



Nanoparticles for Manipulation of the Developmental Wnt, Hedgehog, and Notch Signaling Pathways in Cancer

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Abstract—The Wnt, Hedgehog, and Notch signaling pathways play a crucial role in early development and the maintenance of adult tissues. When dysregulated, these developmental signaling pathways can drive the formation and progression of cancer by facilitating cell survival, proliferation, and stem-like behavior. While this makes these pathways promising targets for therapeutic intervention, their pharmacological inhibition has been challenging due to the substantial complexity that exists within each pathway and the complicated crosstalk that occurs between the pathways. Recently, several small molecule inhibitors, ribonucleic acid (RNA) molecules, and antagonistic antibodies have been developed that can suppress these signaling pathways *in vitro*, but many of them face systemic delivery challenges. Nanoparticle-based delivery vehicles can overcome these challenges to enhance the performance and anti-cancer effects of these therapeutic molecules. This review summarizes the mechanisms by which the Wnt, Hedgehog, and Notch signaling pathways contribute to cancer growth, and discusses various nanoparticle formulations that have been developed to deliver small molecules, RNAs, and antibodies to cancer cells to inhibit these signaling pathways and halt tumor progression. This review also outlines some of the challenges that these nanocarriers must overcome to achieve therapeutic efficacy and clinical translation.

Keywords—Small molecule, RNA interference, Antibody, Multivalency, Drug delivery, Gene regulation, Nanocarrier, Nanomedicine.

INTRODUCTION

The developmental Wnt, Hedgehog (Hh), and Notch signaling pathways guide early development through precise control of cell proliferation, differentiation, migration, and cell-to-cell communication,^{6,53} and they also preserve tissue function by regulating adult stem cell populations.⁹² Given the important and complex role these pathways play in controlling cellular function, it is unsurprising that their aberrant expression has been associated with the formation and progression of cancer.^{11,89,105,106} In fact, many of the “hallmarks” of cancer are directly influenced by Wnt, Hh, and Notch signaling (Fig. 1), and thus cancer cells have commandeered these developmental pathways to support their ability to grow, resist treatment, metastasize, and recur.^{34,35,51} Indeed, overactive developmental signaling facilitates cancer cells’ evasion of growth suppressors and the immune system, and supports the formation of new blood vessels to increase nutrient supply, contributing to unchecked disease progression.^{34,35} Additionally, the Wnt, Hh, and Notch signaling pathways have a significant impact on cancer stem cell populations,^{12,38,63,82,120} which facilitates disease recurrence if tumors are not fully removed.^{64,82,132} Overall, the integral role that Wnt, Hh, and Notch signaling play in cancer progression makes them extremely promising targets for new therapeutic strategies. By inhibiting these pathways, cancer progression may be dramatically slowed or even reversed.

An overview of the Wnt, Hh, and Notch signaling pathways, and the cancers in which they are implicated, is provided in Fig. 2. In brief, each pathway is

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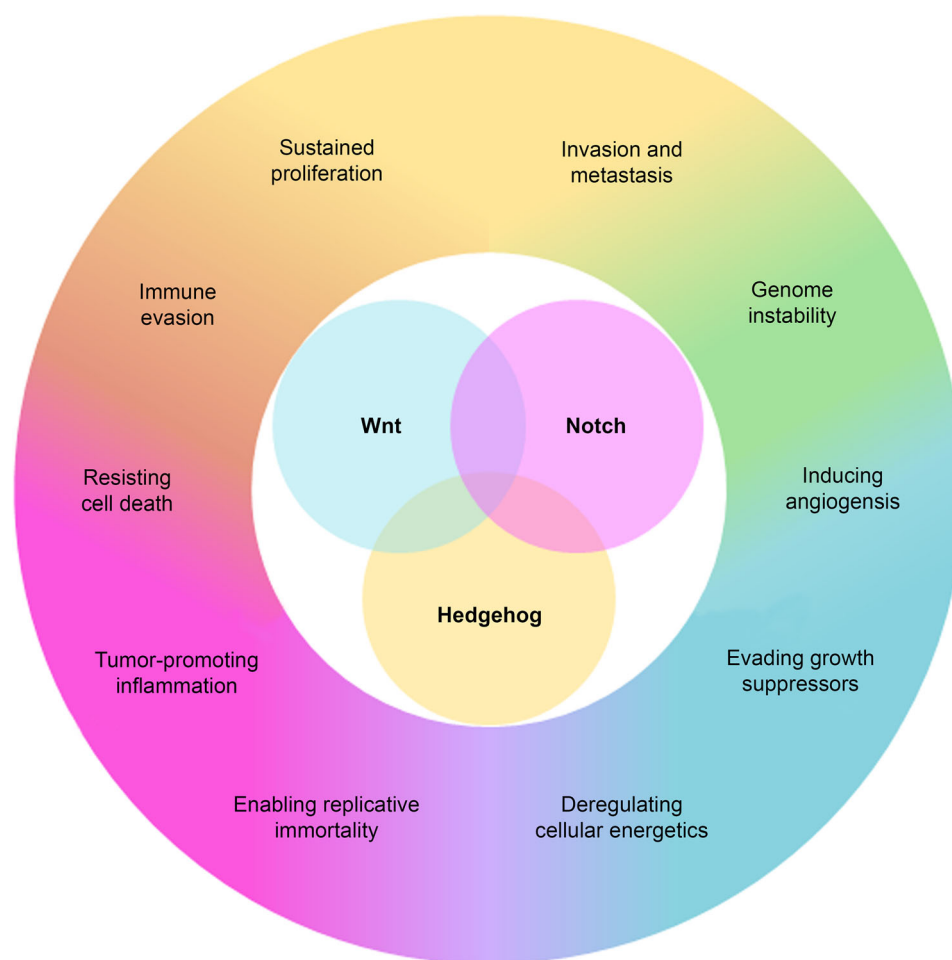


FIGURE 1. Dysregulation of the developmental Wnt, Notch, and Hedgehog signaling pathways is implicated in all of the “hallmarks” of cancer.

activated when extracellular soluble or cell-bound ligands interact with specific transmembrane receptors on the receiving cell. These ligand-receptor interactions initiate downstream intracellular signaling that leads to the nuclear translocation of specific molecules that subsequently promote the transcription of genes associated with tumorigenesis. Though not depicted in Fig. 2, there is substantial crosstalk between the signaling pathways and they often work in concert to support tumor progression. This makes their pharmacological inhibition challenging, as expanded upon in the following paragraph.

There are many components of the Wnt, Hh, and Notch signaling pathways that are amenable to pharmacological regulation, and a variety of therapeutic moieties have begun to be developed and tested (Fig. 3; Table 1). Unfortunately, while inhibiting Wnt, Hh, and Notch signaling is promising in theory, there are many challenges associated with accomplishing this goal. First, given the complexity of each pathway, it is often difficult to determine which receptor(s)/ligand(s)/

intracellular molecule(s) to target in different cancer subtypes. Second, resistance is an issue, as cancer cells may activate one pathway to compensate for inhibition of another. Third, since Wnt, Hh, and Notch are active in some adult tissues, side effects are a concern, particularly when using therapeutic moieties that lack specificity. Finally, some of the key mediators of these developmental pathways are “undruggable” because they lack effective binding sites for small molecule therapeutics.⁴⁸ Overcoming these challenges will revolutionize the treatment of many types of cancer.

There are three main classes of agents that can be used to suppress developmental signaling pathways in cancer: small molecules, nucleic acids, and antibodies. While small molecule inhibitors have been the most widely explored (Table 1), they often encounter issues with solubility, bioavailability, targeted cellular uptake, and systemic toxicity that hinder their clinical translation. The use of small interfering RNA (siRNA) or microRNA (miRNA) molecules to suppress desired targets through RNA interference (RNAi) is a

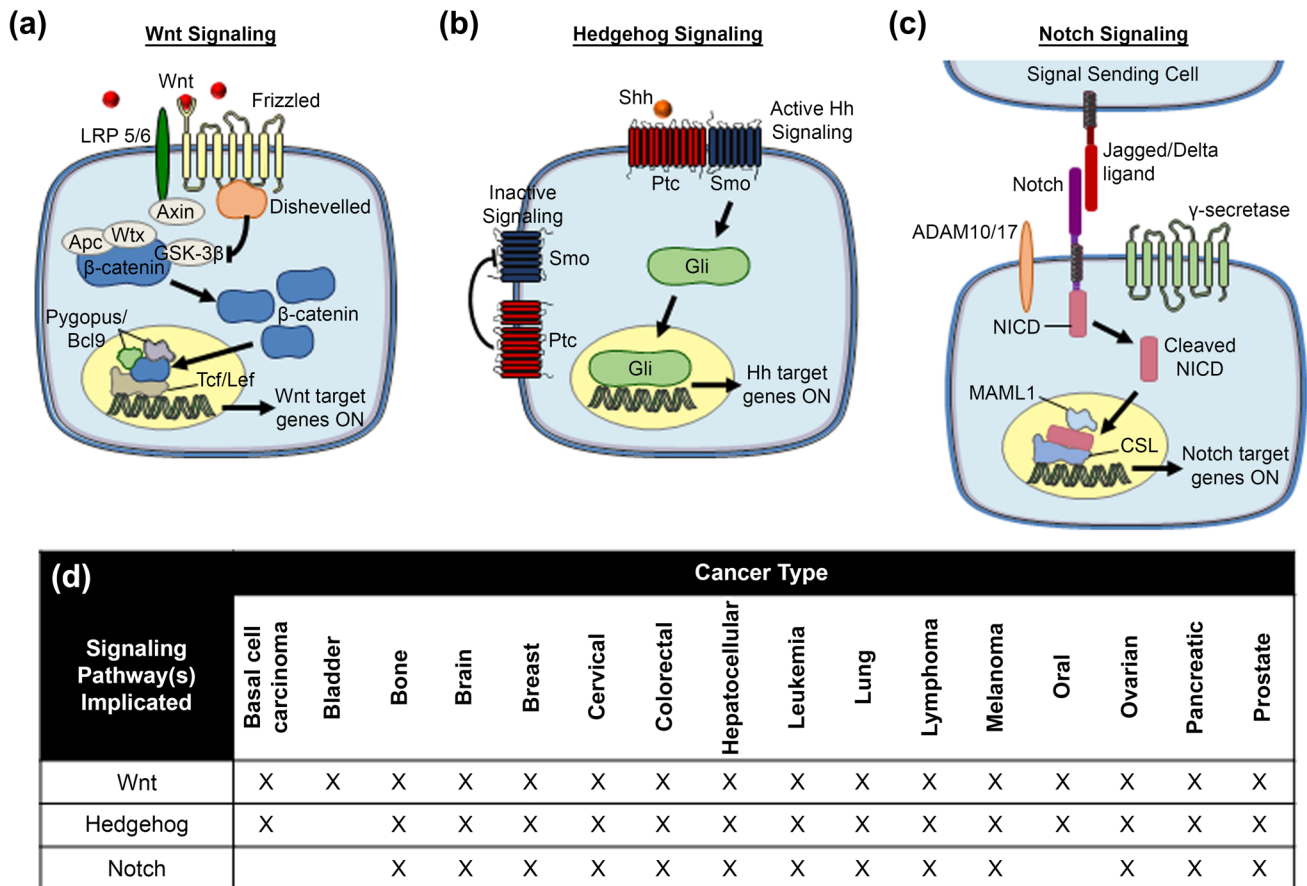


FIGURE 2. Schematic of activated Wnt, Hh, and Notch signaling pathways and the cancers in which they are implicated. (a) Canonical Wnt signaling is activated in cancer cells when extracellular Wnt ligands bind Frizzled and LRP5/6 co-receptors that are amplified on cancer cell surfaces. This leads to inhibition of the β -catenin destruction complex, allowing β -catenin to accumulate in the cytoplasm and then translocate to the nucleus where it activates the transcription of downstream oncogenes. Reproduced with permission from Ref. 73, Riley et al. Small. Copyright 2017 Wiley. (b) Simplified scheme of inactive versus active canonical Hedgehog signaling. In inactive Hh signaling, Patched (Ptc) suppresses Smoothened (Smo) to prevent downstream signaling activity. In active Hh signaling, Sonic hedgehog (Shh) ligand binding to Ptc relieves its suppression of Smo, resulting in the activation and translocation of Gli proteins to the nucleus where they promote transcription of target genes. (c) Canonical Notch signaling is activated when Jagged or Delta ligands on a signal-sending cell bind the extracellular domain of the Notch receptor on a signal-receiving cell. The extracellular domain of Notch is subsequently cleaved by ADAM family proteases, then the intracellular domain (NICD) is cleaved by the gamma secretase complex. Released NICD translocates to the nucleus, where it acts as a promoter for the transcription of downstream target genes that support cell survival, proliferation, stemness, and other oncogenic behaviors. (d) List of cancers with known involvement of Wnt, Hh, and Notch signaling.

promising alternative treatment strategy that may enable reduction of cancer cell survival, proliferation, and stemness. However, the clinical translation of RNA therapeutics is limited due to their susceptibility to nuclease degradation, rapid clearance from the bloodstream, and their inability to passively enter cells.^{31,55,96} For RNA and small molecules to reach their potential, more effective delivery systems must be developed and shown to provide a therapeutic advantage over current standards of care in cancer treatment. Lastly, antagonistic antibodies offer a third way to manipulate developmental signaling pathways. Typically, antagonistic antibodies are designed to bind

extracellular ligands or transmembrane receptors to block ligand/receptor interactions, thereby suppressing downstream signaling. Unfortunately, the high required dosages and cost of antagonistic antibodies have limited their translation. As with RNA and small molecule inhibitors, novel delivery vehicles are needed to enhance the efficacy of antibody therapeutics.

Nanoparticles (NPs) offer substantial promise as carriers to enhance the delivery of small molecules, RNAs, and antibodies to cancer cells to manipulate Wnt, Hh, and Notch signaling and halt disease progression. Encapsulating these molecules inside NPs or loading them on NPs' exterior can improve their sta-

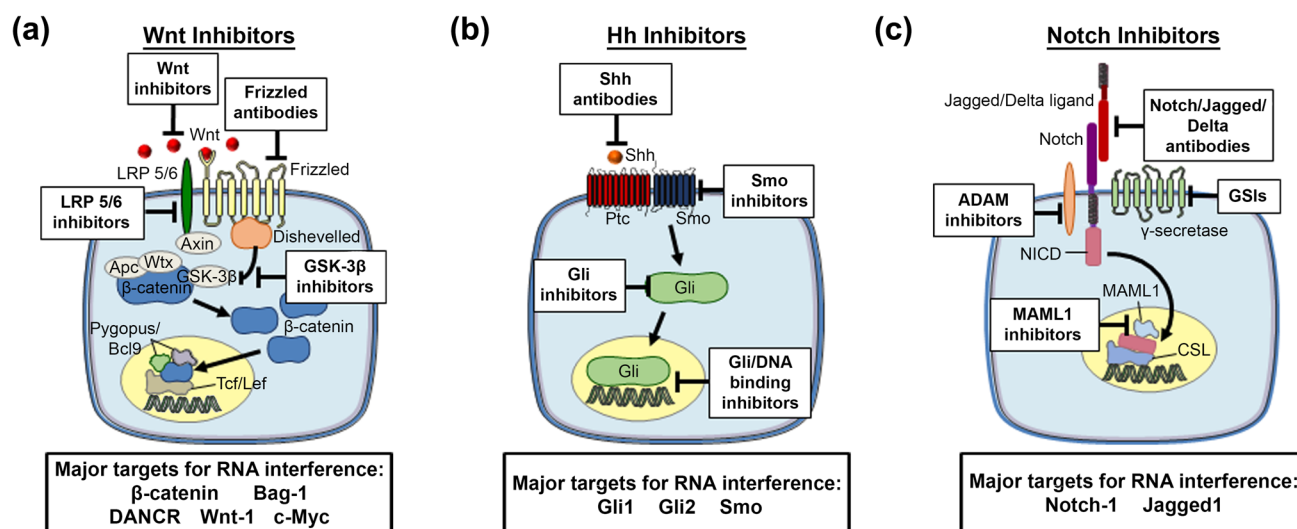


FIGURE 3. Components of the (a) Wnt, (b) Hh, and (c) Notch signaling pathways that are amenable to inhibition by antibodies, small molecules, and RNA molecules.

bility, pharmacokinetics, biodistribution, and tissue/cell-specific delivery.^{28,31,65,66} This enables the nanoformulations to be much more effective than their freely delivered counterparts.^{7,61,65,88,111} This review summarizes nanocarriers that have been developed to date to deliver small molecules, RNA molecules, and antibodies to cancer cells to suppress the Wnt, Hh, and Notch signaling pathways (Table 2). These new therapeutics offer substantial promise as tools for improved treatment of many types of cancer, and a summary of barriers to address in clinical translation is provided at the end of this review.

NANOTHERAPEUTICS TO SUPPRESS WNT SIGNALING IN CANCER

Wnt Signaling in Cancer

After the discovery of the *wingless* gene in a mutagenesis screen for visual phenotypes, many components of the Wnt family of signaling proteins were identified as key mediators of patterning decisions during embryonic development.⁷⁹ The Wnt pathway was connected to cancer when it was observed that activation of Wnt1 resulted in mammary hyperplasia and tumors.¹⁰⁹ Now, Wnt signaling is known to be a key regulator of development and stemness, and its aberrant activity has been implicated in many cancers, including cutaneous melanoma, pancreatic ductal adenocarcinoma, and breast carcinoma, among others.¹²⁹ Indeed, Wnt signaling has been linked with all stages of cancer development, making its inhibition an exciting strategy to combat tumor progression, drug resistance, metastasis, and recurrence.

The Wnt signaling pathway is divided into β -catenin dependent (canonical) and β -catenin independent (non-canonical) pathways.¹²⁹ The two non-canonical pathways include the Wnt/Ca²⁺ and the Wnt/planar cell polarity (Wnt/PCP) pathways.^{5,20,52,108} Their role in cancer is less established than that of the canonical pathway, and thus the majority of therapeutics under development for Wnt inhibition target the canonical pathway. Accordingly, the canonical Wnt signaling pathway is described in detail below, followed by a discussion of nanotherapeutics designed to suppress this pathway.

In healthy cells with inactive canonical Wnt signaling, the absence of extracellular Wnt ligands to activate the pathway results in the phosphorylation of β -catenin by a destruction complex comprised of Axin, Apc, Wtx, and the kinase GSK-3 β . This phosphorylation of β -catenin leads to its proteasomal degradation, and the resulting lack of nuclear β -catenin allows the repressive complex containing Tcf/Lef and Groucho to recruit histone deacetylases to repress Wnt pathway target genes. Conversely, in the cancer microenvironment where there is an abundance of secreted Wnt ligands, the canonical Wnt pathway is activated when the ligands bind to Frizzled (FZD) receptors and LRP co-receptors that are over-expressed on cancer cell surfaces (Fig. 2a). This activation initializes a signaling cascade in which LRP receptors are phosphorylated by kinases CK1 α and GSK-3 β . This is then followed by the recruitment of Dishevelled (Dvl) proteins to the plasma membrane where they polymerize and are activated.¹⁵ The Dvl polymer inhibits the destruction complex, which allows β -catenin to stabilize and accumulate in the cytoplasm. Following translocation

TABLE 1. Therapeutic moieties available to inhibit Wnt, Hh, and Notch signaling.

Pathway	Target	Therapeutic moiety
Wnt	Extracellular ligands	SFRP1
		Wnt5a
	Frizzled receptor	Wnt-C59
		Anti-Frizzled7 antibody
	LRP 5/6 receptor	iWnt peptide
		Niclosamide
		IWR-1
		KYA1797K
		FH535
		siCTNNB1
		siBag-1
		iCRT 3/14
		PNU-74654
		Gigantol
		DANCR lncRNA
	β -catenin and c-Myc	miR-105
		IWP-2
		DK419
		miR-200c
	c-Myc	c-Myc shRNA
		Cromolyn
	GSK-3 β	Pyriminium pamoate
		siWnt-1
	Wnt-1	Adavivint
		Wogonin
Hh	Shh	Robotnikinin
		Anti-Shh 5E1 antibody
		Anti-Shh MEDI-5304 antibody
	Ptc1	Ant-Patched-1 antibody
		HhAntag
	Smo	Cyclopamine
		Vismodegib
		DHCEO
		SMANT
		DY131
		Itraconazole
		ALLO-1/2
		Compound 5
		Jervine
		Cur-61414
		SANT 1-4
		Saridegib
		Sonidegib
		TAK-441
		BMS-833923
		LEQ506
		PF-04449913
		LY2940680
		Vitamin D ₃
		BRD-6851
		SEN450
		MRT-83
	Gli1	Quinacrine
		GANT61
		GANT58
		Arcyriaflavin C
		Physalins F
		siGli1
	Gli1/2	HPI1-4
		Anthothecol

TABLE 1. continued

Pathway	Target	Therapeutic moiety
Notch	ADAM 10/17	Glabrescione B
		Arsenic trioxide
		INCB7839
		INCB3619
		INCB8765
		KP457
		GM6001
		GI254023X
		8C7 antibody
		D1(A12) antibody
		A9(B8) antibody
		MEDI3622 antibody
	MAML1	ADAM10 prodomain
		Rapamycin
		MAM peptide antagonist
		SAHM1
	Notch receptors	Notch-1 shRNA
		Soluble Notch1
		Soluble DLL1
		Soluble Jagged1
		Genistein
		Sulforaphane
		Quercetin
		Curcumin
		Resveratrol
		miR-34a
		Anti-Notch-1-3 antibody
	Jagged/Delta	siJagged1 antibody
		Anti-DLL1-4 antibodies
		Nicastrin antibody
		DAPT
		MRK-560
		MRK-003
		PF-03084014
		MK-0752
		BMS-906024
		DBZ (LY411575)
		LY450139
		RO4929097

to the nucleus, β -catenin forms an active complex with Tcf/Lef by displacing Groucho. This new complex activates the transcription of Wnt target genes, including Axin2, Cyclin D1, and c-Myc, to drive disease progression (Fig. 2a).⁵⁸

Therapies designed to suppress canonical Wnt signaling in cancer cells can either attempt to block ligand/receptor interactions to prevent signal cascade activation or attempt to inhibit downstream molecular targets within the pathway, such as β -catenin or its transcriptional targets (Fig. 3a). The following sections summarize NPs that have been developed to deliver small molecules, RNA therapeutics, or antibodies to cancer cells to suppress canonical Wnt signaling.

TABLE 2. Nanoparticle systems designed to inhibit the Wnt, Hh, and Notch signaling pathways.

NP name	Pathway (target)	Molecule carried	Cancer targeted	Stage of development; key results	Material	Size; charge
Chi-Au NC-Alg NPs ³⁰	Wnt (Wnt ligands)	sFRP1	Cervical	<i>In vitro</i> : 73-fold increase in phosphorylated β -catenin	Gold	767 nm;
Wnt5a trap ⁵⁷	Wnt (Wnt5a)	Frizzled7 receptor plasmid DNA	Metastatic melanoma	<i>In vivo</i> : combination with DOX increases median survival from 39 to 65 days	Lipid-protamine-DNA	– 15.8 mV 100 nm; N/A
CP-NIC ⁷	Wnt (β -catenin)	Niclosamide	Colon	<i>In vivo</i> : extended median survival from 13 to 26 days	Chimeric polypeptide	81.5 nm; N/A
CCSNPs ⁷³	Wnt (GSK-3 β)	Chromolyn	Colorectal	<i>In vivo</i> : decreased tumor occurrence by 67%	Chitosan	112.4 nm; + 39.9 mV
EnCore/CTNNB1 ²⁹	Wnt (β -catenin)	siCTNNB1	Colorectal, hepatocellular	<i>In vivo</i> : tumor growth inhibition of 82%	Lipids	70–100 nm; N/A
Nano-plasmiid ⁴³	Wnt (β -catenin)	siBag-1	Colorectal	<i>In vivo</i> : 70% reduction in tumor volume compared to control	Gold	95.38 nm; + 35.03 mV
RGD-PEG-ECO/siDANC ¹¹⁰	Wnt (β -catenin)	siDANC	Breast	<i>In vivo</i> : 20–40% reduction in tumor volume	Amino lipid ECO, cyclic RGD peptide-PEG	109.12 nm; + 24.67 mV
PEG-PEI-Ce6/siRNA ⁵⁹	Wnt (Wnt-1)	siWnt-1	Oral	<i>In vitro</i> : 58.55% reduction in cell viability	PEG-PEI-Ce6	90nm; + 10 mV
shRNA + NP ¹⁰⁷	Wnt (c-Myc)	c-Myc shRNA	Breast, colorectal	<i>In vivo</i> : more than doubled median survival	PGMA, PEI	150 nm; + 75 mV
FZD7-NS ⁸⁸	Wnt (Frizzled7)	anti-FZD7 antibodies	Breast	<i>In vitro</i> : 25% reduction in cell viability	Gold	178.3 nm; – 27.4 mV
iWnt-ATF ₂₄ -IONPs ⁷¹	Wnt (LRP5/6)	iWnt peptide	Breast	<i>In vivo</i> : 94% reduction in tumor volume compared to control	Iron oxide	25.8 nm; – 34 mV
CCPM- ¹⁷⁷ Lu ¹²⁶	Hh (Smo)	Cyclopamine	Breast, pancreatic	<i>In vivo</i> : 70% reduction in tumor weight compared to control	Lecithin	48.2 nm; N/A
HA-SS-PLGA-DOX-CYC ⁴⁰	Hh (Smo)	Cyclopamine	Breast	<i>In vivo</i> : complete remission in all mice	HA-SS-PLGA	245.3 nm; N/A
P-CYP ¹²⁴	Hh (Smo)	Cyclopamine	Prostate	<i>In vivo</i> : significantly inhibited tumor growth	mPEG-b-PCC-g-DC	76.37 nm; N/A
M-CPA/PTX ¹³⁰	Hh (Smo)	Cyclopamine	Pancreatic	<i>In vivo</i> : tumor growth suppressed in 80% of mice	PEG-PMA-PCL	45.2 nm; N/A
CMNP ⁵⁰	Hh (Smo)	Cyclopamine	Pancreatic	<i>In vivo</i> : 75.3% reduction in tumor volume compared to control	PLGA	66.1 nm; – 26.1 mV
SN38 NPs/GDC-0449 ¹¹⁶	Hh (Gli1)	GDC-0449	Pancreatic	<i>In vivo</i> : induced apoptosis in 50% of tumor cells	PEG _{sk} -P(HE-MASN38) _x	70 nm; N/A
PEG-DB ⁸⁶	Hh (Gli1)	GDC-0449	Pancreatic	<i>In vitro</i> efficacy and <i>in vivo</i> accumulation; 70% reduction in cell viability	PEG-b-poly (carbonate)	149 nm; N/A
Antho-NPs ¹¹⁴	Hh (Gli-DNA binding)	Anthothecol	Pancreatic	<i>In vitro</i> : significantly inhibits EMT in stem cells	PLGA	190.52 nm; 0.02 mV
Mang-NPs ¹¹³	Hh (Gli-DNA binding)	α -Mangostin	Pancreatic	<i>In vivo</i> : complete elimination of liver metastases	PLGA	186.3 nm; 0.03 mV

TABLE 2. continued

NP name	Pathway (target)	Molecule carried	Cancer targeted	Stage of development; key results	Material	Size; charge
NC-GlaB ⁴⁶	Hh (Gli1)	GlaB	Pancreatic	<i>In vitro</i> efficacy and <i>in vivo</i> biodistribution; 30.2% of cells arrested in G2 phase	PLGA	159.4 nm; – 39.8 mV
NQC ⁷⁶	Hh (Gli1)	Quinacrine	Cervical	<i>In vivo</i> ; 10 mm ³ reduction in tumor volume compared to control	PLGA	291.4 nm; + 2.38 mV
NQC ⁷⁷	Hh (Gli1)	Quinacrine	Oral	<i>In vitro</i> ; 17% increase in cell death	PLGA	100 nm; + 2.38 mV
NanoHHI ¹²²	Hh (Gli1)	HPI-1	Hepatocellular carcinoma	<i>In vivo</i> ; 67% reduction in tumor weight compared to control	PLGA-PEG	60 nm; N/A
NanoHHI ¹⁷	Hh (Gli1)	HPI-1	Pancreatic	<i>In vivo</i> ; 90% reduction in tumor weight compared to control	PLGA-PEG	60 nm; N/A
GANT61 PLGA NPs ⁹	Hh (Gli1)	GANT61	Colorectal, breast	<i>In vitro</i> ; significant reduction in tumorsphere formation ability	PLGA	250 nm; – 20 mV
DMP-Gli1si ¹³¹	Hh (Gli1)	Gli1 siRNA	Brain	<i>In vivo</i> ; induced apoptosis in 67.5% of tumor cells	DMP	35.6 nm; + 32.7 mV
GSI-MSNPs ⁶²	Notch (γ -secretase)	DAPT	Breast	<i>In vivo</i> ; 95% reduction in tumor volume compared to control	Silica	350 nm; + 50 mV
MSN-PEI-GAorg-DAPT ⁶¹	Notch (γ -secretase)	DAPT	Breast	<i>In vivo</i> ; 85% reduction in cancer stem cells	Silica	216 nm; + 27.8 mV
CF-NP-EB/DART ¹¹⁵	Notch (γ -secretase)	DAPT	Breast	<i>In vivo</i> ; 79% reduction in tumor volume compared to control	PLA	100 nm; – 24.3 mV
NPs/PEI-FA/shRNA ¹²³	Notch (Notch-1)	Notch-1 shRNA	Breast	<i>In vitro</i> ; threefold increase in cellular apoptosis	Iron oxide, silica	64 nm; + 17.5 mV
Jagged siRNA-CH ¹⁰⁰	Notch (Jagged1)	Jagged1 siRNA	Ovarian	<i>In vivo</i> ; 87.5% reduction in tumor weight compared to control	Chitosan	N/A
CNPs ²³	Notch (multiple)	miR-34a	Breast	<i>In vivo</i> ; 80% reduction in tumor weight compared to control	Hyaluronic acid-chitosan	214 nm; – 33 mV

Nanoparticle Delivery of Small Molecules to Regulate Wnt Signaling

Small molecule inhibitors of Wnt signaling include both naturally occurring compounds^{30,57} and synthetic agents^{7,73}. Regarding natural products, researchers have either utilized nanoparticles to deliver Wnt inhibitors that are produced by cells, or they have used nanoparticles to “trap” Wnt ligands and regulate ligand-mediated activation of the signaling pathway.³⁰ One such naturally produced Wnt inhibitor is SFRP1. Healthy cells secrete SFRP1 upon oxidative stress or DNA damage, but its expression is silenced or mutated in cancer cells.²⁶ Upon secretion, SFRP1 blocks Wnt signaling by binding extracellular Wnt ligands so they can no longer bind Wnt receptors,^{18,112} or it directly binds the FZD receptors to form an inhibitory complex.¹⁸ By replenishing SFRP1 content in the microenvironment of cervical cancer cells through NP delivery *in vitro*, Ghosh and team achieved anti-proliferative effects that were mediated through reduction in the expression levels of the Wnt target proteins β -catenin, cyclin D1, and survivin.³⁰ In an alternative approach, Huang and colleagues used NPs to trap Wnt5a molecules that are produced by cancer cells in the tumor microenvironment and demonstrated that this approach could treat metastatic melanoma by preventing signaling feedback.⁵⁷ Their formulation consisted of cationic lipid-protamine NPs that could deliver plasmid DNA encoding a trimeric trap protein containing the extracellular domain of Frizzled7 receptors to tumors. This caused the tumors to express the trap protein, which bound Wnt5a to reduce its intratumoral expression and limit tumor growth, particularly when combined with doxorubicin chemotherapy.⁵⁷ Notably, Wnt5a has an innate ability to control the immunosuppressive tumor microenvironment. As a result, Huang and colleagues demonstrated that NP-mediated trapping of Wnt5a could enhance dendritic cell function and T-cell infiltration into tumors, resulting in immunogenic cell death within the tumors.⁵⁷

In addition to natural Wnt inhibitors, synthetic Wnt inhibitors like Niclosamide (NIC)⁷ and cromolyn⁷³ have also been incorporated into NP carriers for anti-cancer therapy. NIC is an orally bioavailable drug that has been approved by the Food and Drug Administration (FDA) for the treatment of tapeworms, and it has also recently been identified as a Wnt/ β -catenin inhibitor by the National Cancer Institute and other independent researchers.² Bhattacharya *et al.* have exploited this therapeutic discovery by conjugating NIC to polypeptides that self-assemble into NPs, thus overcoming its low solubility and bioavailability and enabling the treatment of colon cancer.⁷ In *in vitro*

studies, HCT-116 human colon cancer cells treated with NIC-loaded NPs exhibited decreased c-Myc and cyclin D1 expression, indicating these particles effectively inhibit cytosolic β -catenin levels and downstream Wnt targets. In further studies, the team demonstrated that these NPs could slow tumor progression in human colon cancer xenograft models.⁷ In a different approach, Motawi *et al.* prepared chitosan NPs incorporating the synthetic Wnt inhibitor, cromolyn, which potently regulates Wnt signaling by inhibiting GSK-3 β .⁷³ They used these NPs to deliver cromolyn to rats with dimethylhydrazine-induced colorectal cancer, and demonstrated they could effectively decrease protein levels of the Wnt-related proteins GSK-3 β and β -catenin and provide a protective approach to colorectal cancer therapy.⁷³ Taken together, the above studies demonstrate that NP-mediated delivery of small molecule Wnt inhibitors has substantial potential as a cancer treatment strategy.

Nanoparticle-Mediated RNA Interference of Wnt Signaling

Although the above examples demonstrate that pharmacological inhibition of Wnt signaling is possible, the key mediator of this pathway, β -catenin, is still considered “undruggable” because it lacks an effective binding site for small molecule therapeutics. An attractive alternative strategy to suppress β -catenin and other key targets in the Wnt signaling pathway is the use of siRNA or miRNA to elicit RNAi-mediated gene silencing. In an elegant study, Abrams and colleagues demonstrated that effective inhibition of β -catenin could be achieved using lipid nanoparticles (LNPs) containing Dicer substrate siRNA (DsiRNA) targeting *CTNNB1*, the gene encoding β -catenin.²⁹ This formulation achieved significant tumor growth inhibition in Wnt-dependent colorectal and hepatocellular carcinoma models in mice, but not in Wnt-independent tumors.²⁹ Excitingly, analysis of histology sections showed the LNPs achieved homogenous DsiRNA delivery throughout tumors, which is impressive given that insufficient NP penetration into tumors is a major barrier that researchers in the field are actively working to overcome.^{21,22,102,118}

Besides targeting β -catenin directly, researchers have also developed NPs to deliver siRNA or miRNA against other genes whose down-regulation can subsequently decrease β -catenin expression. For example, Huang *et al.* developed magnetic gold NPs to deliver Bag-1 gene-silencing plasmids to colorectal cancer.⁴³ Bag-1 is a positive regulator of the anti-apoptotic gene Bcl-2, so the researchers expected silencing Bag-1 would increase cellular apoptosis. LoVo cells treated with the NPs exhibited reduced Bag-1 mRNA expres-

sion, and the team demonstrated a corresponding notable decrease in β -catenin protein expression in LoVo tumors treated with the NPs through immunohistochemistry and Western blotting of excised tumor tissue. Similarly, Vaidya *et al.* developed polymeric NPs to deliver long noncoding RNA (lncRNA) targeting the gene DANCER to triple negative breast cancer (TNBC).¹¹⁰ These NPs achieved 80-90% knockdown of DANCER expression in MDA-MB-231 and BT549 TNBC cells for up to 7 days, and simultaneously induced a significant decrease in the expression of β -catenin and other proteins involved in epithelial-to-mesenchymal transition and apoptosis regulation.¹¹⁰

Other researchers have also developed platforms to enable RNAi of Wnt signaling by inhibiting Wnt proteins themselves or downstream targets like c-Myc.^{59,107} For example, Ma *et al.* developed polyethylene glycol-polyethylenimine-chlorin e6 (PEG-PEI-Ce6) NPs to deliver Wnt-1 siRNA to oral cancer cells and enable simultaneous photodynamic therapy (PDT; Ce6 is an established photosensitizer).⁵⁹ The rationale for this approach was that PDT is hindered by activation of epithelial-to-mesenchymal transition (EMT), which is regulated by Wnt signaling. Accordingly, the researchers hypothesized that silencing Wnt-1 would enhance the efficacy of PDT by minimizing EMT. Indeed, their *in vitro* studies confirmed the NPs could inhibit Wnt-1, β -catenin, and vimentin expression in KB oral squamous carcinoma cells, and the combined application of the Wnt-1 siRNA with PDT effectively enhanced cell killing.⁵⁹ In an alternative approach, Tangudu *et al.* developed PEG-polyglycidyl methacrylate (PEG-PGMA) NPs to deliver short hairpin RNA (shRNA) targeting c-Myc or various types of miRNA to different types of cancer.¹⁰⁷ NPs containing c-Myc shRNA, miR-105, or miR-200c silenced the expression of both c-Myc and β -catenin in Jurkat cells *in vitro*. Further, oral delivery of c-Myc shRNA-containing NPs to a mouse model of colorectal cancer reduced c-Myc mRNA expression in tumors and resulted in β -catenin relocation from the nucleus to the cytoplasm, indicating inhibition of Wnt signaling.¹⁰⁷ Survival was dramatically improved in these mice compared to all other controls.¹⁰⁷

Overall, enabling selective inhibition of β -catenin and other Wnt pathway targets could transform cancer management, but is challenging to achieve. As new and improved NP platforms for RNA delivery are developed, the use of RNAi to regulate Wnt signaling will likely yield impressive results against cancer.

Antibody-Nanoparticle Conjugates for Modulation of Wnt Signaling

While small molecule therapeutics and RNA molecules must be delivered into cells to elicit their effects, antagonistic antibodies can function from outside the cell, circumventing many delivery barriers. As noted earlier, antagonistic antibodies typically elicit signal cascade interference by binding their targeted receptor on the cell surface to lock it in a ligand-unresponsive state. Recently, researchers have shown that antibody-NP conjugates are drastically more effective than freely delivered antibodies because they can exhibit multivalency by engaging multiple receptors simultaneously, resulting in increased overall binding strength and enhanced signaling inhibition (Fig. 4a).^{88,95} Besides targeting cellular receptors, antibody-NP conjugates can also be designed to bind ligands in the extracellular environment and sequester them from cellular interactions. Additionally, peptide-NP conjugates can be used as an alternative to antibody-NP conjugates.⁷¹ Examples of the use of these strategies to suppress Wnt signaling in cancer are described below.

Recently, Riley and Day demonstrated that Wnt signaling could be suppressed in TNBC cells using Frizzled7 (FZD7) antibodies conjugated to gold nanoshells (NS).⁸⁸ As FZD7 overexpression drives hyperactive Wnt signaling and disease progression in TNBC cells, Riley and Day hypothesized that blocking Wnt3a-mediated activation of FZD7 could inhibit cellular viability. *In vitro* studies showed that FZD7-NS had a lower effective dissociation constant (i.e. greater binding avidity) for TNBC cells than freely delivered FZD7 antibodies, demonstrating the importance of multivalency. Further, MDA-MB-231 cells that were treated with FZD7-NS in the presence of Wnt3a (to competitively bind the receptors) exhibited dramatic reductions in β -catenin expression and nuclear localization, and these effects were not observed when 50X higher doses of free FZD7 antibodies were administered to the cells (Fig. 4b). Moreover, only the FZD7-NS-treated cells exhibited decreased expression of the downstream Wnt-target gene Axin2 by qRT-PCR and reduction of cellular metabolic activity by MTT assay.⁸⁸ Taken together, these findings indicate that FZD7-NS conjugates have substantial potential as a treatment for TNBC and other cancers driven by Wnt signaling.

Notably, peptides offer a cheaper alternative to antibodies that also offer excellent binding strength. Accordingly, using peptide-NP conjugates to block

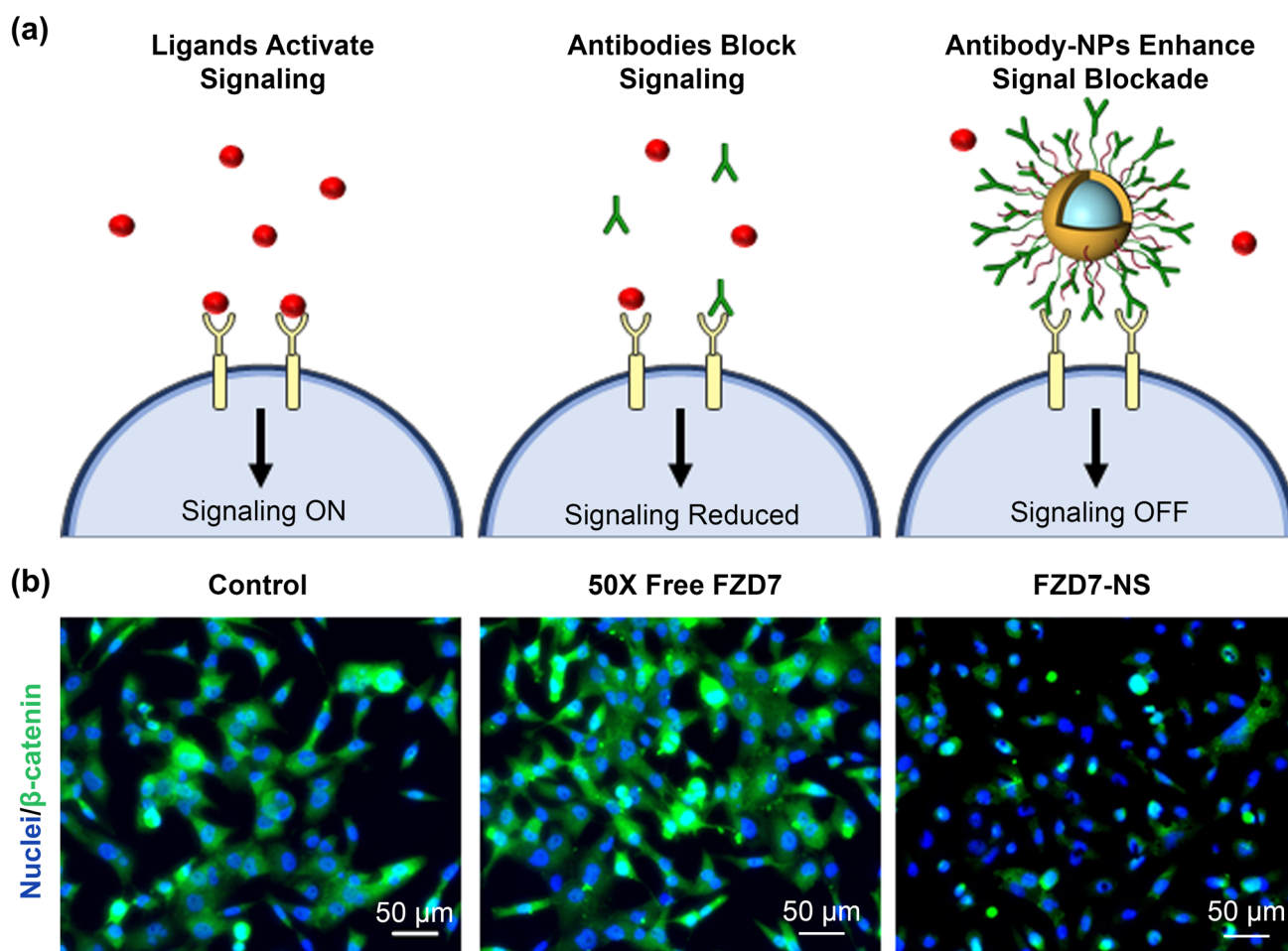


FIGURE 4. Comparison of Wnt signaling inhibition mediated by freely delivered antibodies versus antibody-nanoparticle conjugates. (a) In cancer cells, ligand/receptor interactions activate downstream oncogenic signaling. Antibodies can reduce signaling by binding receptors to lock them in a ligand unresponsive state, and antibody-NP conjugates can enhance signaling blockade by engaging multiple receptors simultaneously. (b) Immunofluorescence analysis of β -catenin expression in MDA-MB-231 triple negative breast cancer cells shows that FZD7 antibody-nanoshell (FZD7-NS) conjugates are more effective than freely delivered antibodies at suppressing β -catenin expression and nuclear localization. Reproduced with permission from Ref. 73, Riley *et al.* Small. Copyright 2017 Wiley.

Wnt signaling is also an attractive therapeutic strategy. Miller-Kleinhenz *et al.* demonstrated this by developing ultra-small magnetic iron oxide nanoparticles (IONPs) coated with peptides that dually target both Wnt/LRP5/6 and uPA receptors.⁷¹ This dual-targeted system successfully inhibited the Wnt/ β -catenin signaling pathway in breast cancer cells, as indicated by reduced protein expression of β -catenin, Axin, GSK-3 β , and Snail.⁷¹ Moreover, the systemic administration of the NPs into mice bearing patient derived xenograft (PDX) tumors led to stronger tumor growth inhibition than non-targeted or single-targeted NPs.⁷¹ Overall, these findings, coupled with those of Riley and Day, demonstrate the utility of exploiting NP multivalency to achieve and enhance antibody-or peptide-mediated Wnt signaling cascade interference and hinder tumor growth. Future studies should continue to explore the

effect of antibody-NP and peptide-NP conjugates against Wnt-dependent tumors, while performing fundamental studies to improve understanding of design characteristics that maximize effect.

NANOTHERAPEUTICS TO SUPPRESS HEDGEHOG SIGNALING IN CANCER

Hedgehog Signaling in Cancer

Hh was initially discovered as a “segment polarity” gene that controls *Drosophila* embryonic cuticle pattern.⁷⁹ Since then, dysregulated Hh signaling has been associated with birth defects⁷⁸ and a diverse spectrum of cancers including basal cell carcinoma (BCC), medulloblastoma, pancreatic ductal adenocarcinoma (PDAC), esophageal and stomach cancer, small-cell

lung cancer (SCLC), breast cancer, and more.^{24,49,103} Therefore, pharmacological blockade of the Hh signaling pathway has emerged as a promising anti-cancer therapeutic strategy. In mammals, three Hh homologues with different spatial and temporal distribution patterns have been identified: Sonic (Shh), Indian (Ihh), and Desert (Dhh), of which Sonic is the best studied.⁴⁷ In the canonical Hh signaling pathway (Fig. 2b), activation is most commonly initiated when an extracellular Hh ligand binds to Patched 1 (Ptc), which relieves its repression of Smoothened (Smo) to drive an intracellular signaling cascade that ultimately results in the activation of three Gli transcription factors, with Gli1 and Gli2 functioning primarily as activators and Gli3 as a suppressor. These transcription factors in turn regulate the expression of genes that promote proliferation, apoptosis suppression, migration, invasion, angiogenesis, DNA damage repair, drug efflux, and cancer stemness.^{24,49,67,91}

A wealth of small molecules, RNAs, and antibodies have been developed and studied for their ability to suppress the Hh signaling pathway (Fig. 3b; Table 1), with some of them undergoing clinical trials or having been approved by the FDA for cancer treatment.¹³ Below, recent examples of NPs that have been developed to deliver these molecules to cancer for Hh signaling pathway suppression are summarized, as well as potential opportunities for future therapeutic development.

Nanoparticle Delivery of Small Molecules to Regulate Hh Signaling

While Hh ligands and Ptc receptors are potential targets for Hh blockade,¹²¹ the majority of small molecules designed to inhibit Hh signaling target Smo. However, clinical applications of these Smo antagonists are restricted by their poor systemic bioavailability, cellular development of resistance through genetic alterations, and additional Gli activation via non-canonical pathways.^{38,83} NP delivery systems that seek to overcome these translational issues focus on two classes of Hh inhibitors, similar to those used in Wnt signaling inhibition: naturally occurring inhibitors and synthetic inhibitors.

The most extensively explored natural inhibitors of the Hh signaling pathway are cyclopamine (CPA) and its analog vismodegib. CPA is isolated from corn lily, and although its inhibition of Hh signaling has been well reported,^{16,104} it faces many barriers to clinical translation, such as poor stability and water solubility, and severe adverse effects. Many NP formulations that incorporate CPA have been designed to overcome these clinical application hurdles. For example, You *et al.* developed a CPA-loaded lipid NP system and

found it effectively enhanced radiation therapy in breast and pancreatic cancer cells.¹²⁶ In addition, Hu *et al.* developed redox-responsive polymeric NPs to co-deliver CPA and doxorubicin and demonstrated remarkable synergistic anti-tumor effects in an orthotopic breast cancer model.⁴⁰ In a similar strategy that combines CPA delivery with chemotherapy, researchers have engineered CPA-paclitaxel (PTX) polymer NPs and utilized them to treat prostate cancer¹²⁴ and pancreatic cancer¹³⁰ in mice. Finally, Jiang and colleagues recently developed a biomimetic NP delivery system in which CPA was encapsulated in erythrocyte membrane-camouflaged poly(lactic co-glycolic acid) (PLGA) NPs,⁵⁰ which enhances CPA delivery by exploiting the long circulation time of the red blood cell-mimetic NPs.¹⁴ Excitingly, combining these NPs with PTX-loaded NPs successfully improved PTX delivery to tumors by disrupting the tumor extracellular matrix, increasing functional vessels, and improving tumor perfusion; consequently, this combination therapy achieved remarkable tumor growth inhibition *in vivo*.⁵⁰

In 2012, the FDA approved vismodegib, a functional analogue of cyclopamine, for basal cell carcinoma therapy, making it the first Shh pathway drug approved for treating cancer. Although vismodegib is more water-soluble than cyclopamine, its solubility is still a limiting factor to its systemic bioavailability. To solve this issue, several research groups have developed polymeric NPs for vismodegib delivery. For instance, Wang *et al.* encapsulated vismodegib in NPs made from SN38 prodrug polymers (SN38 is the active metabolite of irinotecan) to treat pancreatic ductal adenocarcinoma, which is among the most fibrotic and difficult to treat tumors.¹¹⁶ Since Hh signaling plays an important role in the communication between tumor cells and stromal cells, the researchers hypothesized that vismodegib-mediated inhibition of Hh signaling could increase intratumoral drug diffusion and prevent drug resistance. Indeed, they demonstrated that their NPs suppressed Gli1 in the tumor microenvironment in a murine xenograft model, which effectively reversed fibroblast-induced resistance to treatment with SN38.¹¹⁶ Similarly, Ray *et al.* developed pH-responsive polymeric NPs for vismodegib/gemcitabine co-delivery to pancreatic cancer.⁸⁶ Both of these studies indicate that NP-mediated delivery of Hh inhibitors has great potential as a strategy to potentiate chemotherapy.

In addition to CPA and vismodegib, several other naturally occurring Hh inhibitors have been reported and incorporated into NPs. Verma *et al.* found that anthothecol-loaded PLGA NPs could effectively reduce cell proliferation and colony formation and induce apoptosis in pancreatic cancer stem cells (CSCs) by disrupting Gli-DNA binding activity.¹¹⁴ In further

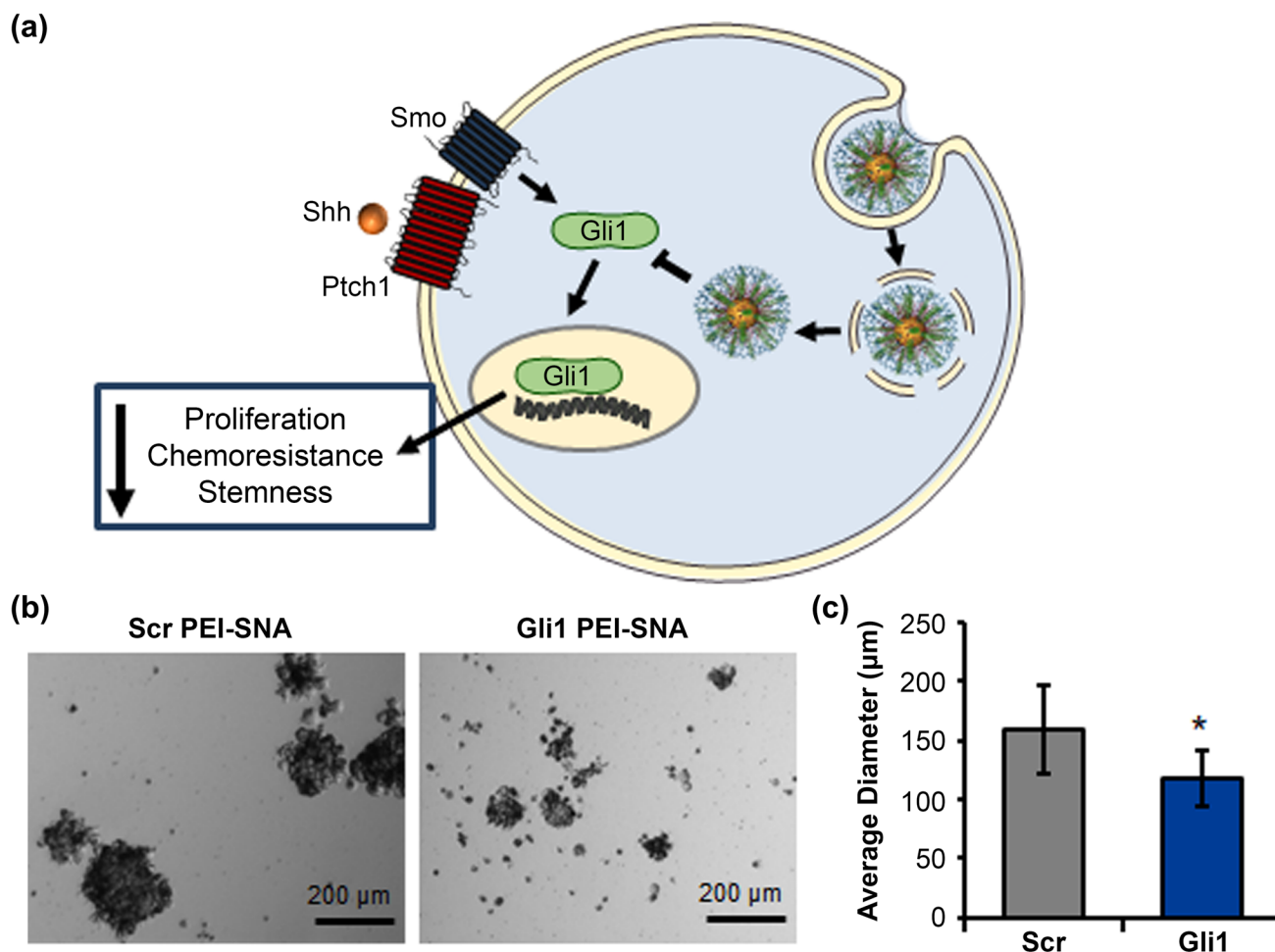


FIGURE 5. (a) Scheme depicting the use of Gli1-targeted PEI-SNAs to suppress Hh signaling in glioblastoma cells and mitigate cellular proliferation, chemoresistance, and stemness. (b) Brightfield images of U87 neurospheres treated with PEI-SNAs carrying Gli1 siRNA or scrambled siRNA. (c) Average diameter of U87 neurospheres treated with PEI-SNAs carrying Gli1 or scrambled siRNA. * $p = 0.03$ by Student's t test. Reprinted with permission from Ref. 65. Copyright 2018 American Chemical Society.

studies, the team also found that α -Mangostin-loaded PLGA NPs could inhibit pancreatic CSC growth, development, and metastasis through disruption of Gli-DNA binding activity.¹¹³ Similarly, Cinzia *et al.* found that glabrescione B encapsulated in PLGA NPs had remarkable cell killing activity against Hh-dependent CSCs and some non-stem-like cancer cells.⁴⁶

NP formulations have also been designed to deliver synthetic Hh inhibitors to cancer. For example, Nayak *et al.* found that NPs loaded with quinacrine (QC), a drug used to treat malaria, could reduce Gli1 mRNA levels by increasing expression of the Hh components GSK-3 β and PTEN, thus inducing apoptosis in CSCs.^{76,77} Similarly, Anders and colleagues found that PLGA NPs encapsulating the Gli1/2 antagonist HPI-1 could significantly inhibit the growth and metastasis of hepatocellular carcinoma models in mice, and significantly decrease the population of CD133-positive cells, which are implicated as CSCs in liver cancers.¹²²

Chenna *et al.* explored these NPs further and showed that when they were combined with gemcitabine, they significantly impeded the growth of orthotopic pancreatic cancer xenografts that have a ligand-dependent, paracrine mechanism of Hh activation.¹⁷ Finally, researchers have loaded the Gli1 inhibitor GANT61 in PLGA NPs, and demonstrated inhibition of Gli1 nuclear translocation, resulting in increased cancer cell death and reduction of CSCs.⁹ These observations support the continued development of NPs as vehicles to deliver small molecule Hh inhibitors to cancer.

RNA Interference of Hedgehog Signaling Mediators

RNAi for Hh signaling inhibition has largely focused on Gli transcription factors, as they are the key oncogenic mediators of this pathway.⁴⁴ In one study, Melamed *et al.* developed a robust platform for Gli1 inhibition in which Gli1 siRNA was conjugated to 13

nm diameter gold NPs to produce spherical nucleic acids (SNAs) that were subsequently wrapped with polyethyleneimine (PEI) (Fig. 5a).⁵⁷ The resultant PEI-SNAs efficiently entered glioblastoma (GBM) cells and suppressed the expression of Gli1 and several of its downstream targets (Smo, cyclin D1, c-Myc, Bcl-2, and ABCG2). Through Hh signaling inhibition, these PEI-SNAs slowed cellular proliferation, induced senescence, and increased sensitivity to temozolomide (TMZ), the frontline chemotherapy for GBM.^{65,67} Additionally, the NPs mitigated the stemness of GBM cells, thereby reducing neurosphere formation (Figs. 5b and 5c).⁵⁷

Zhou *et al.* also demonstrated the ability to deliver siGli1 to cancer cells using NPs formed by self-assembly of 1,2-dioleoyl-3-triethylammonium-propane (DOTAP)-conjugated methoxy-PEG-poly(lactide) copolymer, a non-viral gene delivery vector.¹³¹ These particles induced apoptosis and inhibited GBM cell growth *in vitro*, and slowed the growth of subcutaneous GBM xenografts *in vivo*.¹³¹ These findings corroborate the observations of Melamed *et al.* that indicate Gli1 inhibition is a promising strategy to combat GBM and other Hh-dependent cancers.

Future Potential for Antibody Modulation of Hedgehog Signaling

Antibody blockade of Hh signaling has only recently begun to be explored. While several antagonistic antibodies have been developed for Hh suppression, such as the anti-Shh antibodies 5E1 and MEDI-530^{4,69,75,80,125} they have not yet been incorporated into antibody-NP conjugates. Given that antibody-NP conjugates targeting Wnt signaling have shown greater efficacy than freely delivered antibodies, it is likely that antibody nanocarriers targeting Hh signaling will emerge in the near future as a therapeutic strategy.^{3,88}

NANOTHERAPEUTICS TO SUPPRESS NOTCH SIGNALING IN CANCER

Notch Signaling in Cancer

The Notch signaling pathway is a key regulator of cell-to-cell communication that is important for embryonic development and the maintenance and renewal of adult tissues.⁹⁴ Notch signaling is activated when one of four canonical ligands (Delta1, 3, or 4, or Jagged 1/2) on signal-sending cells bind the extracellular region of Notch receptors 1-4 on signal-receiving cells (Fig. 2c).²⁷ This initiates a downstream cascade of cleavages, first by ADAM family proteases that remove the extracellular portion of Notch receptors from

their transmembrane domain, and subsequently by the gamma secretase complex that removes the intracellular portion of Notch receptors from the transmembrane domain. Ultimately, this cascade leads to the nuclear translocation of the Notch intracellular domain (NICD) to the nucleus.¹²⁷ In the nucleus, NICD complexes with MAML1 and CSL to promote the transcription of genes associated with cell survival, proliferation, and stemness (Fig. 2c). In addition to this ligand dependent activity, the Notch signaling pathway can also undergo ligand independent activation that is mediated by ADAM17.⁸²

As noted before, Notch signaling is frequently dysregulated in cancer.¹²⁷ Depending on the type of cancer, different Notch receptors have varying levels of expression and prognostic implications. In certain cases, Notch can even serve as a tumor suppressor,^{42,45,117} but its overactivation is largely considered oncogenic.¹ While overexpression of Notch 2-4 has been implicated in a variety of cancers,^{1,36,37,99} Notch-1 in particular is frequently overexpressed in cancer and serves as a promising target for therapeutic manipulation.^{27,64,82,90,101} The following sections review the major small molecule, RNA, and antibody-based therapies that utilize nanocarriers to treat cancer through regulation of Notch signaling.

Small Molecules to Regulate Notch Signaling

Small molecule therapies are being developed to inhibit several different components of the Notch signaling pathway (Fig. 3c). ADAM inhibitors block the first of the two cleavages of the Notch receptor that occur after signaling activation. As noted above, ADAM family proteins cleave off the extracellular domain of the Notch receptor. ADAM inhibitors prevent this extracellular cleavage to halt further signaling.²⁷ More specifically, ADAM10 inhibitors prevent ligand-dependent activation of Notch signaling, while ADAM17 inhibitors interfere with ligand-independent receptor activity.⁸² Further downstream, MAML1 inhibitory peptides block the activity of the NICD-CSL-MAML1 complex, thereby preventing the transcription of oncogenic Notch target genes.^{27,68,81} The most widely explored class of small molecule inhibitors of Notch signaling are gamma secretase inhibitors (GSIs), which block cleavage of the NICD and prevent its translocation to the nucleus (Fig. 6a).^{27,127} Neither ADAM inhibitors nor MAML1 inhibitors have been incorporated into NP formulations to date, but GSIs have begun to be explored in nano-formulations.

While GSIs are potent against Notch-dependent cancers, they commonly exhibit severe gastrointestinal

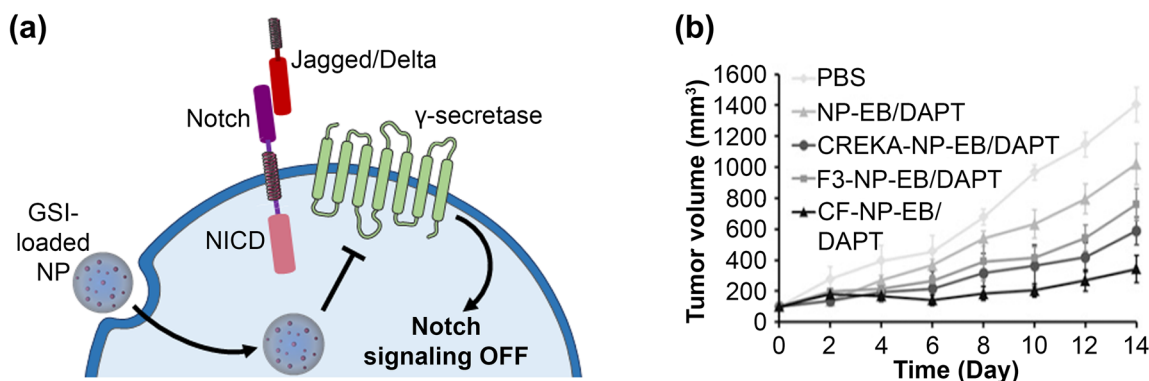


FIGURE 6. (a) Scheme depicting the use of nanoparticles carrying a gamma secretase inhibitor (GSI) to suppress Notch signaling in cancer cells. (b) Nanoparticles carrying the GSI DAPT and the drug erlotinib that were coated with both CREKA tumor-homing peptides and F3 cell-penetrating peptides reduced the growth of triple negative breast cancer tumors to a greater extent than NPs coated with either targeting agent individually. Reproduced with permission from reference ¹¹⁵ under the Creative Commons Attribution License. Copyright 2019 Xu Wan, Chaoqian Liu, Yinan Lin, Jie Fu, Guohong Lu, and Zhengmao Lu. Published by Informa UK Limited, trading as Taylor & Francis Group.

side effects when delivered freely as they are typically delivered orally due to low solubility in aqueous conditions.¹²⁷ One effective method to minimize these toxic off-target effects is to encapsulate GSIs within NPs. In an intriguing study, Mamaeva *et al.* developed mesoporous silica NPs to deliver the GSI DAPT to breast cancer.^{61,62} In testing this NP formulation against MDA-MB-231 breast cancer cells transplanted on chorioallantoic membranes, Mamaeva *et al.* found that NP-mediated delivery of DAPT successfully inhibited Notch signaling to subsequently reduce the CSC population. Lu and colleagues also delivered DAPT, in combination with the EGFR-inhibitor erlotinib, to triple negative breast cancer (TNBC) using polylactic acid-based NPs.¹¹⁵ When the NPs were coated with both CREKA, a tumor-homing peptide, and F3, a cell-penetrating peptide, they could achieve greater anti-tumor effects *in vivo* than NPs coated with either external functional agent alone or no coating (Fig. 6b).⁹⁹ Besides DAPT, several other GSIs are in clinical development (e.g., MRK-560, MK-0752, BMS-906024) that may benefit from delivery via NP carriers, as these hydrophobic drugs could be encapsulated in NPs to enhance their systemic tumor delivery and limit the side effects they induce.^{19,33,72,85}

While small molecule inhibitors of Notch like those described above are effective, they also face limitations. For example, GSIs are pan-Notch inhibitors that affect Notch 1–4 simultaneously, which can elevate toxicity versus targeting the receptors individually.¹¹⁹ Further, given that specific receptors play distinct roles in driving the progression of different types of cancer, it would be beneficial to inhibit these receptors individually. Two methods that lend themselves to specific

Notch inhibition are RNA interference and antibody-based therapeutics, as discussed below.

Nanoparticle-Mediated RNA Interference of Notch Signaling

RNAi directed at Notch signaling largely targets the Notch receptors and their ligands, rather than attempting to suppress downstream pathway mediators. Liu and colleagues have developed iron oxide-silica NPs to effectively deliver shRNA targeting Notch-1 to TNBC.¹²³ Through their work, they demonstrated that these NPs effectively reduce Notch-1 expression and cell proliferation, and induce apoptosis in MDA-MB-231 TNBC cells. Similarly, Steg *et al.* employed chitosan NPs to deliver siJagged1 to treat ovarian cancer.¹⁰⁰ These NPs reduced angiogenic behavior in endothelial cells and inhibited chemoresistance and proliferation of tumor cells in an orthotopic mouse model of ovarian cancer.⁸⁴ In a less direct approach, Deng *et al.* examined the effect of delivering the tumor suppressive microRNA miR-34a to TNBC using chitosan-based NPs.²³ They showed that miR-34a delivery to MDA-MB-231 TNBC cells via these NPs inhibited cell migration and reduced protein expression of Notch-1. Overall these studies demonstrate that interfering with Notch signaling, particularly at the receptor/ligand level, can significantly reduce the proliferation of cancer cells and halt tumor progression.

Antibody Nanocarriers to Modulate Notch Signaling

As with the Wnt and Hh signaling pathways, several antibodies have been developed and tested to antago-

nize Notch signaling, with the majority of research focusing on Notch-1 inhibition.^{4,27,97,119} However, these antibodies have not yet been incorporated into NP systems, which would likely increase their potency due to multivalency. This is an area with great potential for future development. Notably, Biktasova *et al.* have demonstrated the potential impact of exploiting multivalency by administering multivalent forms of the Notch receptor ligand DLL-1 to preclinical mouse models of lung cancer.⁸ They created DLL-1 clusters by biotinylating the Fc portion of IgG antibodies fused with the extracellular domain of DLL-1 and mixing them with NeutrAvidin. These clusters were able to regulate Notch signaling and activate a tumor-specific T-cell response for enhanced anti-tumor effect.⁸ In the future, antibody nanocarriers designed to modulate Notch signaling could transform the management of Notch-dependent cancers.

CHALLENGES AND OPPORTUNITIES

As highlighted in this review, various NP formulations have been designed to deliver small molecule, RNA, and antibody inhibitors of the developmental Wnt, Hh, and Notch signaling pathways to cancer to halt disease progression (Table 2). Although these pathways are not expressed in all cancer types, they are highly implicated in many aggressive cancers, making them valuable targets for NP-mediated inhibition. While the NP formulations discussed in this review can improve the pharmacokinetics and safety profiles of their designated cargo⁹³ and often reduce the administered dose required,⁸⁸ there are still challenges to overcome both in achieving significant levels of therapeutic efficacy and in reaching clinical translation.

One key challenge in targeting Wnt, Notch, or Hh signaling is that cells can develop resistance by exploiting complexity within the pathway. For example, as discussed previously, Smo inhibitors for the Hh signaling pathway can encounter resistance when Gli1 is upregulated through noncanonical pathways.⁸³ A further mechanism of resistance is that there is substantial crosstalk between the Wnt, Notch, and Hh signaling pathways, and a molecule that acts as a tumor-promoter in one pathway may play a tumor-suppressive role in another pathway. GSK-3 β , for example, is a key mediator of the Wnt signaling pathway and serves as a popular target for inhibition,⁷³ but in the Hh signaling pathway, this protein can serve as a suppressor and its expression is thus desired.^{76,77} NPs afford the opportunity to counter some of this developed resistance to treatment with the possibility for co-encapsulation or delivery of multiple therapeutic molecules, but this presents additional

challenges for future clinical evaluation and implementation. For a more in depth review of the crosstalk between these pathways, readers should refer to the review by Borggreffe *et al.*¹⁰

In addition to the challenges associated with cellular resistance, there are also barriers to effective tumor delivery of NPs that target the Wnt, Notch, and Hh signaling pathways. Recent studies have indicated that only 0.7% of injected NP doses actually reach solid tumors.¹¹⁸ This means off-target toxicity could be a concern, as Wnt, Notch, and Hh signaling play a regulatory role in some adult tissues.^{39,128} For example, Wnt signaling plays an important role in bone repair and regeneration.³⁹ Inhibiting Wnt signaling can lead to bone loss, but the delivery of bisphosphonates can mitigate these side effects.⁶⁰ Moving forward, researchers should develop strategies to increase the fraction of NPs that reach tumors versus healthy organs to minimize toxicity, and also explore the possibility of delivering concurrent therapeutics such as bisphosphonates to alleviate any side effects that do occur.

Besides the challenge of reaching tumors versus healthy organs, once in tumors, NPs encounter a variety of cell types and extracellular matrix components that limit their ability to penetrate deeply beyond vessel walls. Chan and colleagues calculated that only 0.0014% of an injected NP dose reaches the desired cancer cells within a tumor.²² Notably, inhibition of Wnt, Notch, and/or Hh signaling may modulate the tumor microenvironment to enhance NP delivery. For example, Jiang *et al.* demonstrated that cyclopamine-loaded NPs that inhibit Hh signaling could disrupt the tumor extracellular matrix and enhance tumor perfusion. Combining this treatment with PTX-loaded NPs significantly improved the delivery of PTX to the tumor to enhance treatment effect.⁵⁰ This work provides evidence that modulating the tumor microenvironment through inhibition of developmental signaling pathways may improve the tumor delivery of NPs, but this should be thoroughly investigated through mechanistic studies in the future.

A further consideration when creating NPs to regulate developmental signaling pathways is the location of the targeted protein or molecule within the cancer cell itself (extracellular, cytosolic, or nuclear), as this will dictate the choice of therapeutic agent that should be carried by the NP. For example, large therapeutic molecules such as antibodies can typically only be delivered to extracellular sites, making antibody-NP conjugates best suited for inhibition of soluble ligands or surface-bound receptors. Notably, substantial progress has been made in recent years towards enabling intracellular antibody delivery through clever nano-engineering or chemical modification of NPs with cell-

penetrating peptides.^{32,56,70,98} With continued optimization, antibody-NP conjugates may be used to suppress both extracellular and intracellular targets in the Wnt, Notch, and Hh signaling pathways.

When designing NPs to attack intracellular targets in developmental pathways, one must also consider that the mechanism of endocytosis can influence the fate of the NP and thus impact the potency of the cargo.⁵⁴ In particular, the delivery of RNA molecules requires endolysosomal escape so that the released RNA cargo can bind the RNA-induced silencing complex and impact gene expression.²⁵ Studies have shown that cargo internalized by clathrin or caveolae-dependent mechanisms can be routed to lysosomes, but caveolae can also fuse with caveosomes in some cases to avoid lysosome accumulation.^{74,84,87} Thus, when designing NP carriers, it is important to consider both the mechanism of cellular uptake and of action of the cargo.

Two final hurdles to clinical translation of NPs targeting Wnt, Notch, and Hh signaling include the processing required for large-scale manufacturing and the cost of this production. While NP delivery systems can limit cost of therapeutic agents by reducing the effective dose, the additional cost of the nanocarrier material, the cost of any product lost during NP synthesis, and the scalability of the synthesis are important considerations for these systems as they move toward the clinic. For a more in depth review of the challenges facing NPs and the current standing of clinical translation of these formulations, readers should turn to the review by Hua *et al.*⁴¹

Overall, there are several barriers remaining to clinical translation of NPs targeting Wnt, Notch, and Hh signaling. Biological barriers to translation include complexities within the signaling pathways that can confer resistance on the cancer cells, and continued challenges in delivery not only to tumors, but also to the targeted cells and molecules within tumors. Production barriers including processing constraints and cost also contribute to the challenges of NP therapeutic clinical translation. Successfully overcoming these barriers will result in improved outcomes for patients with a variety of debilitating cancers.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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