



REVIEW ARTICLE

Nanomedicine-mediated alteration of the pharmacokinetic profile of small molecule cancer immunotherapeutics

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The advent of immunotherapy is a game changer in cancer therapy with monoclonal antibody- and T cell-based therapeutics being the current flagships. Small molecule immunotherapeutics might offer advantages over the biological drugs in terms of complexity, tissue penetration, manufacturing cost, stability, and shelf life. However, small molecule drugs are prone to rapid systemic distribution, which might induce severe off-target side effects. Nanotechnology could aid in the formulation of the drug molecules to improve their delivery to specific immune cell subsets. In this review we summarize the current efforts in changing the pharmacokinetic profile of small molecule immunotherapeutics with a strong focus on Toll-like receptor agonists. In addition, we give our vision on limitations and future pathways in the route of nanomedicine to the clinical practice.

Keywords: cancer immunotherapy; small molecule drugs; pharmacokinetic profile; nanomedicine; toll-like receptors; stimulator of interferon genes (STING)

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INTRODUCTION

Among anticancer therapeutics, immunotherapy is the rising star dominating the current research field. Multiple immune checkpoint blockade (ICB) antibodies, chimeric antigen receptor (CAR)-T cells and other immuno-oncology drugs have received approval, and even more are in the pipeline or being investigated in clinical trials against different types of cancer [1]. Despite all the successes, some drawbacks or limitations have emerged over time. These limitations can be related to low response rates in a substantial fraction of patients or in certain cancer types and to severe immune-related adverse events [2, 3]. Moreover, the majority of these treatments are antibody or cell-based therapies and come with an immense financial burden, as high as \$500,000 for CAR-T cell therapy. To relieve some financial pressure, current therapies can be improved or side effects can be eliminated through the development of advanced nanotechnology-based delivery systems. Antibodies and nanobodies are expensive and sensitive, making chemical modification cumbersome. An interesting alternative would be the use of small molecule ligands, as they are generally easier and less expensive to produce and more robust to chemical modification or conjugation. Furthermore, they do not face limited tumor penetration, as is the case for high molar mass or very high affinity antibodies. Small molecules are less likely to induce an immune response against the injected compound, as can be the case for the Fc part in antibodies [4–6].

However, for many applications, small molecule agents do not possess the most desirable pharmacokinetic profile. Upon injection into the body, a fast systemic distribution is often observed together with rapid clearance from the body, resulting in the loss of useful compound at the target site. For immunomodulating compounds, systemic exposure can give rise to severe

immune-related adverse events, such as cytokine release syndrome and autoreactive T cells [7]. Different delivery-based strategies originating from the chemotherapy field can be applied to alter the pharmacokinetic profile of small molecule immuno-oncology agents. These include the use of nanoscale or macroscale delivery systems, conjugation to macromolecules such as polymers or proteins and lipidation [8, 9]. A multitude of factors can be engineered based on a desired pharmacokinetic profile. A macromolecular or nanoscale carrier device can aid in the solubilization of often poorly water-soluble compounds, yielding a stable dispersion in aqueous medium. Tailoring of the surface chemistry allows for enhanced circulation time by evading opsonization, for example, by PEG-ylation [10]. Modifications in the surface properties can also be applied to modulate biodistribution, enabling preferential accumulation in a specific organ or cell type. In a nonspecific way, these strategies can lead to drug delivery to immune cells and within the tumor microenvironment. Alterations in size and shape allow for enhanced uptake by phagocytotic immune cells [11]. The combination of a highly permeable vasculature and decreased lymphatic drainage of tumors leads to a passive accumulation of macromolecules inside the tumor environment, a phenomenon known as the enhanced permeability and retention (EPR) effect [12, 13]. Alternatively, decorating the surface with targeting moieties allows for specific cellular delivery. The release profile can be further controlled by using stimuli-responsive nanocarriers or linker chemistries, thus allowing for environment-specific release [14–17]. For intratumoral or peritumoral injections of immunotherapies, nanoparticles can aid in the retention of drugs at the tumor site and ensure efficient transport to the draining lymph nodes, thereby minimizing systemic toxicity and increasing activity in the tumor region [18, 19].

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We will focus here on the application of these delivery tools to alter the pharmacokinetic profile specific for small molecule immunomodulating compounds. For a more general overview of the use of nanomedicine [20–22], polymer conjugates [23–25] and antibody-drug conjugates [26, 27], readers are directed to the respective reviews. We will provide a summarized overview of recent developments and preferential delivery techniques for agonists of Toll-like receptors (TLRs) as some examples of small molecule immunomodulators. In addition, we provide what we consider limitations and future paths for research leading nanomedicine to clinical practice.

PATTERN RECOGNITION RECEPTOR AGONISTS

Next to cytokines, the most important signal for potent activation of innate immune cells is pathogen-associated molecular patterns that bind to pattern recognition receptors. These include TLRs, intracellular sensors of nucleic acids such as the stimulator of interferon genes (STING) and C-type lectin receptors, among others [28]. TLRs are generally expressed by immune cells. Antigen-presenting cells (APCs), such as B cells, DCs and macrophages, express all TLRs with a higher prevalence than non-APCs depending on the cell type. TLRs are also expressed by T cells, although mixed information is reported on which TLRs are generally expressed [29–31]. In addition to immune cells, TLRs can be found on cells that make up physical barriers against pathogenic intruders, such as some endothelial and epithelial cells. Finally, multiple tumor cells also express TLRs [32].

An overview of currently known agonists or each TLR is provided in Table 1, together with the clinical status and the type of formulation that reached clinical phase testing. TLR agonists are mostly employed as vaccine adjuvants because of their stimulating effect on APCs in lymphoid organs. Receptor–ligand interaction results in a cascade of pro-inflammatory responses, finally leading to an improved immune response with augmented antibody titers and increased antigen-specific cellular immunity. The discussion will be expanded here with the application of TLR agonists as immune-modulating compounds directed to the tumor microenvironment and draining lymph nodes. Due to the close relation between cancer development and inflammation, stimulation of TLR receptors can have both positive and detrimental outcomes on tumor progression. Tumor-promoting immune effects have been mainly linked to TLR2 and TLR4 activation [33–35]. Furthermore, the expression of TLRs on tumor cells has to be taken into consideration when activation might lead to protumoral effects [4, 36, 37]. For example, TLR7 stimulation in primary lung carcinoma could promote tumor growth through the recruitment of myeloid-derived suppressor cells [32, 38, 39].

TLR2

The most studied ligands for TLR2 are diacetylated or triacetylated lipopeptides and their synthetic analogs. The two or three palmitic acid groups are located at the N-terminal cysteine residue of the short SK₄ peptide motif, Pam_{2/3}CSK₄, which enhances the solubility and adjuvant potency (Fig. 1). The presence of either two or three palmitoyl chains determines the dimerization of TLR2 with TLR6 or TLR1, respectively. Intraperitoneal or intravenous (i.v.) injection of soluble Pam₃CSK₄ in mice results in a fast systemic release of pro-inflammatory cytokines and nitroxide, leading to a multitude of adverse events [40, 41]. In addition, the hydrophobic nature of triacetylated lipopeptides limits their practical applicability due to solubility issues. Chua et al. prepared a stable aqueous formulation by complexing branched Pam₂CSK to an antigen protein by electrostatic interactions [42]. In a subsequent study, they further improved the system by using a PEG conjugate of the TLR2 agonist (R₄Pam₂Cys-PEG₁₁). The presence of hydrophilic PEG chains

increased solubility, leading to smaller complexes ranging from the micrometer range to 400 nm (Fig. 2a). They attributed the improved CD8⁺ T-cell responses to altered lymphatic trafficking (Fig. 2b). After injection, microparticles are transported to the draining lymph nodes by macrophages and DCs, while nanosized particles migrate with the lymphatic flow to reach immune cells in the lymph nodes within hours after injection [43]. Alternative routes toward solubilization of lipopeptides can be achieved by incorporation into PLGA nanoparticles or covalent conjugation to a protein antigen [44, 45].

Multiple groups have reported on the design of TLR2-peptide conjugates by linking an antigenic synthetic long peptide to Pam₃CSK₄ [46–49] or the derivative Amplivant [50], where the N-terminal amide is replaced with a urea linkage. Interestingly, the uptake mechanisms is not mediated by the TLR2 agonist-receptor interaction but occurs via clathrin-dependent endocytosis [51], a typical uptake pathway for particles from 10 to 300 nm [52]. An important consideration here is the contribution of the hydrophobic lipid chains, as they might induce self-assembly into nanoparticles or interaction with serum proteins, leading to increased cellular uptake and lymphatic drainage. In all cases, greatly increased lymphatic trafficking and cellular uptake were observed for the antigen-adjuvant conjugate compared with the antigen alone, resulting in increased activation of DCs capable of priming antigen-specific CD8⁺ and CD4⁺ T cells.

Macrophage-activating lipopeptide (MALP-2), a ligand for TLR2/6, was evaluated in a phase I/II clinical trial in 2007, but no follow-up studies have been reported [53]. Interestingly, Castelletto et al. studied the self-assembly behavior of MALP-2 and found that MALP-2 self-assembled into a β -sheet-based bilayer at a concentration above 20 μ g/mL [54]. Although aggregation into macromolecular structures would aid in macrophage uptake, it is not certain that this would be the case in vitro/in vivo, as measurements were performed in water, and the aggregation concentration is well above typical concentrations used for in vitro stimulation [55].

Another lipid agonist developed by Pizzuto et al. has TLR2 and/or TLR4 agonist behavior. They synthesized cationic lipopolyamines that were able to self-assemble into cationic liposomes, strongly aiding in their lymphatic transport, cellular uptake and potential antigen codelivery to APCs in the draining lymph nodes [56].

In addition to these lipidic TLR2 agonists, some novel non-lipidic agonists have also been discovered. A high-throughput screen by Guan et al. led to the discovery of five new compounds with TLR2 agonistic properties [57]. To date, their use has been limited. The Boger laboratory recently discovered diprovocims as an interesting and very potent new class of TLR2 agonists [58]. Their lead molecule discovery was based on receptor dimerization, and subsequent structure-activity optimization resulted in compounds with agonistic activity at concentrations as low as 110 pM. The diprovocim is a distinct class of molecules sharing no structural similarity with Pam₃CSK₄ or any other TLR agonist. Although their most potent compound, diprovocim-1 (Fig. 1), does not allow for easy conjugation, the introduction of more reactive chemical moieties is possible, albeit with a small loss in potency. Evaluation of the adjuvant properties in vitro and in vivo yielded promising results, encompassing a strong boost in secretion of pro-inflammatory cytokines and inducing a strong TLR2-dependent humoral and cellular (CD4⁺ and CD8⁺ T cells) immune response. In melanoma-bearing mice, tumor growth could be inhibited with anti-PD-L1 checkpoint inhibition [59]. Of note, due to the hydrophobic nature of the compound, aqueous solubility is limited, and a mixture containing DMSO:Tween 80:saline was needed to solubilize the drug prior to administration. Hydrophobic encapsulation into nanoparticles might both offer a solubilization platform and enhance delivery to local APC populations.

Table 1. Overview of TLRs and some example of their respective agonists

TLR	Agonist	Example	Formulation	Clinical status	Ref.
TLR1/2	Lipids, lipopeptides	Pam ₃ CSK ₄		/	NCT02821494
		Amplivant®	Peptide conjugate	Phase 1	Starting
TLR2/6	Lipids, lipopeptides	Pam ₂ CSK ₄	Solution	Phase 1	Complete [161]
		MALP-2	Peptide conjugate	Phase 1/2	Complete [53]
TLR2	lipophilic	Cationic lipopolyamines	Liposomes	/	[56]
	Non-lipophilic	CU-T12-9		/	[57, 162]
TLR3	dsRNA	Diprovocims	/	/	[58, 59]
	Small molecule	Poly I:C/Poly ICLC		Phase 1/2	[60, 97]
TLR4	Lipopolysaccharides	LPS, MPLA, GLA	AS04	Approved	
		Pyrimido-[5,4- <i>b</i>]indoles			[108]
		Ugi compounds			[83]
		Neoseptins			[164, 165]
	Small molecules	Amphotericin B, nystatin	Liposomes	Approved for other indications	[84, 85]
		Euodenine A			[166]
		Paclitaxel, docetaxel	Nanoparticles, Liposomes	Approved for other indications	[90, 91]
Nanostructures	Self-assembling peptide (TR-433)			[79]	
TLR5	Bacterial	Flagellin (Entolimod)		Phase 1	Complete [167]
TLR7/8	Nucleic acids	ssRNA			
	Imidazoquinolines	Imiquimod, Resiquimod, "IMDQ"	Topical cream	Approved	[98, 113, 114]
	Guanosine analogs	TOG, loxoribine			[168]
	Adenine analogs	UC-1V150			[106, 107]
TLR9	ssDNA	CpG SD-101		Phase 1/2	Ongoing

TLR3
The endosomal receptor TLR3 is triggered by the negatively charged backbone of double-stranded RNA. The most studied agonist is poly(inosinic)-poly(cytidylic acid) (poly I:C). The *in vivo* use of poly I:C is limited due to severe toxicity and rapid enzymatic degradation, emphasizing the need for a carrier device. Most currently ongoing clinical trials make use of Hiltonol® as a TLR3 agonist [60]. Hiltonol, poly-ICLC, is a derivative of poly I:C stabilized with poly(lysine) with increased resistance against degradation and reduced toxicity. Multiple systems primarily making use of charge interactions between negatively charged poly I:C and positively charged polymers or lipid-forming polyplexes of liposomes are reported in the literature [61]. We will not discuss this in detail, as this review is focused on the delivery of small molecule ligands. The development of small molecule alternatives such as CU-CPT17e has seen limited success, and CU-CPT17e is the only known small molecule agonist. The low potency has limited enthusiasm so far.

TLR4
The TLR4 receptor is of particular interest due to its capability to signal via both the MyD88 and TRIF pathways, inducing pro-inflammatory cytokines and type I IFNs, respectively. TLR4 is primarily a cell surface TLR but is also found in endosomal compartments after endocytosis [62]. Lipopolysaccharide (LPS), the major component of the bacterial cell membrane, is a natural ligand for TLR4 and TLR2; however, due to toxicity, its use is restricted in the clinic. In contrast, monophosphoryl lipid A (MPLA, Fig. 3), the detoxified derivative of LPS, has been widely applied in clinical trials and is a component of the FDA-approved Adjuvant System 04 (AS04) together with alum [60, 63].

MPLA remains the most popular TLR4 adjuvant in preclinical and clinical research. A large variety of nanotechnology-based delivery systems are employed to improve the (passive) targeting of APCs and limit unwanted systemic inflammation. Reports on hydrophobic encapsulation of MPLA into polymer nanocarriers are primarily based on poly(lactide-co-glycolide) acid (PLGA) [64–67], although other options have been explored [68]. Apart from polymers for hydrophobic encapsulation, liposomal formulations make up the majority of reported nanocarriers [69–73]. One example is the glycoliposomes containing MPLA designed by Bok et al. They encapsulated MPLA into liposomes containing the glycan Lewis(Le)^X (MPLA-Le^X), which is a binding ligand for the C-type lectin receptor DC-SIGN expressed by DCs. They showed that Le^X modification resulted in preferential uptake by DCs over macrophages (Fig. 4a). Furthermore, targeting DC-SIGN resulted in particle endocytosis, which was not the case for nontargeted liposomes (Fig. 4b). When comparing the resulting CD8⁺ T-cell responses, MPLA liposomes outperformed soluble MPLA, but the response was further improved by DC-SIGN-targeted liposomes (Fig. 4c) [74]. In a different strategy, conjugation of MPLA to OVA aided solubilization and resulted in 20 nm sized aggregates. Furthermore, the OVA-MPLA conjugate induced stronger immune activation than the unconjugated version [75]. Other derivatives have been developed, such as glucopyranosyl lipid adjuvant (GLA) and GSK1795091 [60]. Fox et al. have shown that formulation of GLA in an oil-in-water emulsion or in liposomes shifted the immune response from a T_H2-type response to a dominantly T_H1-biased response in the context of vaccination [76].

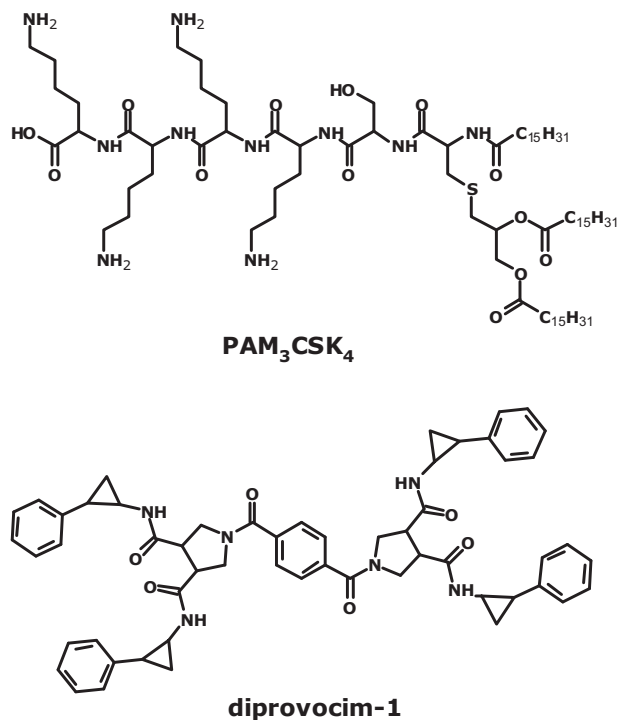


Fig. 1 Chemical structure of TLR2 agonists Pam₃CSK₄ and diprovocim-1

Nanoparticles are mainly presented here as a means to improve the delivery to immune cells and decrease systemic exposure of adjuvants. However, multiple examples exist of nanostructures being immune-stimulating on themselves, of which some of these work by triggering of TLR4 [77, 78]. An example of these nanostructures with TLR4 agonist properties is the self-assembling peptide developed by Tandon et al. [79]. Although more complex in synthesis than small molecules, these nanostructures do bear the advantage of having strongly improved targeting of APCs without the need for additional formulation methods.

Over the past decade, research toward alternative small molecule TLR4 agonists has resulted in the discovery of pyrimido-[5,4-*b*]indoles, Ugi compounds, neoseptins, euodenine A, amphotericin B and paclitaxel (Fig. 2). A high-throughput screen by Chan et al. resulted in the discovery of pyrimido-[5,4-*b*]indoles as selective TLR4 agonists [80]. A subsequent structure-activity relationship study *in vivo* linked agonist potency to produced cytokine levels in sera. These experiments point toward systemic immune activation, thus providing preliminary evidence for an advanced delivery platform to avoid systemic exposure upon *i.v.* injection [81, 82]. The “Ugi compounds” were only recently discovered by Marshall et al. from a high-throughput screen against human TLR4 [83]. Their naming was derived from the used Ugi coupling reactions, which allowed for easy variation of their structure. Lead optimization was performed to increase potency and hydrophilicity, yielding AZ617 with activity in the nanomolar range. Although its hydrophilicity makes it favorable for administration, its high potency increases the risk for immune-related toxicity upon systemic exposure, again pointing toward the need for a delivery system. The evaluated compounds strongly increased the secretion of pro-inflammatory cytokines but provoked only limited secretion of type I IFNs. This would favor their use as vaccine adjuvants over tumor-modulating immunotherapies.

In addition, a library screen performed by the Sunil David laboratory suggested amphotericin B (ampB) as a TLR4 agonist. To

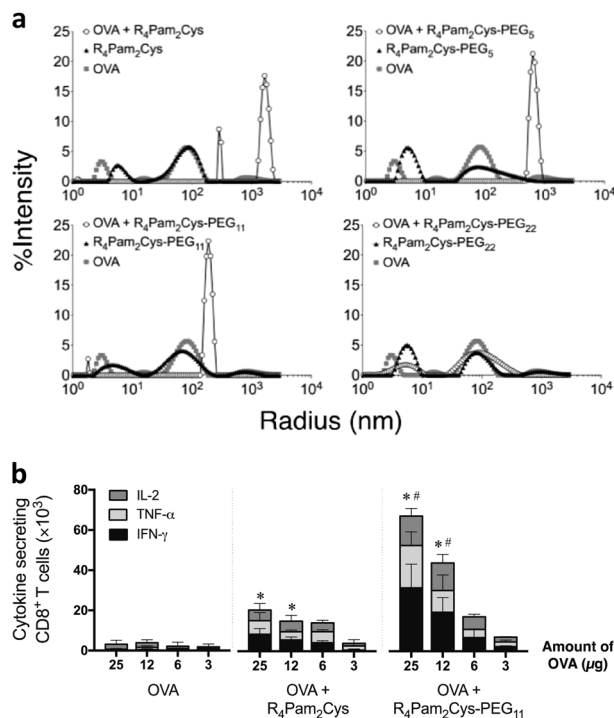


Fig. 2 Protein antigen complex of OVA with branched TLR2 agonist R₄Pam₂Cys-PEG₁₁. **a** Size distribution profiles of electrostatic complexation of OVA with different pegylated agonists. Increasing PEG length helped in solubilization and decreased particle size. **b** CD8⁺ T cell responses were measured by inoculating C57BL/6 mice (*n* = 3 per group) with OVA alone or OVA formulated with lipopeptide at 1:3 molar ratio. Splensens were obtained 10 days later and OVA257-264-specific IFN-γ, TNF-α, and IL-2 secreting CD8⁺ T cells enumerated by ICS assay. Each bar graph depicts the total number (±SD) of CD8⁺ T cells secreting each cytokine. Reproduced from reference [43]. Reprinted with permission from Elsevier

date, experimental data on ampB in a vaccine adjuvant context are limited to reports published by the David group and our laboratories [84, 85]. Nevertheless, a vast amount of research has been done on formulating ampB due to its notoriously high toxicity and poor aqueous solubility [86]. This resulted in the approved formulations Fungizone and Ambisome. Ambisome is the preferred treatment against fungal infections in the clinic. The most relevant work on altering the toxic profile of ampB itself and simultaneously investigating potential conjugation sites was done by the Burke laboratory [87, 88]. Regarding nanomedicine-based solutions, Van Herck et al. developed an acid-degradable thermoresponsive block copolymer for the encapsulation of ampB via hydrophobic interactions. This polymer system was shown to be an excellent carrier for delivery of a hydrophobic payload to the endosomal compartments (Fig. 5a). Encapsulation of ampB in these polymer NPs reduced cellular toxicity and stimulated the antigen-specific immune response by localizing activity (Fig. 5b, c) [89]. The taxanes paclitaxel (PTX) and docetaxel are mostly known for their chemotherapeutic activity, but they are reported to have adjuvant potential through TLR4 stimulation [90, 91]. The vast amount of research on nanomedicine-based delivery of taxanes as chemotherapeutics is discussed in other reviews [92]. Thomas et al. incorporated PTX via hydrophobic encapsulation into pluronic NPs of ~30 nm [90]. In addition to solubilization, this allowed for localized delivery in the draining lymph node, leading to DC activation and decreased tumor growth, which were not observed with free PTX. Encapsulation can modulate localized delivery on organelle and intracellular level. In comparison to free drugs, which can be taken up via passive diffusion, particles are

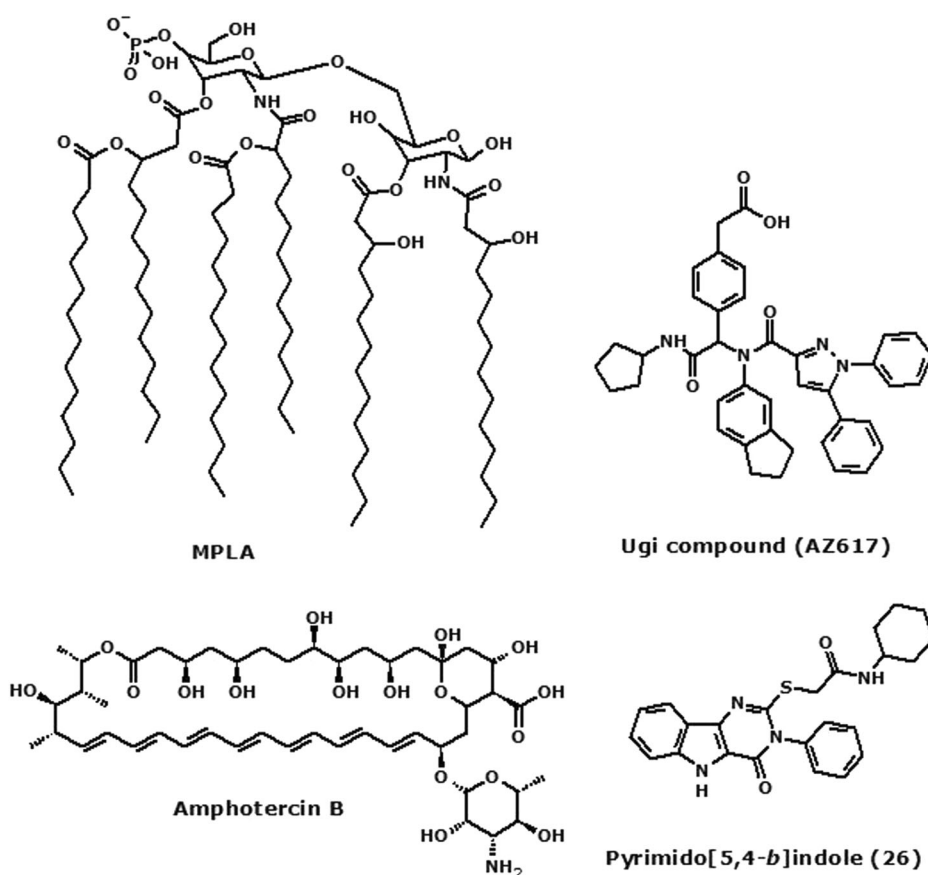


Fig. 3 TLR4 ligands MPLA, Ugi compound AZ617, pyrimido [5,4,b]indole 26 and amphotericin B

taken up via endocytic mechanisms leading to accumulation of agonists in lysosomes, where TLR4 is also located.

Not only do agonists offer a useful treatment modality, they have also been broadly explored as antagonizing ligands for TLRs, although less so in the setting of cancer therapy. Since TLR antagonists also modulate immune responses, it is equally important to control physiological exposure. The TLR4 antagonist curcumin is used to treat inflammatory bowel disease. Delivery difficulties involve limited solubility, poor aqueous stability and harmful systemic exposure. Kesharwani et al. complexed curcumin to a hydrophilic polymer, thereby aiding in aqueous solubility and stability. By using a pH-responsive polymer with a release profile at pH above 6.7, systemic release via the intestine was avoided, and delivery was localized to the colon. Additional charge interactions between the polymer and the site of inflammation further improved site-specific delivery [93].

TLR5

Due to successes with other TLR agonists and the lack of potent synthetic small molecule agonists for TLR5, the use of TLR5 as a vaccine adjuvant has been limited so far. Delivery strategies developed for TLR5 activation involve liposomal [94] or polymer [95]-based nanocarriers and microneedle [96] delivery. Interestingly, Faham et al. decorated the surface of liposomes with the TLR5 agonist flagellin [94]. In this case, flagellin aided in cellular delivery due to the promoted interaction with DCs. In addition, delivery of antigen and adjuvant was localized to the same cell, increasing the immune response.

TLR7/8

ssRNAs are natural ligands for TLR7/8 but are in research mostly replaced by one of multiple small molecule agonists, such as

adenine or guanosine analogs and imidazoquinolines. Of the imidazoquinolines, imiquimod has been approved by the FDA for the treatment of basal cell carcinoma by topical administration. TLR7/8 agonists exert their antitumour effect via recruitment of tumor-infiltrating plasmacytoid DCs and macrophages [97]. Next to imiquimod, the most studied agonist is resiquimod (R848, Fig. 6). Both agonists are used in clinical trials as vaccine adjuvants and as direct anticancer therapeutics, mostly in the setting of combination therapy [60]. The separate administration of adjuvant and antigen can be practically unattractive for vaccination, and alternative formulations are needed. A plethora of formulation methods to alter the pharmacokinetic profile of TLR7/8 agonists are being developed based on encapsulation or conjugation [98].

Kranz et al. combined the utility of RNA to serve as both an encoded antigen and agonist [99]. Delivery of naked RNA via i.v. injection is extremely inefficient due to rapid degradation by nucleases. In addition, the negative charge provokes repulsion at the cell membrane, resulting in poor uptake (Fig. 7). In contrast, a liposomal formulation RNA-LPX protected RNA from serum degradation and altered the pharmacokinetic profile. The biodistribution profile could be tuned to preferential accumulation in the spleen by alterations in the lipoplex composition. RNA-LPX accumulated in major lymphoid structures but was also found in lymph nodes as well as in bone marrow. In all organs, RNA-LPX was primarily found in DCs and macrophages.

Hydrophobic encapsulation into nanoparticles has proven to be a versatile formulation method. Formulation of resiquimod (R848) in PLGA nanoparticles has resulted in localized immune activation with minimal systemic cytokine production [100]. Kasturi et al. have shown a synergistic effect of combining MPLA and R837 in PLGA nanoparticles on antibody production [101]. Many more examples exist of encapsulating adenine and imidazoquinoline

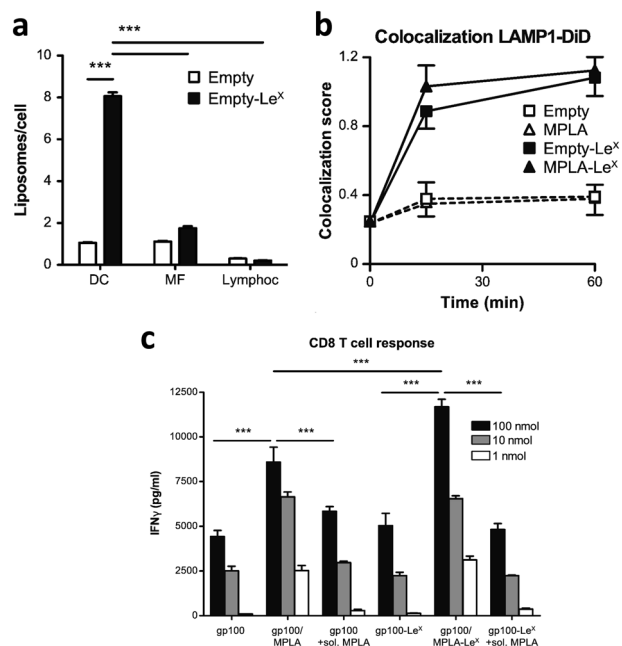


Fig. 4 Influence of Le^X modified liposomes on cellular uptake and MPLA delivery. **a** Preferential uptake of Le^X-liposomes by DCs in comparison to other cell lines. **b** Intracellular fate of liposomes. Colocalisation with lysosomal stain LAMP1-DiD indicated endocytosis. **c** Targeting DC with Lewis^X- and MPLA-modified liposomes enhances antigen presentation to CD8⁺ T cells. HLA-A2⁺ DC were exposed to various concentrations of non-modified or Le^X- and/or MPLA-modified liposomes, all loaded with gp100_{280–288} peptide, for 1 h, in the presence or absence of soluble MPLA. Data reproduced from reference [74]. Reprinted with permission from Elsevier

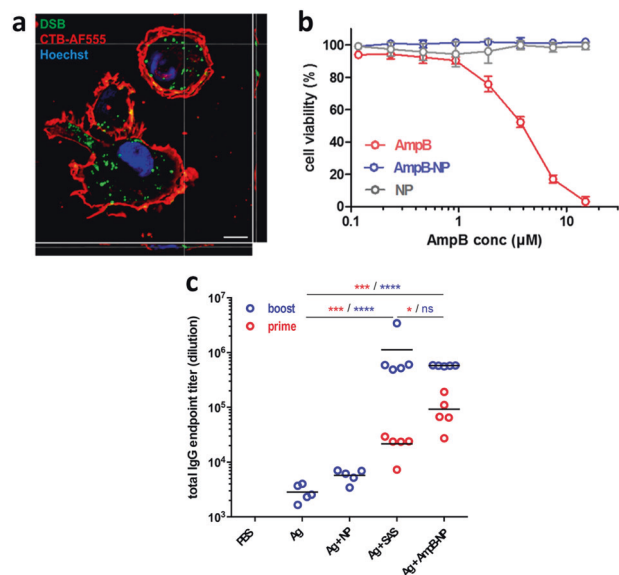


Fig. 5 **a** Intracellular destination of hydrophobic payload encapsulated in responsive NPs imaged by confocal microscopy. **b** Cytotoxicity analysis of free and encapsulated ampB incubated with RAW264.7 macrophages. Cell viability was analysed via MTT assay after 24 h incubation. **c** End-point titers after prime and boost immunization against SHe peptide. Reprinted with permission from ACS [89]

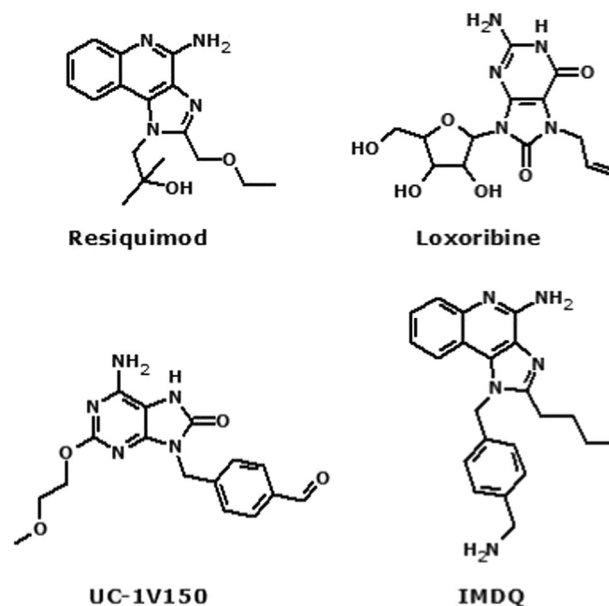


Fig. 6 TLR7/8 agonists resiquimod, loxoribine, UC-1V150, IMDQ

TLR agonists into nanocarriers [98, 102, 103]. Fewer studies have been performed on the delivery of guanosine analogs such as 7-thia-8-oxoguanosine (TOG) and loxoribine (Fig. 6), probably due to their lower potency compared with other TLR7/8 agonist groups. The *in vitro* immunostimulatory and cytotoxicity profiles of TOG were improved by conjugation to carbon nanotubes, and these effects were linked to a drastic increase in the intracellular concentrations achieved by nanocarrier delivery compared with soluble agonists [104]. In another construct the commercially available loxoribine was used as one arm of a TLR4/7/9 trivalent adjuvant system [105].

TLR7/8 derivatives based on the adenine analog SM360320 are highly potent agonists and hence are prone to increased side effects due to systemic production of cytokines and off-target inflammation. To limit this, derivatives were synthesized with an aldehyde (UC-1V150, Fig. 6) or carboxylic acid at the N1-benzyl position to allow for conjugation. The Carson laboratory conjugated UC-1V150 to mouse serum albumin, resulting in a staggered increase in potency *in vitro* and prolonged localized immune activation *in vivo* due to the altered circulation time for macromolecules compared with small molecules [106]. Conjugation to protein was further investigated by Gao et al., who conjugated UC-1V150 with varying N1-linker groups to different proteins and found that conjugation dramatically increased potency even to the extent that an inactive compound turned active upon conjugation [107]. The Carson laboratory simplified this concept by designing phospholipid conjugates (1V270) with or without PEG spacers. The phospholipid conjugate induced strong and prolonged IL-6 and type I IFN release; although the PEG-containing conjugate had decreased potency, its hydrophilicity and pharmacokinetic profile make it a potentially interesting construct [108]. Upon intratumoral injection of 1V270, the biodistribution is modulated by the phospholipid moiety, resulting in immune activation focused at the injection site and draining lymph node. This is demonstrated by the absence of systemic cytokine release seen with other agonists (Fig. 8a) [109]. After systemic administration of the phospholipid 1V270, the strongest immune activation was observed for DCs in the tumor and draining lymph nodes, resulting in a tumor-specific systemic immune response capable of eliminating tumor cells at distal

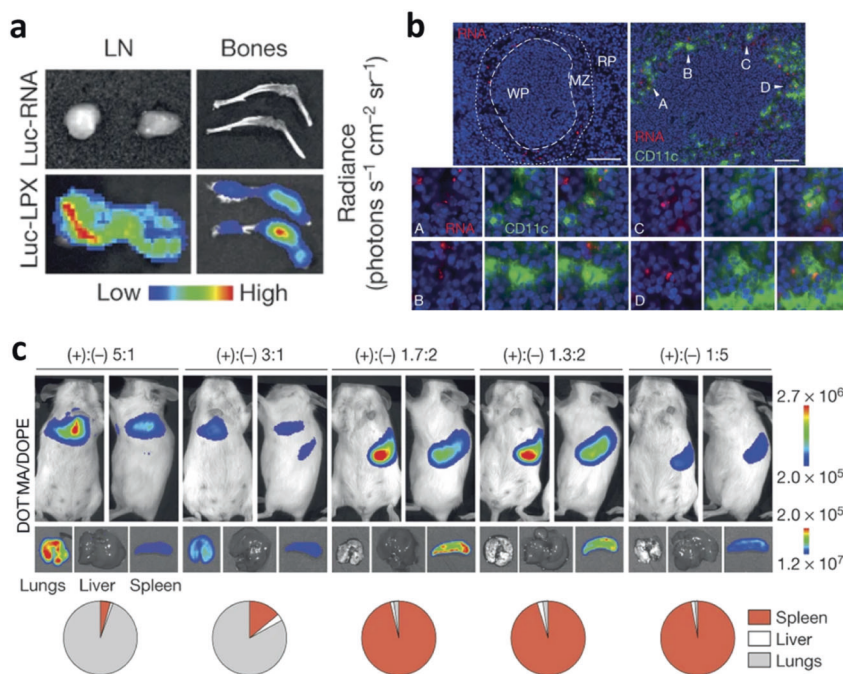


Fig. 7 **a** Bioluminescence imaging of inguinal lymph nodes (LN), femur, and tibia in BALB/c mice ($n = 3$) after i.v. injection of Luc-LPX or naked Luc-RNA. **b** Splenic localization of CD11c and Cy3 double-positive cells in BALB/c mice ($n = 2$) 1 h after i.v. injection of Cy3-labeled RNA-LPX. Scale bar, 100 μm . MZ marginal zone; RP red pulp; WP white pulp. **c** Bioluminescence imaging of BALB/c mice ($n = 3$) after i.v. injection of Luc-LPX at various charge ratios. Pie charts show relative contribution of each organ to total signal. Data reproduced from reference. Reprinted with permission from Springer Nature [99]

sites [39]. Furthermore, phospholipid 1V270 has been extensively incorporated in liposomes as a vaccine adjuvant [110, 111].

Altering the pharmacokinetic profile via incorporation of a lipid tail has also been explored for imidazoquinolines, with 3M-052 as an example. 3M-052 is a small molecule agonist modified with a C18 fatty acid tail that features a slow release from the injection site, thereby limiting fast systemic exposure. Novartis has used this concept on their first-generation TLR7 small molecule immune potentiators (SMIPs), benzonaphthyridines. These lipophilic TLR7-SMIPs had a favorable distribution profile, with limited systemic exposure and excellent adjuvant potency (Fig. 8b). Formulation and manufacturing hurdles resulted in the second generation of more soluble SMIPs that were absorbed onto Al(OH)₃ to have the best characteristics of both generations of SMIPs [112].

The De Geest laboratory covalently linked the TLR7/8 agonist IMDQ (Fig. 6) to acid-degradable nanocarriers, thereby modulating the biodistribution of the agonist from systemic to the lymph nodes (Fig. 9a) [113, 114]. They showed that by conjugation of the agonist to a hydrophilic polymer systemic exposure was avoided and activation focused to the injection site, while conjugation to a nanoparticle forming polymer gave an efficient delivery to the draining lymph nodes. A similar effect was achieved using an amphiphilic cholesterol-polymer-agonist conjugate. The enhanced lymphatic trafficking was affected by the serum-binding properties of the lipid motif and subsequent lymphatic drainage of serum proteins [115]. The formulation admixed with an RSV antigen induced strongly increased IgG2a titers compared with free IMDQ and a control adjuvant, the sigma adjuvant system (SAS, containing MPLA). This indicates an improved delivery and a shift toward a T_H1-biased immune response [116]. In addition, upon intratumoral injection of the IMDQ-containing nanogels, immune activation was restricted to the tumor site and the draining lymph nodes, limiting systemic exposure (Fig. 9b). The combined administration of anti-PD-L1 checkpoint blockade and Flt3L, a ligand that expands and mobilizes DCs from the bone marrow, improved the therapeutic outcome and inhibited further

tumor growth [117]. In the context of immunotherapy, Rodell et al. have shown that targeting of a TLR7/8 agonist, R848, containing cyclodextrin nanoparticles to tumor-associated macrophages can transform these cells from an immune-suppressive M2-like phenotype to an antitumorigenic M1-like phenotype. Again, combination with ICBs further improved the therapeutic outcome [118, 119].

TLR9

The TLR9 agonist CpG-oligodeoxynucleotides (CpG-ODNs) are potent inducers of type I IFN secretion and are widely applied as vaccine adjuvants as well as for tumor immunomodulating monotherapy in both preclinical development and clinical trials [97]. CpG-ODNs are known to induce systemic inflammation-related side effects upon intratumoral injection [109]. For this reason, advanced delivery platforms are required in the context of anticancer vaccination to ensure localized delivery to the APCs in draining lymph nodes. Nuhn et al. employed a negative charge to formulate CpG-ODNs into cationic hydrogel particles together with tumor antigen epitopes, resulting in a strongly increased immune response [120]. Formulation is also possible in cationic liposomes [121–123], virus-like particles [124] and other nanoparticles [125, 126]. Furthermore, CpG-ODNs have been conjugated to peptides [51] or lipids for improved delivery.

The Irvine group made use of a concept referred to as ‘albumin hitchhiking’ to alter the pharmacokinetic profile of CpG dinucleotides. Lipid motifs can bind in the hydrophobic pockets of serum proteins, which aid in circulation time and lymphatic trafficking. They evaluated a series of lipid motifs and found a positive relationship between albumin binding ability and lymph node accumulation, with the best results for a dialkyl lipid, providing evidence for the suggested ‘album hitchhiking’ mechanism (Fig. 10) [127].

Direct triggering of TLR9 in the tumor microenvironment can elicit both protumoral and antitumoral effects. These effects can be manifested via a multitude of pathways signaling through the activation of TLR9 on tumor cells or the triggering of immune cells

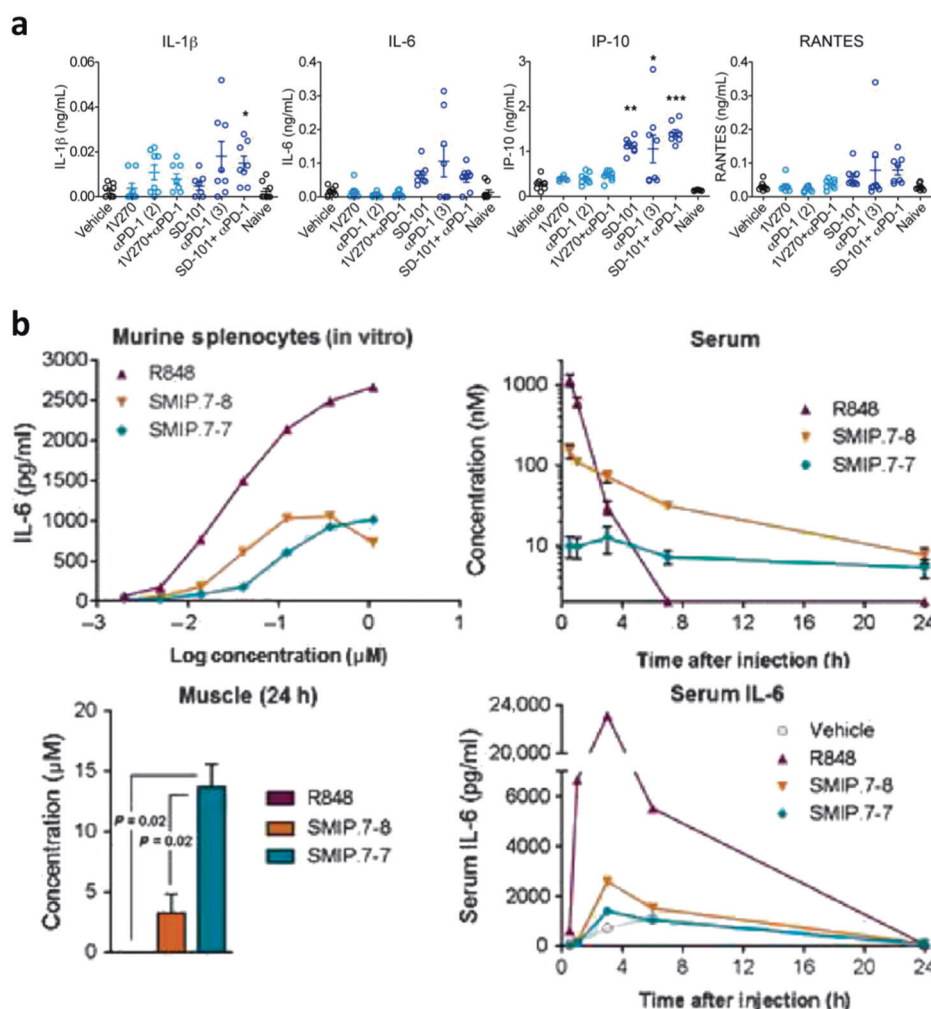


Fig. 8 **a** Systemic cytokines after intratumoral injection of 1V270 [109]. **b** Assessment of pharmacokinetic properties of SMIPs after intramuscular injection. Reprinted with permission from The American Society for Clinical Investigation

in the tumor microenvironment. For a detailed discussion on this, we direct the reader to the following reviews [32, 37]. Delivery of CpG-ODNs to the tumor site is primarily performed by intratumoral injection. CpG-ODNs used for an in situ vaccination approach can be improved by using a delivery vehicle that restricts distribution to the tumor site and draining lymph node after injection. This can be achieved by using a conjugation or nanoparticle approach [128, 129]. Furthermore, the therapeutic efficacy of the vaccination is improved by combination with ICB or radiotherapy [130, 131]. In addition to improving lymphatic drainage, amphiphile-CpG conjugates can be applied for increased tumor targeting, again via albumin trafficking. Appelbe et al. were able to induce a potent cellular immune response via an in situ vaccination using these amphiphile-CpG conjugates in combination with radiotherapy [132].

STING

STING is an endoplasmic reticulum-associated signaling molecule that is broadly expressed in multiple cell lines and is triggered by cyclic dinucleotides (CDNs) or aberrant DNA. CDNs are generated when cytosolic dsDNA is detected by cyclic GTP-AMP synthase (cGAS), which forms the CDN cyclic 2'3'-GMP-AMP (cGAMP, Fig. 11) from ATP and GTP. cGAMP binds and activates STING. Alternatively, the bacterial CDN c-di-AMP can directly trigger STING. Like TLR signaling, STING signaling can also be a double-edged sword in cancer. STING-induced chronic inflammatory signaling can lead to

the development of cancer via stimulation of cellular proliferation and survival and the promotion of angiogenesis, as is the case in skin cancer [133]. In Lewis lung carcinoma, STING-induced enhanced tumor development is related to the activation of indoleamine 2,3-dioxygenase (IDO), an immune checkpoint [134]. STING triggering is also linked with antitumoral effects, as it can recognize early DNA damage resulting in the production of wound repair-initiating cytokines. Most importantly, STING is a very potent inducer of type I IFN production and other pro-inflammatory cytokines that mediate the cross-presentation of tumor antigens and the mobilization of tumor-specific CD8⁺ T cells [133, 135–137].

The use of STING agonists as immune modulators has received an increased amount of attention in recent years. Administration of STING agonists has been limited to the use of CDNs, which is challenging due to their rapid systemic distribution and off-target inflammation. Additional delivery hurdles include poor serum stability and poor intracellular accumulation, linked to the hydrophilic and negatively charged nature of CDNs. Very promising results have been obtained with intratumoral STING agonist injection, which led to an increased antitumor immune response by stimulating both APCs and T cells [138, 139]. As intratumoral injection is not possible for all tumors, an advanced delivery platform would be desired. Hanson et al. formulated cyclic di-GMP (cdGMP) in PEGylated lipid nanoparticles in a vaccine adjuvant setting. The influence of the liposomal formulation on the pharmacokinetic profile is demonstrated in Fig. 12.

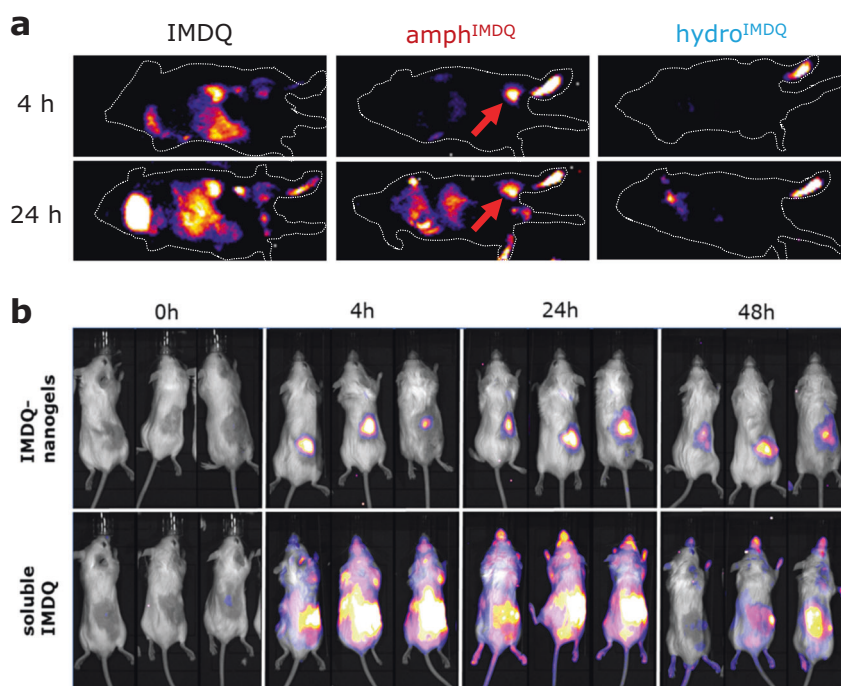


Fig. 9 Biodistribution visualized by luminescence images of luciferase reporter mice ($IFN\beta + \Delta\beta\text{-luc}$). **a** images taken 4 and 24 h after footpad injection of soluble IMDQ, amphiphilic polymer conjugated IMDQ ($\text{amph}^{\text{IMDQ}}$), or hydrophilic polymer conjugated IMDQ ($\text{hydro}^{\text{IMDQ}}$) [114]. Reprinted with permission from ACS. **b** Images taken before and 4, 24, and 48 h after peritumoral injection of soluble IMDQ ($\text{IMDQ}^{\text{soluble}}$) or nanogel conjugated IMDQ ($\text{IMDQ}^{\text{nanogel}}$) [117]. Reprinted with permission from Wiley

Subcutaneous injection of cdGMP resulted in an almost absent accumulation in the draining lymph nodes; hence, the intracellular concentration in lymph node-resident APCs is very low. Most of the cdGMP leaked into the blood vessels and was rapidly cleared. In contrast, formulation in lipid NPs strongly augmented accumulation in lymph nodes and induced a marked increase in APC intracellular levels [140]. Localized delivery can also be achieved with other nanomedicine formulations used for cGAMP, such as liposomes [141], PLGA [142], polymerosomes [143] or other polymer particles [144]. Shae et al. incorporated cGAMP in the hydrophilic core of polymerosomes that aided in cellular uptake and transport to the cytosol by inducing endosomal disruption after uptake [143].

The search for alternative synthetic ligands has seen a setback in the development of the promising synthetic murine STING ligand DMXAA (Fig. 11), as this molecule failed a phase II clinical trial due to inactivity against human STING [133]. GSK discovered amidobenzimidazole (ABZI) compounds as novel synthetic small molecule STING agonists through a high-throughput screen. Further optimization and linking of compounds yielded the dimeric ABZI compound 3 (Fig. 11) as the most potent agonist operating in the nanomolar range [145]. The current interest in the STING pathway and the discovery of synthetic ABZI agonists that allow for easy conjugation suggest a bright future for STING-related research in the coming years. More recently, Zhang et al. reported on another novel small molecule modulator of the cGAS-STING pathway, BNBC. Their most potent compound showed activity from a concentration of $2\ \mu\text{M}$. No detailed structure-activity relationship study was performed, so little can be said on potential sites for macromolecule conjugation [146].

SMALL MOLECULE CHECKPOINT INHIBITORS AND OTHER IMMUNE MODULATORS

Small molecule immune checkpoint blockers offer several advantages over their larger biological counterparts. These include

the potential to reach intracellular targets, easier preparation and a strongly reduced cost from production to clinical development and registration.

The PD1/PD-L1 axis has proven to be very successful in immune checkpoint inhibition, with multiple antibodies for PD1 or PD-L1 approved to date. The search for small molecules interfering with this axis is spurred by the success of monoclonal antibodies, but the development is still lagging behind compared with protein based blockers [147]. To date, three classes of PD-L1 small molecule ligands have been developed: (cyclic) peptides with direct competitive antagonist activity, small molecules disrupting the PD1/PD-L1 interaction by inducing PD-L1 dimerization, and small molecules acting via an unknown mode of action [148]. A multitude of compounds have been discovered by researchers at Bristol Myers Squibb, such as BMS-8, BMS-37, BMS-200 and BMS-202. The development is still in early stages, and the reported binding activities were determined via homogenous time-resolved fluorescence. The most progress has been made in regards to CA-170, a small molecule reported by researchers at Aurigene and licensed by Curis. CA-170 is a direct target for PD-L1 and is currently in a phase I clinical trial [147, 148]. No reports were found in the literature on conjugation or formulation strategies to alter the pharmacokinetic profile.

Small molecules have been developed for a wide range of other immune checkpoint targets, such as IDO, different kinase pathways (the transforming growth factor β (TGF- β) and vascular endothelial growth factor (VEGF) pathways) and chemokine receptors (CXCRs and CCRs) [4, 5]. TGF- β is an important immunosuppressive cytokine in the tumor microenvironment that affects cell proliferation and T-cell differentiation. Systemic antagonist exposure is related to severe side effects and toxicity. Tumor-targeted delivery of the TGF- β antagonist LY2109761 has been improved by encapsulation in polyelectrolyte capsules [149]. Alternatively, Zheng et al. have “backpacked” galunisertib onto tumor-targeting T cells using T cells targeting PEGylated liposomes, which allowed for sustained release of galunisertib in

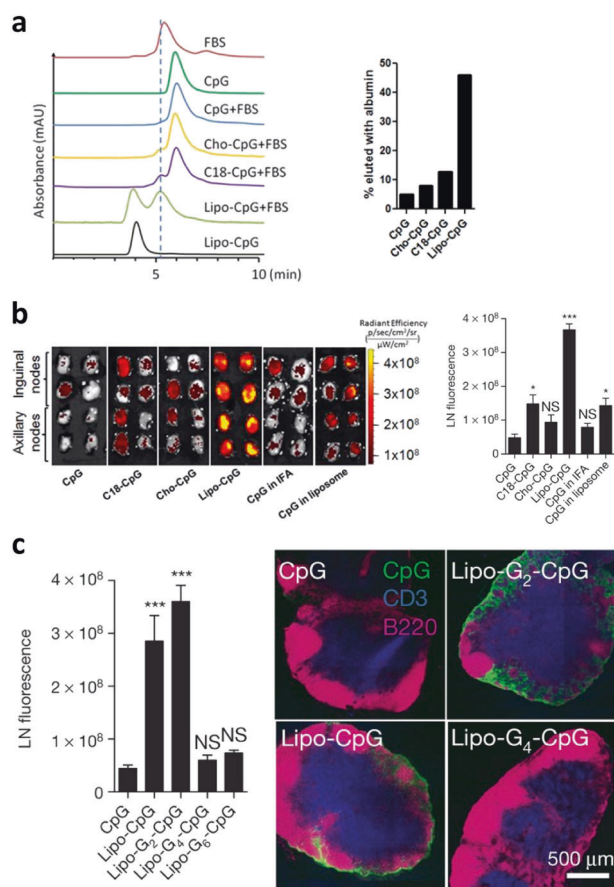


Fig. 10 **a** Size exclusion elugram of CpG-lipids alone or after incubation with serum proteins. **b, c** IVIS fluorescence imaging of excised draining LNs from C57BL/6 mice ($n = 4$ LNs per group) injected with Lipid-CpGs (3.3 nmol) in soluble form, emulsified in IFA, entrapped in liposomes, or as amphiphile conjugates. **b** IVIS images and quantification from inguinal and axillary nodes at 24 h. **c** left: IVIS quantification of CpG in LNs 24 h after injection of G-quadruplex-forming Lipo-Gn-CpGs. Right: Immunohistochemistry of inguinal LNs 24 h after injection (CD3, blue; B220, pink; CpG, green). Data reproduced from reference [127]. Reprinted with permission from Springer Nature

the tumor environment and reversal of immunosuppressive TGF- β activity, thereby improving the activity of effector T cells [150]. Indoximod, an IDO inhibitor, has been conjugated to phospholipids by Lu et al. and formulated into silica nanoparticles or liposomes in combination with the immunogenic cell death inducers oxaliplatin or doxorubicin, respectively. In both cases, the in situ vaccine-induced immune response was strongly increased by the IDO inhibitor [151, 152]. VEGF stimulates angiogenesis, and inhibition has a normalizing effect on the tumor vasculature, thereby removing a physical barrier for cell immune cell infiltration, leading to an increased amount of intratumoral effector T cells and reduced amounts of T_{reg} cells [153]. Axitinib has been formulated in silica nanoparticles with a PEGylated lipidic bilayer [154], in dual pH-responsive nanoparticles [155] and in liposomes to improve tumor-directed delivery [156].

FUTURE OUTLOOK

In the amalgam of great successes and unexpected failures, there is some underlying consistency in the use of immunotherapy, which is mostly related to the complexity of the immunosuppressive and immune evasion mechanisms in the tumor microenvironment and the influence of metabolic alterations. First, the future

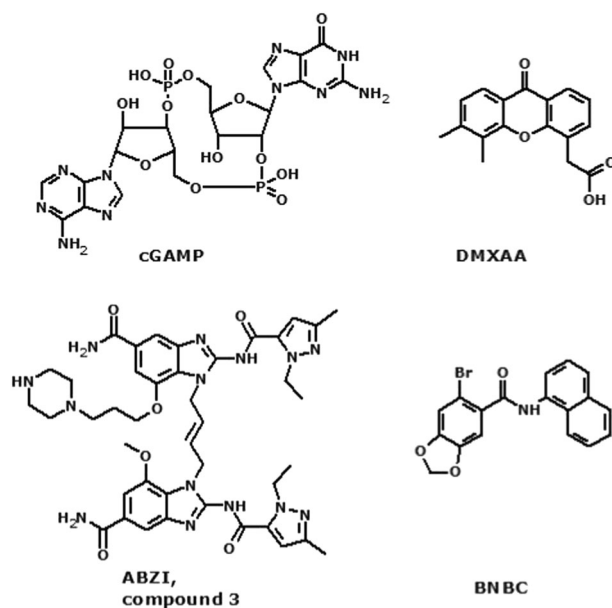


Fig. 11 STING agonists cGAMP, DMXAA, amidobenzimidazole (ABZI) compound 3 and BNBC

of immunotherapy is progressing toward combination therapy. This mindset has already infiltrated the clinical trial landscape, as the majority of new immunotherapy studies use a combination treatment. The treatment outcomes of CAR-T cells can be drastically improved in combination with immune checkpoint inhibition. Similarly, 'immune cold' tumors respond poorly to checkpoint inhibition. Hence, combination therapies that increase effector T cell infiltration positively alter therapeutic outcomes. The second important consideration relates to intertumoral heterogeneity and even interpatient variations. Heterogenous tumors cannot be treated similarly, and selecting the appropriate immunotherapy combination should be related to the expression pattern and secreted cytokines in the tumor environment. This of course requires the characterization of easily detectable biomarkers [157].

After the choice of therapy comes the issue of delivery. Systemic exposure to immune-modulating therapeutics can result in severe inflammation-related adverse events and in some exceptional cases even death. Different strategies have been developed over the past decade based on conjugation or encapsulation principles to alter the pharmacokinetic profile of therapies. Despite the progress made in nanotechnology and the plethora of examples showing advantages in preclinical studies, the number of nanoscale or macroscopic delivery systems in clinical trials is limited. This demonstrates the need for further development in versatile delivery systems that allow for simplified clinical translation.

Barriers that limit the progression of nanomedicine into the clinic can be related to the nanoformulation itself, the production process and the regulatory requirements. One limitation on nanomedicine effectiveness has been pointed out in a prospective article by the Chan group stating that even with nanocarriers, the actual delivery of injected payload was below 1% [158]. In an answer to this paper, Torrice et al. wanted to qualify the message and present a different perspective on nanomedicine [159]. Although the analyzed data do not represent the full body of performed research, they still shed light on an important limitation of nanomedicine delivery related to the EPR effect. EPR effect-mediated accumulation of macromolecules inside the tumor is a generally accepted principle but might not hold true in all cases, and a large variety of molecules can be found in the tumor region,

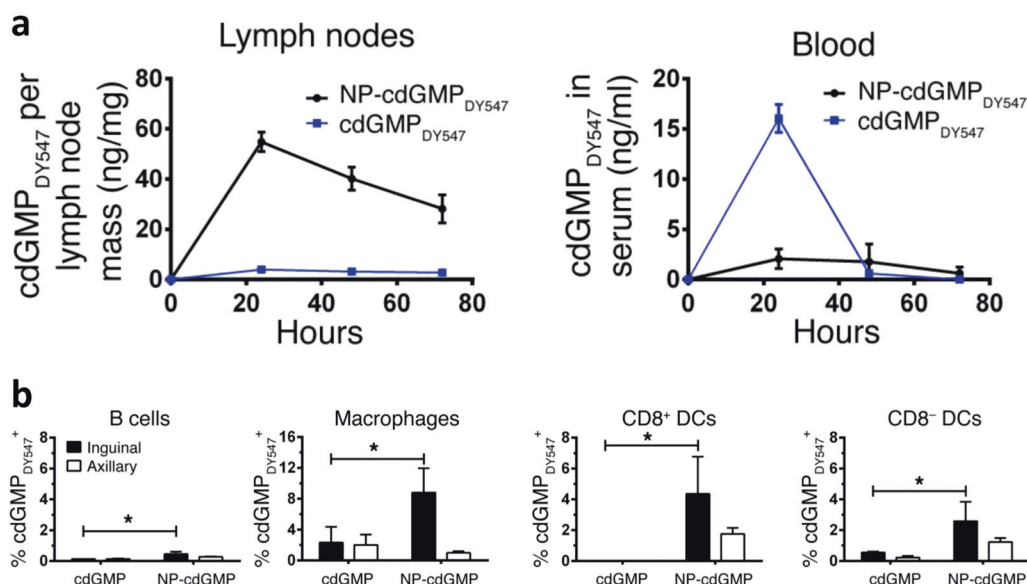


Fig. 12 NP-cdGMP enhances LN uptake of CDNs. **a** Distribution of cdGMP after injected in soluble form or liposomal NP measured by fluorescence spectroscopy. **b** Percentage of cdGMP positive APCs in the inguinal and axillary lymph node [140]. Reprinted with permission from The American Society for Clinical Investigation

depending on the level of tumor vascularization and patient differences. This situation might, however, be different for immunotherapy, especially when highly endocytic cells such as DCs, macrophages and B cells are targeted, as uptake is more efficient, and immune training can be done in a more accessible place than the tumor. Tumor-specific accumulation could be improved by using targeting moieties. This, however, introduces difficulties on other levels. Barriers in the production process and regulatory affairs also exist. On a regulatory basis, nanomedicines are treated as multicomponent systems, thereby making them a much more complex new medical entity than single-agent therapies. Quality assurance requires consistency in all aspects of the nanocarrier, which might involve polymer composition, loading capacity, self-assembly and modified ligand density. All these factors place pressure on the production process. Reproducibility might be possible on a small laboratory scale but remains difficult for large-scale production due to the complexity, as upscaling introduces challenges at every step. A more efficient translation from the laboratory to clinical practice requires efforts from all branches. As researchers develop novel materials, it is important to consider the balance between complexity and simplicity. As the field further matures, alterations in the regulatory pathways can open doors for more nanomedicine-based therapies [160].

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ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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