral transduction and transplantation of murine hematopoietic stem cells (HSCs) [143]. These platelets produced an anticancer effect in a murine metastasis model using the human prostate cancer cell line, PC3. Likewise, TRAIL- and von Willebrand factor-coated liposomes have effectively killed CTCs isolated from patients with cancer with metastatic disease through interconnection with cancer cells via platelets [144]. Zhao et al. used paclitaxel and IR-780-containing P-selectin-targeting nanoparticles and adopted a similar strategy [145]. The

therapeutic efficacy of the drug-containing nanoparticles was further enhanced by PTT, which increased platelet accumulation at the tumor site. This 'hitchhiking' strategy was also exploited by Li et al., who used P-selectin-binding nanoparticles loaded with ticagrelor and the anti-inflammatory agent celecoxib to inhibit metastasis in breast cancer-bearing mice through modulation of the tumor microenvironment [146].

Using platelets as a drug delivery system may be a double-edged sword because platelets also have protumoral properties. This effect can be reduced by adding the sulfated polysaccharide fucoidan to the system. Guo et al. used micelles containing fucoidan and DOX that could bind to platelets through P-selectin, thus targeting platelets *in vivo* that are further connected to cancer cells [147]. The addition of fucoidan, when compared to dextran containing (control) micelles or free DOX, further decreased tumor volume in a murine 4T1 breast cancer metastasis model, possibly through reduction of tumor TGF-ß levels leading to a favorable immune response with increased levels of pro-inflammatory cells and decreased levels of anti-inflammatory cells.

5.2.1. Platelet-based immunotherapy

The introduction of checkpoint inhibitors represents a paradigm shift in treating solid cancers, resulting in durable remission in previously untreatable diseases. Briefly, this treatment modality seeks to disrupt checkpoint signaling in T cells to stimulate an anticancer immune response. Recently, many studies have used platelets as mAb carriers to optimize this technology further.

Wang et al. developed a drug delivery system in which antibodies against programmed death-1 protein (PD-1) were conjugated to platelets for use in melanoma and breast cancer models [148]. This system uses the ability of platelets to infiltrate tumors and then release the drug *in situ* by shedding PEVs. Anti-PD-1-conjugated platelets were superior to free anti-PD-1 treatment, increasing the circulation half-life and yielding a stronger local anticancer immune response and tumor reduction. Lu et al. used a different approach to link tenfold the amount of anti-PD-1 antibody per platelet compared to the previous study [149]. This was managed by conjugating antibody-crosslinked nanogel backpacks that dissolved in response to the reduction activity of the platelet surface upon platelet activation. Furthermore, Hu et al. used anti-PD-1-conjugated platelets to treat acute myeloid leukemia (AML)-bearing mice by conjugating platelets to HSCs [150]. HSC-conjugation was necessary for sufficient BM infiltration; however, adding platelets to the system substantially increased the anti-leukemic inflammatory response and survival. This study thus illustrates insufficient BM infiltration as a potential weakness of unmodified platelets as a drug delivery system. The same group combined anti-PD-1-conjugated platelets with chimeric antigen receptor (CAR)-T cells in a biodegradable hydrogel for implantation into the tumor resection cavity of melanoma-bearing mice [151]. Anti-PD-1 antibodies are released upon activation *in situ*, increasing the efficacy of CAR-T cell treatment. Zhang et al. engineered murine PD-1 expressing platelets from MK progenitors that accumulated in partially resected malignant melanoma tumor beds [152]. The authors observed an anticancer effect by increasing tumor-infiltrating cytotoxic CD8+ T cells, as observed in the previous studies with anti-PD-1-conjugated platelets. Moreover, the anticancer effect was increased when PD-1-expressing platelets were loaded with cyclophosphamide to deplete the tumor microenvironment of regulatory T cells.

The inflammatory anticancer effect of anti-PD-1-conjugated platelets was also potentiated by incorporating them into a hydrogel loaded with the tyrosine kinase inhibitor pexidartinib [153]. This incorporation depleted the resection sites of tumor-associated macrophages in tumor-bearing mice with different cancer types by blocking CSF1R signaling. Furthermore, concomitant treatment with vadimezan increases the recruitment of anti-PD-1-conjugated platelets through tumor vascular disruption, resulting in substantially improved survival in several murine cancer models [154]. Lastly, PTT has been combined with anti-PD-1-conjugated platelets to enhance the immune stimulatory effect through 'wounding' of the tumor to increase platelet infiltration and tumor antigen release [155,156].

Thus, platelets may not only play a role in developing resistance to different immunotherapies, they can also be engineered into potent immunomodulatory anticancer cell fragments using existing drugs.

5.3. Platelet hybrid vesicles

Several researchers have developed platelet-inspired synthetic hybrid vesicles. Possible benefits of implementing platelet proteins or membrane elements in nanocarriers include improved cancer-specific targeting and slowed clearance by macrophages. PLGA nanoparticles cloaked with platelet membranes mimic several aspects of platelets, including platelet antigen expression, collagen binding, and endothelial cell binding [157]. Macrophage phagocytosis *in vitro* was also inhibited in a CD47-specific manner. Wang et al. used this method by coating chitosan-modified PLGA nanoparticles loaded with the anticancer drug bufalin with PEVs to increase cancer cell apoptosis without excessive off-target effects [158]. The tumor infiltration and anticancer effects of platelet-coated hybrid vesicles loaded with docetaxel were further enhanced by incorporating them with recombinant VAR2CSA [159]. This peptide can attach to chondroitin sulfate expressed on melanoma cells with different phenotypes after EMT and mesenchymal-to-epithelial transition, thus increasing platelet recruitment in both primary tumors and metastases. Combining the vascular-disrupting agent vadimezan with anti-PD-1-conjugated platelets may increase tumor platelet accumulation and therapeutic efficacy [154], as described in the previous sections. Similar effects were observed when vadimezan was combined with paclitaxel-loaded platelet membrane-coated nanocrystals in breast cancer-bearing mice [160]. Pan et al. coated DOX-loaded liposomes with ligands to mimic platelet-tumor binding and increased apoptosis induction in co-cultures with breast cancer cell lines [161]. In addition to the drug-loading strategy, conjugating platelet membrane-coated silica particles with the death receptor ligand TRAIL has shown potential for treating murine breast cancer metastatic disease [162]. Consequently, combining TRAIL

expression and DOX loading in platelet hybrid vesicles may provide additional therapeutic benefits [163].

Kim et al. used red blood cells and platelet membranes to coat AuNSs with hybrid vesicles, which were then loaded with curcumin [164]. Thus, AuNS-directed PTT was combined with a cytotoxic drug. Platelet membranes were integrated for the selective targeting of cancer cells, and adding red blood cell membranes proved crucial for further evasion of macrophage phagocytosis. Coating PTA-containing hybrid vesicles with platelet membranes may increase tumor uptake and penetration [165]. Multiple studies have shown an increased *in vivo* anticancer efficacy in breast cancer-bearing mice [165–167]. Similar to the beneficial effects of platelet coating in PTT systems, coating gold nanocages with PEVs and loading them with cisplatin has been shown to sensitize breast cancer-bearing mice to low-dose radiotherapy [168].

5.3.1. Platelet hybrid vesicle-based immunotherapy

Platelet hybrid vesicles have been manufactured to induce favorable immune responses in the tumor microenvironment. A variation of this method was developed by Yu et al. in which vesicles from genetically engineered HEK-293T cells and platelets were fused and loaded with oxaliplatin [130]. HEK-293T cells were infected with the gene encoding the T cell immunoreceptor with immunoglobulin and the ITIM domain (TIGIT), which binds to the CD155 antigen on cancer cells. Thus, the hybrid vesicles blocked TIGIT-mediated immune evasion and restored CD8+ T cell activity. In a recent study, Bahmani et al. coated nanoparticles containing the TLR agonist resiquimod with platelet membranes, which yielded promising efficacy, especially in MC-38 tumor-bearing mice, where all tumors were eradicated even after two tumor rechallenges compared to the 28.6% survival rate of treatment with free and naked nanoparticle-encapsulated drugs [169]. This superior therapeutic efficacy was attributed to enhanced immune activation and increased T-cell infiltration of the tumor.

5.3.2. Combinatory platelet hybrid vesicle platforms

Platelets or platelet hybrid vesicles have been used in drug delivery systems to increase selective uptake by cancer cells. However, there are numerous strategies for further optimizing tumor infiltration, thereby increasing the therapeutic efficacy of platelet-inspired drug delivery. Hu et al. developed a double-hit relay system [131]. First, a nanogel encapsulating TNF-α, decorated with an Arg-Gly-Asp peptide, was administered to increase tumor vascular inflammation in breast cancer-bearing mice. Then, platelet-membrane-coated dextran nanocarriers loaded with paclitaxel were administered to induce apoptosis. Paclitaxel uptake and therapeutic efficacy considerably increased when tumor vascular inflammation was concomitantly induced. Multiple myeloma (MM) is a hematological malignancy usually confined to the BM. Thus, linking the bisphosphonate alendronate, which can bind to hydroxyapatite in bones, to platelet membrane-cloaked bortezomib-loaded nanocarriers increases tumor uptake and improves the survival of MM-bearing mice [170]. Zhou et al. used metal-organic nanostructures loaded with α-methyl-DL-tryptophan to block glutamine uptake in the breast cancer cell line ZR-75-1 [171]. After drug loading, the nanocarriers were coated with MnO_2 to prevent drug leakage and increase their glutathione scavenging properties, followed by loading into platelets. This combination resulted in glutamine deprivation and increased reactive oxygen species levels in cancer cells owing to reduced glutathione levels. Treatment was supplemented with ultrasonography of the tumors, causing the drug-loaded platelets to release the nanocarriers and to aggregate and form thrombi, yielding synergistic anticancer effects.

In addition, multiple studies have investigated combinations of cytotoxic drugs, death ligands, adhesion molecules, antibodies, and some variants of tumor disruption in either platelet or platelet hybrid vesicle-based drug delivery systems to optimize these platforms, as described in the previous sections and summarized in Figure 4.

6. Challenges and future perspectives

Using platelets for drug delivery faces several challenges. First, platelets must be loaded with drugs. Larger molecules that cannot cross the lipid bilayer plasma membrane, such as antibodies or complex receptor ligands, generally need to be attached to the plasma membrane, as described in the previous sections. However, some evidence indicates that platelets can internalize mAbs and release them upon activation [172]. Smaller molecules can permeate the platelet membrane if they are sufficiently lipophilic. DOX is a relatively small drug with a theoretical diameter of 1.5 nm [173], equal to the inner diameter of the large aquaporin 4 [174]. Thus, diffusion across channels and pores for drugs is not likely an important mechanism; however, this field has been poorly investigated. Consequently, the influx of hydrophilic drugs might require transporter proteins or endocytosis. Girardi et al. used a systematic solute carrier (SLC)-focused CRISPR/Cas9 library to screen the cellular uptake of 60 commonly used cytotoxic drugs and found that 47 were functionally dependent on one or more SLC transporters [175]. Nucleoside analogs such as decitabine and azacitidine are frequently used to treat myelodysplastic syndrome and AML. These hydrophilic drugs are transported by human equilibrative nucleoside transporters (hENTs) or human concentrative nucleoside transporters (hCNTs) [176–178]. Although hCNTs have not been identified in platelets, hENT1 (SLC29A1) has been [25]. hENT1 also is an important transporter for the nucleoside analog cytarabine, a mainstay chemotherapeutic agent in AML, and its expression levels correlate with the survival of patients with AML receiving intensive chemotherapy [179]. Various single-nucleotide polymorphisms in the gene encoding the hENT1 protein may also affect the treatment response [180]. Likewise, protein expression correlates with survival after treatment with gemcitabine, another nucleoside analog, in biliary tract and pancreatic cancers [181,182]. Moreover, recent research has identified another SLC-transporter, OCTN1 (SLC22A4), as a potent transporter for several nucleoside analogs, and its expression levels, which are dependent on DNA methylation [183], correlated independently with survival in patients with AML receiving intensive chemotherapy [184]. Numerous potential drug transporters exist within the SLC family of proteins, such as OCT1 and OCTN1, which are not found in

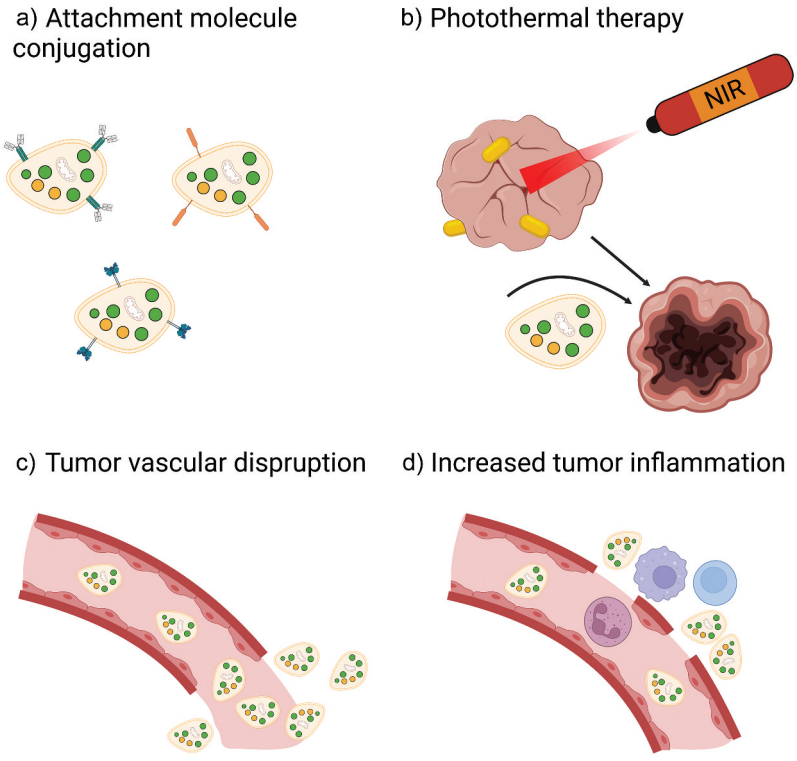

Figure 4. Strategies to optimize platelet-based drug-delivery systems. In order to enhance therapeutic efficacy, drug-loaded platelets, drug-bound platelets, or platelet hybrid vesicles can be conjugated with attachment molecules to increase retention in tumors a). Platelet infiltration of tumors may also be increased through combination with PTT b), tumor vascular-disrupting agents c), and tumor-inflammation increasing agents d). NIR, near infrared.

the platelet proteome [25,185]. Therefore, the ability of platelets to load hydrophilic drugs may be limited.

Although sonication, electroporation, and extrusion can facilitate drug loading of PEVs [157,158,166,168,186], these strategies may not be feasible for whole platelets. Using electroporation, Rao et al. loaded platelets with relatively large AuNSs, approximately 12 nm in diameter and 50 nm in length [137]. The electroporated platelets could hold the AuNSs for up to 48 h in a buffer; however, platelet function, viability, and circulation time were not tested. Although the non-immunological mechanisms for platelet clearance are not entirely understood, platelets are prone to apoptosis through the BCL2 antagonist/killer (BAK) and BCL2-associated X and apoptosis regulator (BAX)-dependent pathways [187]. Furthermore, loss-of-function mutations in B-cell lymphoma extra-large (BCL-X_L) or treatment with ABT-737, which antagonizes BCL-X_L, shortens the platelet half-life, and ABT-737 even causes acute thrombocytopenia [188]. Thus, the loading procedure should not affect the viability of platelets to function as a drug delivery system.

Platelets have scavenging properties as they can engulf large molecules such as fibrinogen, or even whole bacteria, which then localize in invaginations of the open canalicular system [134]. However, platelets can also phagocytose proteins, process them into peptides, and present them to naïve T cells via MHC-1 [87,189]. Although limited data support the scavenging of nanoparticles for drug-loading, MnO_2-coated nanocarriers with an average diameter of 146 nm could be loaded into unperturbed platelets, suggestively through endocytosis, but the exact mechanism was not investigated in detail [171].

Following drug loading or binding, platelets transfer the drugs to target cancer cells, which depends on the ability of platelets or shed PEVs to infiltrate the tumor microenvironment. After loading into platelets or platelet hybrid vesicles, different drugs are released into the cell culture medium or buffer over time, and this release depends on pH [124,126,131,161,170]. However, if a drug is only released passively in the plasma swiftly after infusion, drug carriers will have little specificity, meaning that this should preferably happen close to the target cells. Wu et al. suggested that cancer cell-derived EVs could activate platelets and stimulate the PEV shedding leading to drug release [126]. However, transfused platelets will regardless shed PEVs, but this will comprise only a small fraction of the circulating PEVs. Thus, they may be outcompeted by non-loaded PEVs *in vivo* unless this mainly occurs in the tumor microenvironment.

Furthermore, much of the present *in vivo* data on platelets as a drug delivery system are derived from solitary tumor models, quantitative analysis of small metastatic foci, or CTC models. If close contact is necessary for drug-loaded or drug-bound platelets to induce apoptosis, one therapeutic dose should preferably contain more than one platelet per cancer cell, as many transfused platelets will likely never encounter or connect with cancer cells. This may be an unrealistic number in cancer with disseminated disease. Thus, platelets may not be effective as a drug delivery system for treating cancer types with typically high tumor loads, such as leukemia and lymphoma. This obstacle may be overcome by using drug-loaded

PEVs or platelet-coated nanoparticles, which are infused in much larger numbers and thus have a greater chance of interconnecting with cancer cells. However, using drug-loaded PEVs or platelet-coated nanoparticles may raise safety concerns because very high numbers of transfused PEVs may theoretically be perilous, as they are generally believed to be thrombogenic [190]. Nonetheless, this function depends on the origin of PEVs [191]. In addition, the exact role of PEVs in hemostasis is controversial, as newer, more sensitive methods reveal a more complex contribution to hemostasis, as they also have fibrinolytic properties [34].

All platelet concentrates contain PEVs, and the number is affected by the production method, storage, and handling [192]. Thus, some amount of PEVs is safe for transfusion, but there is very little data on the infusion of large amounts in humans other than some small clinical trials from the 1990s where 'infusible platelet membranes,' i.e. PEVs, were tested as a substitute for platelet transfusion [193]. However, there is evidence suggesting that a high number of PEVs in platelet concentrates is associated with the risk of transfusion reactions [194]. Another aspect of whether PEVs are better suited for drug delivery than whole platelets is their circulation time. Transfused murine PEVs bind almost completely to blood cells or extravasate within minutes [38]. A study on platelet transfusion in patients with different hematological diseases showed that the half-life of transfused PEVs from one apheresis-derived platelet concentrate was 5.3 h – considerably shorter than the platelet half-life of 24 h [195]. Thus, platelets may have critical advantages over PEVs as drug vectors, including beneficial clearance kinetics.

There is also a question regarding platelet preparation. The reviewed papers calculated the efficiency of drug loading or binding using different techniques, including liquid chromatography, mass spectrometry, fluorescence spectrophotometry, and enzyme-linked immunosorbent assay [125,126,135,149]. The amount of drug per platelet can then be calculated, and individual treatment doses with the correct number of platelets equivalent to the desired concentration of free drug can be prepared by washing the platelets and resuspending them in a buffer or plasma before treatment. This method can be referred to as the 'wash' strategy (Figure 5). However, as every product in clinical use needs to be quality controlled, post-quantitation handling and subsequent storage could potentially decay the product, as washing will lead to platelet loss and potentially platelet activation with subsequent drug release. In contrast, platelets will be transfused directly after incubation when implementing a 'no wash' strategy. Thus, doses are not calculated by the platelet number but by the drug concentration and suspension volume. Excellent encapsulation efficiency of 86.6% for DOX at 100 μM has been reported [124], meaning only a small fraction of the suspension will be free drug if adapting a 'no wash' strategy. This method simplifies the workflow, but it may not be feasible for drug-bound platelets or for loading hydrophilic drugs, which may yield lower encapsulation efficiency.

Generally, strategies using platelets for drug delivery presuppose tumor infiltration, although circulating platelets loaded or bound to cytotoxic compounds will in most cases also target CTCs as long as normal platelet functionality is preserved. The only exceptions will be the strategies that depend on the tumor microenvironment for drug release or killing. However, there are strategies for selectively targeting of CTCs, which include platelet [196] and platelet hybrid vesicle decoys [197]. These decoys compete with normal platelets for CTC binding. Otherwise, they have no platelet functionality that may aid immune evasion or CTC extravasation. As there are no cytotoxic elements, these strategies may have good tolerability; however, more studies are necessary

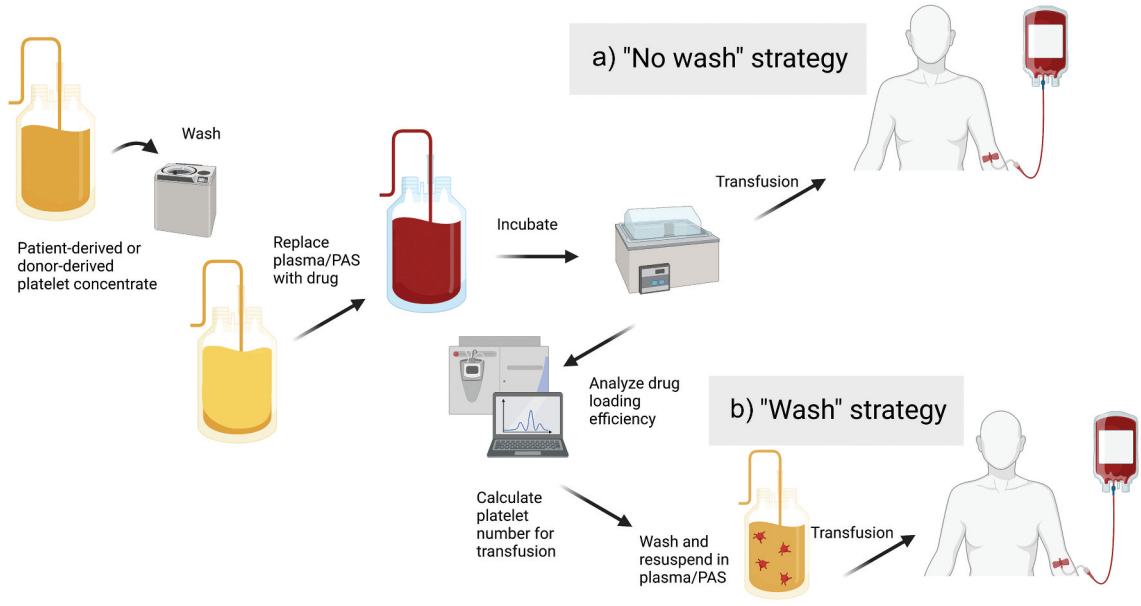

Figure 5. Clinical implementation of platelet-based drug-delivery systems. Platelets may be harvested from the patients themselves by thrombapheresis or supplied from blood bank storage. Following washing and resuspension in drug solution for loading or binding of drug, platelets are incubated and either transfused immediately a) or after washing and quantitation to calculate equivalent doses of free drug b). PAS, platelet additive solution.

to assess their efficacy, which will be limited to metastatic potential.

7. Conclusion

The interplay between platelets and cancer cells is essential in cancer biology. Thus, using platelets or other platelet-engineered hybrid technologies as drug delivery systems is an increasingly interesting strategy to improve drug efficacy and reduce harmful adverse events in cancer. Preclinical data are promising, and this technology may provide therapeutic benefits through selective targeting of cancer cells, improved cancer cell drug uptake, and slowed drug elimination. Platelet concentrates are also readily available from blood banks, relatively cheap to manufacture, and safe for transfusion. Thus, platelets have many advantages as drug delivery systems over synthetic drug carriers. However, unlike synthetic formulations, there is no clinical data on platelet- or platelet-inspired drug delivery systems in humans. Thus, the clinical relevance of this technology remains to be determined.

8. Expert opinion

Our understanding of platelet function beyond its role in hemostasis, especially in cancer biology, has changed profoundly in recent years. This knowledge has inspired platelet-based drug delivery systems to use the exceptional features of platelets to target the tumor microenvironment. The drug delivery systems reviewed in this paper represent some of the great potentials of this platform. Although clinical data in humans are lacking, plenty of studies using animal models show good tolerability and have generally implemented well-proven and safe drugs such as DOX or other cytotoxic compounds. Thus, clinical trials in humans will likely follow, and there is reason to believe that using platelets as drug vectors will improve the therapeutic efficacy of existing therapies for many cancers, possibly with only minor additional financial costs. Although this may be limited to cancer types with a high degree of platelet-cancer interaction, using platelet-based drug delivery systems are promising for prevalent cancer types such as breast, pancreatic, and colorectal cancer. However, as with any novel treatment, the initial clinical trials in humans will likely include heavily treated patients with refractory diseases. In this context, profound drug resistance may mask the primary benefits of using a platelet-based drug delivery system, i.e. selective targeting of cancer cells and optimized pharmacokinetics. Therefore, further development of more complex platelet-based drug delivery systems using a combination of drugs, strategies to improve platelet infiltration, and immunotherapeutic components for optimum anticancer efficacy are required.

This combined approach shows promise in preclinical trials, which underlines the greatest of a number of advantages of platelet-based drug delivery systems over synthetic nanocarriers, namely the dynamic properties of platelets. Not only can platelets be modified, but they can also respond to modifications in the tumor microenvironment, substantially increasing the complexity and potential of the treatment platform. Thus, although multiple studies using platelet-based drug delivery systems have been published recently, the optimal platform is probably not yet developed.

Drug modifications of platelets in platelet concentrates will likely not be performed by blood bank services, at least not more complex modifications such as the conjugation of different molecules to the plasma membrane or manufacturing of platelet hybrid vesicles. Clinical studies, and eventually clinical implementation, will require the availability of advanced good manufacturing practice (GMP) cell-therapy laboratories, which may be challenging in the near future, because this capacity is generally limited. However, these services are likely to become more available with the emergence of advanced cellular therapies, such as CAR-T cells, dendritic cell cancer vaccines, and tissue engineering techniques.

Medical cancer treatment generally requires multiple treatments over extended periods, regardless if the aim is disease stabilization or curation. Thus, treatment centralization is generally limited to rare diseases or complex protocols such as allogeneic hematopoietic stem cell transplantation. In a scenario with insufficient accessibility to GMP cell therapy laboratories and therefore the need for centralized manufacturing, some technical obstacles must be overcome for widespread application. The evidence for the release of drugs from drug-loaded platelets suggests that storage longer than a couple of hours is not advisable, at least for lipophilic drugs. However, loading of hydrophilic drugs and especially the use of membrane-conjugated cytotoxic compounds, may theoretically yield better stability. Still, this may render centralized manufacturing and treatment at local hospitals impossible in many settings owing to logistical challenges. In contrast, as described earlier, there is some evidence for making 'off-the-shelf products' of platelet-based drug delivery systems through cryopreservation, meaning production facilities could still be localized far from the patient. However, cryopreservation may introduce challenges with altered platelet functionality [198]; therefore, this remains to be studied in more detail in the setting of platelets as drug carriers.

Another strategy for developing platelet-inspired drug delivery systems and circumventing storage and improper drug loading issues is to use platelet-targeting nanoparticles, which may have superior *ex vivo* stability. Platelets are then 'loaded' or drug bound *in vivo* before infiltration of the tumor microenvironment, as described in the previous sections [142,144–147]. Although this confers several benefits, including selective nanoparticle cancer cell targeting, it will increase complexity and thus financial cost and may not elude the issues with the immunogenicity of synthetic drug carriers.

In a scenario where the necessary cell therapy facilities are readily available, apheresis-derived autologous platelets will be the likely source for platelet-based drug delivery systems. Subsequently, harvesting, manufacturing, and treatment can be performed in local hospitals without draining blood donor resources. This treatment strategy may be feasible for most cancer patients because medical cancer treatment is generally not commenced with concomitant thrombocytopenia, which impedes platelet harvesting. However, some patients with hematological

malignancies may require treatment despite of being thrombocytopenic, and thus there is need for allogenic platelets for manufacturing of the drug delivery systems. Alloimmunization is not uncommon in multi-line treated hematological patients, who generally have received numerous platelet transfusions, and accordingly will require the use of human leukocyte antigen-matched single-donor apheresis-derived platelets. Otherwise, the more available pooled buffy coat-derived platelet concentrates may be supplied from blood banks.

In conclusion, platelet-based drug delivery systems will likely represent a future treatment platform in hematology and oncology, which are in the later stages of preclinical testing and thus may be available for patients within the next decade. This treatment strategy can potentially improve the survival of patients with various cancers without developing new and expensive drugs. However, there still are technical and logistical challenges that has to be overcome before platelet-based drug delivery systems, if proven to be an effective platform in human clinical trials, can be available for the general population.

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