REVIEW ARTICLE

Promising molecular mechanisms responsible for gemcitabine resistance in cancer

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Abstract   Gemcitabine is the first-line treatment for pancreatic ductual adenocarcinoma (PDAC) as well as acts against a wide range of other solid tumors. Patients usually have a good initial response to gemcitabine-based chemotherapy but would eventually develop resistance. To improve survival and prognosis of cancer patients, better understanding of the mechanisms responsible for gemcitabine resistance and discovery of new therapeutic strategies are in great need. Amounting evidence indicate that the developmental pathways, such as Hedgehog (Hh), Wnt and Notch, become reactivated in gemcitabine-resistant cancer cells. Thus, the strategies for targeting these pathways may sensitize cancer cells to gemcitabine treatment. In this review, we will summarize recent development in this area of research and discuss strategies to overcome gemcitabine resistance. Given the cross-talk between these three developmental signaling pathways, designing clinical trials using a cocktail of inhibitory agents targeting all these pathways may be more effective. Ultimately, our hope is that targeting these developmental pathways may be an effective way to improve the gemcitabine treatment outcome in cancer patients.

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Introduction

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine; dFdC)[1] is a novel deoxycytidine analogue. Despite of some similarities with other nucleoside analogues such as cytosine arabinoside (AraC), gemcitabine has several unique properties and specific spectrum of activity.[2,3] The cytotoxic lesions caused by gemcitabine include killing cells with active DNA synthesis (S phase) and blocking cell cycle progression at the G1/S phase boundary.[4] Gemcitabine was originally used for its antiviral effects but is now widely used as an anti-cancer chemotherapeutic agent. Gemcitabine is recommended as a single agent for first-line chemotherapy for patients with advanced pancreatic cancer.[5] It is also used for chemotherapy for patients with non-small cell lung cancer,[6] breast cancer,[7] bladder cancer[8] and ovarian cancer.[9]

Patients usually have a good initial response to gemcitabine-based chemotherapy but develop resistance by time. Gemcitabine resistance can be either intrinsic or acquired. Resistance can result from molecular and cellular changes, including nucleotide metabolism enzymes, inactivation of the apoptosis pathway, high expression of drug efflux pumps, activation of the cancer stem cells or epithelial-to-mesenchymal transition (EMT) pathway, up- or down-regulated expression of microRNA (miRNA) (Fig. 1). It has been demonstrated that pathways such as Hedgehog (Hh), Wnt and Notch, which regulate embryonic development and somatic stem cells (SCs), can be reactivated in gemcitabine resistance cancer cells (Fig. 2). Herein, we will describe recent advances towards targeting these pathways with a goal to overcome gemcitabine resistance.

Gemcitabine metabolism and mechanism of action

Gemcitabine is transported into cells by sodium-dependent (concentrative nucleoside transporter hCNTs) and sodium-independent (equilibrative nucleoside transporter hENTs) mechanisms.[10,11] Once inside the cell, gemcitabine undergoes a series of phosphorylation by deoxycytidine kinase (dCK) to the monophosphate (dFdCMP) and then by pyrimidine nucleoside monophosphate kinase (UMP–CMP kinase) to give gemcitabine diphosphate (dFdCDP), resulting in formation of gemcitabine triphosphate (dFdCTP) mediated by nucleoside diphosphate kinase (NDPK).[12] The first phosphorylation by dCK is considered the rate-limiting step for dFdCDP and dFdCTP production.

Gemcitabine can get inactivated through deamination by cytidine deaminase (CDA), and when in the monophosphate form by deoxycytidylate deaminase (dCTD). Gemcitabine may also become inactivated by dephosphorylation of the monophosphate form by 5′-nucleotidases (5′-NTs), converting nucleotides back to nucleosides. On the other hand, gemcitabine exhibits a unique property called self-potentiation to enhance its activation. The diphosphate form (dFdCDP) inhibits ribonucleotide reductase M1 or M2 (RRM1/RRM2) that convert CDP to dCDP, leading to depletion of dCTP pools and facilitating incorporation of dFdCTP into DNA.[13]

Known mechanisms of gemcitabine resistance

One mechanism responsible for gemcitabine resistance is the dysregulation of the proteins participating in gemcitabine metabolism pathways, including deficiency of hENT1,
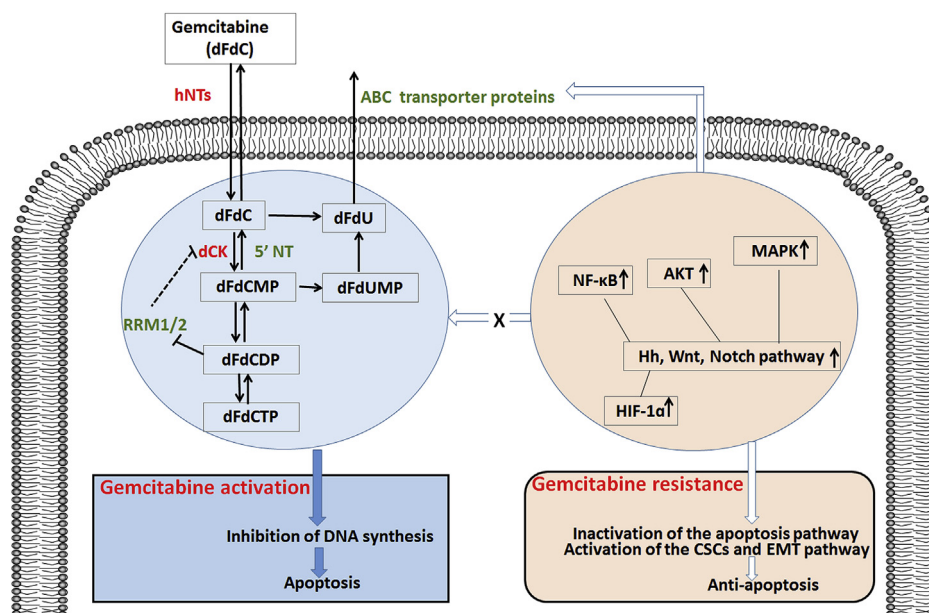

Fig. 1   A diagram of gemcitabine metabolism and proposed mechanisms of gemcitabine resistance. Gemcitabine (dFdC) is a pro-drug that requires cellular uptake and serial phosphorylation to become pharmacologically active. Gemcitabine can interrupt DNA synthesis and then induce cancer cells apoptosis. Gemcitabine resistance can be either intrinsic or acquired. Resistance can result from molecular and cellular changes, including nucleotide metabolism enzymes, apoptosis pathway, ABC transporter proteins, activation of the CSCs and EMT pathway.
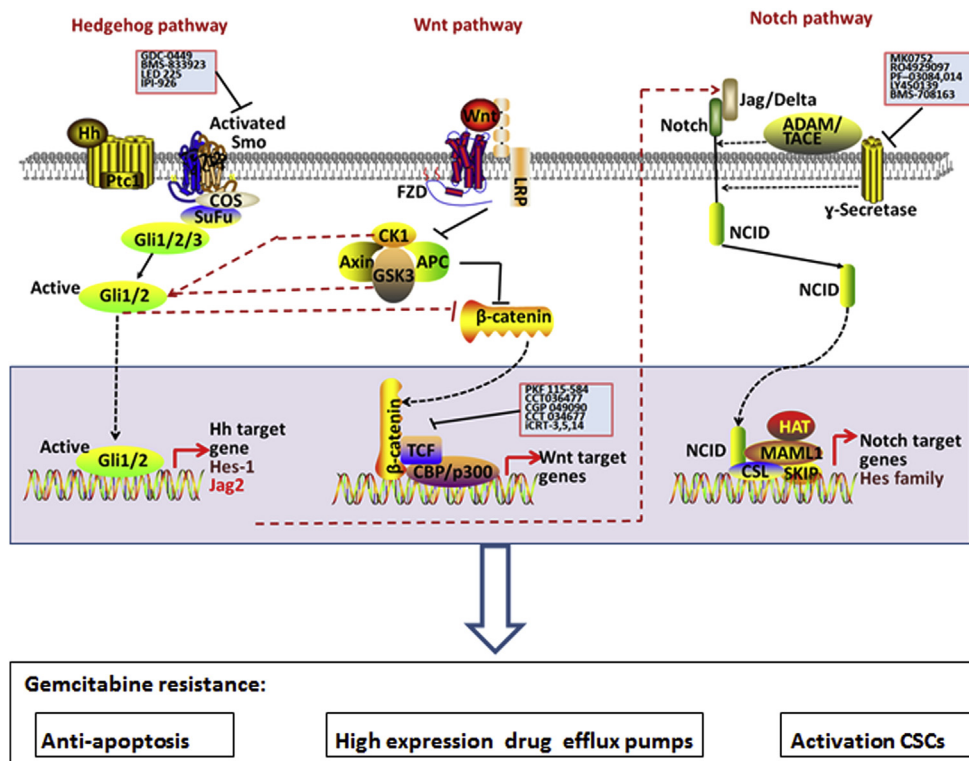
Fig. 2 **Diagrams of the Hedgehog, Wnt, Notch pathways and their roles for gemcitabine resistance**. Hedgehog (Hh), Wnt and Notch signaling, which regulate embryonic development and somatic stem cells, may be reactivated in gemcitabine-resistant cancer cells. Elevated expression of the target genes in these pathways may result in inactivation of the apoptosis pathway, high expression of drug efflux pumps or activation of the cancer stem cells (CSCs), ultimately leading to gemcitabine resistance.

down-regulation of rate-limiting enzyme dCK, and up-regulation of RRM1/RRM2.[14,15] Hu antigen R (HuR), which is an RNA-binding protein that post-transcriptionally regulates dCK, is also associated with gemcitabine efficacy. High levels of HuR correlated with a better overall survival in gemcitabine-treated patients.[16]

Another gemcitabine resistance mechanism is high expression of drug efflux pumps, such as (ABC) transporter family proteins.[17] These proteins are highly expressed in cancer stem cells (CSCs) and have been shown to protect CSCs from chemotherapeutic agents.[18] Recent evidence has supported the link of gemcitabine resistance and EMT with stem cell phenotype.[19] In general, therapeutics directed against CSCs and EMT offer new strategies to chemoresistance.

Gemcitabine resistance is also associated with multiple genetic and epigenetic abnormalities. Changes in one or a few genes remain crucial for maintaining drug resistance cell survival and malignant phenotype. There is evidence to indicate that NF-κB,[20–23] AKT,[20,24,25] MAPK,[25,26] HIF-1α[27] pathways are involved in gemcitabine resistance *in vitro* and some *in vivo* models (Fig. 1). However, the functional significance of these molecules for gemcitabine resistance remains largely untested.

Relevance of hedgehog signaling to gemcitabine resistance

As an essential developmental signaling pathway, the Hh pathway is critical for maintaining tissue polarity and

stem cell population. Hedgehog signaling molecules in mammal include three ligands (Sonic hedgehog-Shh, Indian hedgehog-Ihh and Desert hedgehog-Dhh), two receptors (Patched1-Ptc1, Patched2-Ptc2), a key signal transducer smoothened (Smo) and three transcription factors (Gli1, Gli2, Gli3). When ligands are not present, Smo function is inhibited by another transmembrane protein (Ptc1, Ptc2). Upon binding of an active Hh ligand, this inhibitory effect is lifted, allowing Smo to signal downstream, eventually leading to active transcription of Gli molecules through binding the specific consensus sequences located in the promoter region of target genes.[28,29] Besides these canonical Hh pathways, there are non-canonical Hh pathways activated directly through Ptc1 or Smo, or other pathways including the PI3K/AKT and MEK signaling cascades.[30] Dysregulated signaling through the Hh pathway is implicated in virtually all human cancers. Furthermore, Hh pathway is now also recognized as a major driver of resistance to a number of chemotherapeutical reagents. Smo inhibitors, including vismodegib (GDC-0449), sonidegib (LDE225), BMS-833923, PF-04449913, and LY2940680, are being tested in a range of advanced cancers (NCI Clinical Trail Database (http://www.clinicaltrials.gov/)). To our disappointment, however, the Smo inhibitors seem to be ineffective in most extracutaneous solid tumors.[31] Therefore, Smo inhibition alone may not provide sufficient efficacy in these cancers, and combinations with other conventional cytotoxic anti-tumor agents might be needed to achieve maximal benefit. Currently, several Phase II trials of hedgehog

pathway inhibitor vismodegib and Phase I trials of LDE225 in combination with gemcitabine are ongoing (http://www.clinicaltrials.gov/).

Activation of Hh signaling may not work alone because cross-talk with other signaling pathways plays critical roles in gemcitabine resistance. For example, MAP3K10 over-expression can result in up-regulation of Gli1 and Gli2, leading to decrease in the gemcitabine sensitivity of in pancreatic cancer cells.[32] Perifosine, a novel Akt inhibitor, through suppressing Gli1 activation, can enhance gemcitabine-induced cytotoxicity in pancreatic cancer cells.[33] Lithium synergistically enhances the anti-cancer effect of gemcitabine, by inhibiting the activity of glycogen synthase kinase 3β (GSK3β), therefore promoting the ubiquitin-dependent proteasome degradation of Gli1.[34] ABCB2, the drug efflux transporters, serves as the downstream target of Shh signaling, and its expression can increase gemcitabine resistance in PDAC cells.[35] Understanding how integration of HH signaling with other pathways and discovery and synthesis of Gli specific inhibitors is in great need for effective suppression of non-canonical Hh signaling in cancer.

Recent reports have also indicated that hedgehog signaling may be critical for maintaining a small cell population with stem cell properties, and thus conferring resistance to chemotherapy. There is evidence to indicate that Hh signaling regulates expression of several cancer stem cell-related markers, such as ALDH1, Bmi1, Snail, Wnt2, PDGFRα, Jagged-1, CD44 and c-MET.[36−42] The level of Hh expression is often higher in the cancer stem cell population.[43−46] Thus, we have reasons to believe that inhibition of Hh signaling may be effective in reducing the number of cancer stem cells, which may play an important role for chemotherapy and radiotherapy resistance. Chloroquine treatment in combination with gemcitabine significantly decreased CSCs, via inhibition of hedgehog signaling in the stroma, which supports CSCs and non-CSCs via a paracrine mechanism.[47] Arsenictrioxide (ATO) is described as a SHH inhibitor. Combination treatment of ATO and low dose gemcitabine inhibits tumor growth, decreases the expression of CD24, CD44, and aldehyde dehydrogenase 1 family member A1 significantly in mouse model.[48] In response to gemcitabine, both Shh and CSCs markers as well as EMT regulators are over-expressed, indicating a protective role of hedgehog signaling against chemotherapeutic drugs.[19] Discovery of a more specific and complete set of cell surface markers may help to further characterize CSCs, and the role that Hh pathway played in drug resistance and the in-depth mechanism require further systemic studies.

The role of Wnt signaling for gemcitabine resistance

The Wnt signaling pathway controls and regulates crucial aspects of development, cell proliferation, survival and polarity.[49] Wnts are a family of 19 secreted glycoproteins, binding to a number of 7-transmembrane receptors of the Frizzled (FZD) family.[50] These receptors transduce signal to several intracellular proteins, such as Dishevelled (Dsh), glycogen synthase kinase-3β (GSK-3), Axin, Adenomatous Polyposis Coli (APC), and β-Catenin. To date, major

signaling branches have been identified including the canonical Wnt/β-Catenin pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway. These pathways are being actively dissected at the molecular and biochemical levels. When FZD co-operates with LRP5/6 (low-density lipoprotein receptor-related proteins 5 and 6), the canonical Wnt/β-Catenin signaling pathway is activated.[51] In the absence of the activating Wnt signals, β-Catenin is phosphorylated by a complex of GSK-3β, proteins APC, β-TRCP and Axin. Non-canonical Wnt signaling can phosphorylate small G-proteins and JNK to transmit the signal to the nucleus, or release calcium affecting Nemo-like kinase (NLK) or nuclear factor of activated T-cells (NFAT).[52]

Emerging evidence indicates that activation of the Wnt pathway is involved in cancer development and drug resistance. Wnt/β-Catenin signaling is activated in gemcitabine-resistant lung cancer cell lines.[53] WNT5A expression leads to resistance to gemcitabine-induced apoptosis in a xenograft model of pancreatic ductal adenocarcinoma (PDAC).[54] The heparan sulfate mimetic PG545 in combination with gemcitabine exerts anti-tumor activity by disrupting Wnt/β-Catenin signaling.[55] Masitinib, a tyrosine kinase inhibitor, can sensitize gemcitabine treatment through down-regulation of the Wnt/β-Catenin signaling pathway in pancreatic cancer cell lines, which is further confirmed in a Phase II clinical study.[56] These therapeutic approaches to target upstream or downstream of Wnt signaling pathway indicate that combination therapy may be required to effectively treat gemcitabine-resistant malignancies.

Recent reports have implicated Wnt pathway may be especially important in maintaining the subpopulation of cancer cells with stem cell properties as well as conferring resistance to chemotherapies. Wnt signaling is increased in pancreatic cancer side population (SP) cells. Furthermore, when cultured with increasing concentrations of gemcitabine, the proportion of SP cells were significantly enriched.[57] EMT has been implicated as a drug resistance mechanism, allowing tumor cells to escape from cytotoxic and pathway targeted therapies. Activation of Wnt signaling up-regulates the EMT activator ZEB1 and determines response to gemcitabine in mantle cell lymphoma.[58] It will be important for future research to unravel the interconnectedness of Wnt pathway with CSCs and EMT in gemcitabine resistance.

The Notch pathway regulates cancer stem cells in gemcitabine resistance

It has been well known that Notch signaling plays important roles for regulating cell—cell communication, cell proliferation, differentiation and apoptosis. Activation of Notch receptors (Notch-1—4) is often achieved by canonical ligands, Delta-like ligands (DLLs) 1, 3 and 4, and Jagged ligands 1 and 2. The binding of ligand—receptor results in γ-secretase-mediated cleavage and nuclear localization of its intracellular domain to regulate notch-specific gene expression.[59]

Evidence suggests that Notch signaling may play a crucial role in the tumor progression and possibly therapy-

resistance.[60,61] Notch-2 and Jagged-1 are highly up-regulated in gemcitabine-resistant pancreatic cancer cells.[62] Notch-3 mRNA expression is predicted to be the key predictive biomarker for gemcitabine effect and sensitivity in pre-treatment endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) samples from patients with PDC.[63] Inhibition of Notch-1 by siRNA enhances the sensitivity to gemcitabine in pancreatic cancer through activating apoptosis activity.[64] Midkine (MK)—Notch-2 interaction increases Notch signaling, elevates EMT, and is associated with gemcitabine resistance.[65] Moreover, inhibition of Notch-3 enhances the sensitivity to gemcitabine in pancreatic cancer through inactivation of the PI3K/Akt-dependent pathway.[66] The γ-secretase inhibitors DAPT and MRK003 effectively inhibit intra-tumoral Notch signaling in PDA, leading to a remarkable increase in chemosensitivity to gemcitabine.[67,68]

Emerging evidence clearly shows that abnormal Notch signaling may contribute to carcinogenesis by deregulating the self-renewal of normal stem cells. Inappropriate activation of Notch signaling could be an early event leading to accumulation of undifferentiated precursor cells in pancreatic cancers.[69] MRK-003, γ-secretase inhibitor, treatment results in down-regulation of nuclear Notch-1 intracellular domain, and a decrease in tumor-initiating cells that are capable of extensive self-renewal.[70] Furthermore, the proportion of CD24$^+$CD44$^+$ CSCs in pancreatic cancer cells line increased after gemcitabine treatment and this increase was mediated by the Notch pathway.[71] A Notch signaling pathway inhibitor (L1790) and Hes-1 siRNA are shown to reverse the chemoresistance induced by PSCs.[72] Neutralizing antibodies against human DLL4 reduces the percentage of CSC in gemcitabine-resistant mouse model.[73] Through down-regulation of Notch-1, Sulforaphane (SF) prevented gemcitabine-induced selection of ALDH-positive cells.[74] As indicated above, Notch signaling may be a promising target to overcome gemcitabine resistance.

Cross-talk among signaling pathways

Cross-talks among Hh, Notch or Wnt signaling pathways have been reported in a variety of cancer cells. The transcription of Jagged-2, a Notch ligand, can be up-regulated in response to Hh pathway activation.[75] Whereas Wnt/β-Catenin can activate Jagged-1,[76] Wnt-1 induces transformation and tumorigenesis in human mammary epithelial cells, through up-regulation of Notch pathway. Notch and Hh signaling both can activate Hes-1.[77] On the other hand, Hh signaling is found to inhibit Wnt signaling via up-regulation of Wnt-inhibitory factor sFRP1, and to suppress β-Catenin transcriptional activity.[78] CK1 and GSK3, the core component of the Wnt pathway, regulate Gli1 proteolytic processing.[79] Therefore, combination therapies targeting more than one pathway in cancer may be more effective. Hh may modulate the tumorigenic property in cooperation with Notch in breast cancer. Phase I and Phase Ib/II trials, combining Hh pathway inhibitor vismodegib and Notch pathway inhibitor RO4929097 are being conducted in patients with breast cancer and sarcoma (http://www.clinicaltrials.gov).

Conclusions and future directions

Gemcitabine still has clinical potential to treat a broad spectrum of human cancers, but resistance is probably the major impediment to achieve satisfactory clinical

Table 1 Summary of combination therapies and their mechanism in regulation gemcitabine resistance.

Pathway	Target	Compound	Mechanism of action
Hh	Smo	Vismodegib, LDE225	Phase II trials of vismodegib and Phase I trials of LDE225 in combination with gemcitabine are ongoing (http://www.clinicaltrials.gov/).
		Chloroquine	Chloroquine treatment in combination with gemcitabine significantly decreased CSCs, via inhibition of hedgehog signaling in the stroma.[47]
	Gli1	Perifosine	Perifosine, an Akt inhibitor, through suppressing Gli1 activation, can enhance gemcitabine-induced cytotoxicity in pancreatic cancer cells[33]
		Lithium	By inhibiting the activity of glycogen synthase kinase 3β (GSK3β), therefore promoting the ubiquitin-dependent proteasome degradation of Gli1.[34]
	SHH	Arsenic trioxide (ATO)	Combination treatment of ATO and low dose gemcitabine inhibits tumor growth, decreases the expression of CD24, CD44, and aldehyde dehydrogenase 1 family member A1 significantly in mouse model.[48]
Wnt	β-Catenin	PG545	PG545 in combination with gemcitabine exerts anti-tumor activity by disrupting Wnt/β-Catenin signaling.[55]
		Masitinib	Masitinib, a tyrosine kinase inhibitor, can sensitize gemcitabine treatment through down-regulation Wnt/β-Catenin signaling pathway.[56]
Notch	γ-secretase	DAPT, MRK003	DAPT and MRK003 effectively inhibit intratumoral Notch signaling in PDA, leading to a remarkable decrease in chemosensitivity to gemcitabine[67,68]
		L1790	L1790 are shown to reverse the gemcitabine resistance induced by PSCs.[72]
	DLL4	Neutralizing antibodies	Neutralizing antibodies against human DLL4 reduces the percentage of CSC in gemcitabine-resistant mouse model.[73]
	Notch-1	Sulforaphane (SF)	Through down-regulation of Notch-1, Sulforaphane (SF) prevented gemcitabine-induced selection of ALDH1 positive cells[74]

outcomes. As evident in the numerous studies, targeting developmental pathways is quite promising to improve gemcitabine efficacy in cancer (Table 1).

There are a number of challenges in overcoming gemcitabine resistance. First, the mechanism of gemcitabine resistance is still largely unknown. Inhibition of a single signaling pathway is unlikely to result in a substantial improvement in gemcitabine resistance, owing to the cross-talk from various pathways. Second, despite exciting data in various tumor and their disease models, it is still too early to know whether any of these therapeutic agents that specifically target the developmental pathway will be efficacious with an acceptable safety profile. Third, further studies are urgently needed to confirm the relevance of these developmental signaling pathways to gemcitabine resistance and to find reasonable combination for therapy. Despite these future challenges, targeting Hh, Notch and Wnt signaling pathways to improve the gemcitabine treatment outcome in cancer patients is clearly the way for future studies.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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