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REVIEW

Overcoming tumor cell chemoresistance using nanoparticles: lysosomes are beneficial for (stearoyl) gemcitabine-incorporated solid lipid nanoparticles

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Abstract: Despite recent advances in targeted therapies and immunotherapies, chemotherapy using cytotoxic agents remains an indispensable modality in cancer treatment. Recently, there has been a growing emphasis in using nanomedicine in cancer chemotherapy, and several nanomedicines have already been used clinically to treat cancers. There is evidence that formulating small molecular cancer chemotherapeutic agents into nanomedicines significantly modifies their pharmacokinetics and often improves their efficacy. Importantly, cancer cells often develop resistance to chemotherapy, and formulating anticancer drugs into nanomedicines also helps overcome chemoresistance. In this review, we briefly describe the different classes of cancer chemotherapeutic agents, their mechanisms of action and resistance, and evidence of overcoming the resistance using nanomedicines. We then emphasize on gemcitabine and our experience in discovering the unique (stearoyl) gemcitabine solid lipid nanoparticles that are effective against tumor cells resistant to gemcitabine and elucidate the underlying mechanisms. It seems that lysosomes, which are an obstacle in the delivery of many drugs, are actually beneficial for our (stearoyl) gemcitabine solid lipid nanoparticles to overcome tumor cell resistance to gemcitabine.

Keywords: gemcitabine, chemoresistance, chemotherapeutic agents, nanomedicine

Nanomedicine and cancer chemotherapy

Cancer is a major public health problem worldwide and the second most common cause of death.[1,2] Cancer chemotherapy, the treatment of cancer with one or a combination of chemotherapeutic agents, is one of the mainstream anticancer therapies.[3–5] Nanomedicines are nanometer-sized medicinal entities. They are actively explored to diagnose, prevent, or treat cancer.[6] Indeed, a few nanomedicines have already been approved by the United States Food and Drug Administration for cancer treatment and more are currently in various stages of preclinical and clinical development.[7] Compared to conventional formulations/medicines, nanomedicines have numerous advantages; for example, they can exhibit prolonged systemic circulation time, sustained drug release kinetics, and increased tumor accumulation.[8,9] Nanomedicines can be prepared using various materials, including liposomes, micelles, polymeric nanoparticles, solid lipid nanoparticles, inorganic nanoparticles, drug–polymer conjugates, drug–antibody conjugates, and supramolecular vesicular aggregates, etc.

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Cancer chemotherapeutic agents and mechanisms of chemoresistance

The first modern cancer chemotherapeutic agent was discovered serendipitously. During World War I (1914–1918), accidental releases of mustard gas led to the discovery of the effect of nitrogen mustard on lymphoma.[10] Historically, anticancer drugs were derived from available chemical sources. Synthetic molecules from the chemical industry, in particular dyestuffs and chemical warfare agents, and natural products from plants, bacteria, and fungi are all sources of anticancer agents.[11] The breadth of cancer chemotherapeutic agents is vast, which is actually beneficial as most cancer patients receive multi-drug regimens. This is due to the inherent complexity of cancer[12] – a non-responder to one chemotherapeutic agent may respond to another. In this review, we focus on traditional cytotoxic chemotherapeutic drugs. Despite the increasing desire by cancer patients for targeted therapies and immunotherapies with reduced adverse effects, cytotoxic drugs still play an indispensable role in systemic cancer therapy, and for many cancers, targeted therapy is not available.

Tumor chemoresistance is a major clinical obstacle to successful tumor therapy.[13] Tumor chemoresistance can be divided into intrinsic resistance and acquired resistance.[14] Intrinsic resistance indicates that before receiving chemotherapy, resistance factors already pre-exist in tumor cells. Acquired resistance develops during treatment.[14,15] Cancer cell resistance to chemotherapy is the main cause of recurrence or relapse and has gained clinical attention.[4] Cancer cells evade chemotherapy efficiently through a number of different mechanisms and strategies, such as decrease in drug uptake, increase in drug efflux, alteration of drug metabolism, activation of DNA repair pathways, and induction of the anti-apoptotic machinery.[14,16,17] In addition, it is increasingly recognized that the tumor microenvironment plays a critical role in tumor cell response, or lack of response, to chemotherapy.[18]

Cytotoxic chemotherapeutic drugs can be roughly divided into alkylating agents, antimetabolites, natural products, hormones and hormone antagonists, and other miscellaneous agents.[10,12,19]

Alkylating agents

Alkylating agents are commonly used as cancer chemotherapeutic agents and have a long history of clinical applications. Alkylating agents, including carmustine, lomustine, and temozolomide, can easily cross the blood–brain barrier and have thus shown the most activity against malignant glioma.[20] The general mode of action of alkylating agents is the in vivo formation of electron-deficient active intermediates, which are highly unstable and form covalent bonds with DNA bases. The most vulnerable to attack is the 7-N-atom of guanine.[21,22] Moreover, alkylating agents can react with other molecules to produce extensive cellular damages.

The cytotoxicity of alkylating agents depends on DNA repair pathways, and thus enhancing DNA-repair capacity can lead to tumor resistance to alkylating agents.[23] Mechanisms of resistance to alkylating agents mainly involve O^6-methylguanine methyltransferase (MGMT), DNA mismatch repair (MMR) pathway, and base excision repair (BER) pathway. One important mechanism of resistance to alkylating agents is mediated by the DNA repair enzyme MGMT, which repairs O^6-methylguanine adducts.[20] MGMT covalently transfers the methyl group from O^6-methylguanine to an internal cysteine residue, yielding an inactive S-alkylcysteine-modified protein and guanine.[24] The effects of alkylating agents on DNA can be repaired by MGMT, leading to alkylating agent resistance. DNA MMR pathway is critical for mediating the cytotoxic effect of O^6-methylguanine, which is programed to correct errors in DNA base pairing, and defects in this system cause resistance to temozolomide.[20] Another mechanism of resistance to alkylating agents is the BER pathway that can repair N^7-methylguanine and N^3-methyladenine DNA adducts. Cells that are defective in MMR are generally resistant to temozolomide.[23]

Antimetabolites

Antimetabolites are widely used for the treatment of many types of cancer. Antimetabolites have molecular structures similar to the substrates of enzymes that are involved in DNA and RNA synthesis. Inhibition of DNA or RNA synthesis ultimately destroys the structure and function of DNA or RNA and leads to tumor cell death. Antimetabolites such as 5-fluorouracil, cytarabine, methotrexate, hydroxyurea, and gemcitabine are generally analogs of the natural building blocks of DNA.[25,26] For example, gemcitabine is a deoxycytidine analog and is widely used in the treatment of solid tumors.[27–29] However, tumor resistance of gemcitabine often seriously limits its effect.[30] These drugs may interact with DNA in two ways: by acting as structural analogs of the precursors and intermediates for the synthetic pathway, and therefore interfering with the synthesis of purines and pyrimidines, or by acting as false bases in the assembly of the DNA double helix during replication and transcription.

Antimetabolites can be divided into pyrimidine analogs, purine analogs, and folic acid analogs. Research on chemoresistance to nucleoside analogs such as pyrimidine analogs and purine analogs shows that deficiency of nucleoside transporters or nucleoside kinases such as deoxycytidine kinase (dCK), increased activity of ribonucleotide reductase (RR) or cytidine deaminase (CDA), and expression of 5′-nucleotidases are related to decrease in the cytotoxicity of nucleoside analogs.[31–33] In addition, folic acid analog resistance may result from decreased cellular influx or increased efflux of the analogs, impaired polyglutamation, increased expression and various alterations in target enzymes, and intracellular accumulation of tetrahydrofolate cofactors.[34,35]

Natural products

Natural products, molecules discovered and isolated from living organisms and possessing biological or pharmacological activity, are commonly utilized for cancer chemotherapy.[36,37] In addition, natural products can also be synthesized, with chemical property equivalent to their natural counterparts. Many anticancer drugs such as paclitaxel, vinblastine, etoposide, and hydroxycamptothecine are all natural products. Anticancer antibiotics, produced by microorganisms, are also valuable natural products.[10,38] These drugs tend to be cell-cycle non-specific and therefore are used in the treatment of slow-growing tumors that have a low growth fraction, including daunorubicin, doxorubicin (DOX), epirubicin, idarubicin, valrubicin, mitoxantrone, bleomycin, and mitomycin c.[10]

Taxanes are important natural product antitumor drugs. Paclitaxel and docetaxel interfere with spindle microtubules, causing cell apoptosis. Paclitaxel enters cells and binds to β-tubulin on the inner surface of microtubules.[39] Paclitaxel resistance is mainly associated with the following factors: multidrug resistance caused by the overexpression of P-glycoprotein (P-gp), tubulin mutations or alterations in microtubule stability, and reduced function of significant apoptotic proteins, such as Bcl-2 and p53.[39–41]

DOX is a widely used chemotherapeutic agent. The most common mechanism of resistance to DOX is the overexpression of ATP-binding cassette (ABC) transporters such as P-gp.[42] In addition, alterations of the drug target, topoisomerase, and modulation of programed cell death pathways are also important contributors to DOX resistance.[43,44]

Hormones and hormone antagonists

Tumors sometimes arise from hormone-sensitive cells. Tumor grows vigorously in the presence of hormones, and even depending on these hormones. Anticancer hormone therapy exploits these features to limit the availability of the hormones to cells in different ways.[10] Drugs in this category include selective estrogen receptor modulators (SERMS), progestins (megestrol acetate), luteinizing hormone-releasing hormone agonists, and androgenic agonists.

Glucocorticoids (GCs) such as dexamethasone (DEX) are a class of steroid hormones frequently used as a supportive care co-medication to suppress the side effects of other chemotherapeutic agents.[45,46] Hormones are carried into cells, where they interact with hormone receptors, regulating transcription and protein synthesis of target genes in tumor cells. Therefore, interference of hormone–hormone receptor interaction can lead to cancer cell death.[10] Main mechanisms of GC resistance include ligand-induced downregulation of the receptors, the dominant-negative inhibition by the β-isoform of the receptors and repression by the transcription factor NF-κB.[47]

Tamoxifen, a SERMS, is widely used to treat estrogen receptor (ER)-positive breast tumors. However, tamoxifen therapy often fails due to de novo and acquired tamoxifen resistance.[48] Tamoxifen resistance is associated with altered ER expression, especially on the plasma membrane, or altered expression of microRNAs and signaling pathways that regulate epithelial–mesenchymal transition in the tumor microenvironment.[48,49]

Miscellaneous agents

This group of agents includes several cancer chemotherapeutic agents that are difficult to categorize, mainly platinum analogs and enzymes. Platinum analogs are widely used in human neoplasia therapy, alone or in combination with other agents.[50] Platinum adducts induce distortion of DNA double helix and cellular DNA damage. Cisplatin, carboplatin, and oxaliplatin are the main platinum analogs used for chemotherapy.[19] The mechanisms of cellular resistance to platinum analogs can be classified in two groups: those that limit the formation of cytotoxic platinum-DNA adducts and those that prevent cell death occurring after platinum-DNA adduct formation.[51,52]

Asparaginase, an enzyme, is an important chemotherapeutic agent for the management of acute leukemia and other blood-related cancers.[53] The mechanisms of asparaginase resistance include increased asparagine synthetase activity, genomic modulations and alterations, epigenetic changes, and so on.[54,55] Other miscellaneous drugs include hydroxyurea, procarbazine, and dacarbazine.[10]

Nanomedicine in overcoming cancer cell chemoresistance

There is evidence that nanomedicines can help overcome cancer cell resistance to all the classes of chemotherapeutic agents mentioned above. Examples of using nanomedicines to overcome tumor cell resistance to representative drugs in different classes of cancer chemotherapeutic agents are showed in Table 1.

Table 1 Examples of using nanomedicines to overcome tumor cell resistance to representative drugs in different classes of cancer chemotherapeutics

Classes of cancer chemotherapeutics	Representative drugs	Common mechanisms of resistance	Example(s) of overcoming the resistance using nanomedicines	Comments	Reference
Alkylating agents	Temozolomide	The DNA repair enzyme MGMT, which repairs O^6-methylguanine adducts; Cells that are defective in MMR are generally resistant to temozolomide	Temozolomide nanoconjugates were synthesized using poly(β-L-malic acid), which comprised LLL and antibody to TfR. Temozolomide-resistant cells were sensitive to the temozolomide-nanoconjugates	The absorption of the temozolomide nanoconjugates was by receptor-mediated endocytosis. The conjugates may have increased the plasma half-life of temozolomide	58, 206
Antimetabolites	Methotrexate	Efflux transporters play a major role in methotrexate resistance	Methotrexate was conjugated to QDs and the resultant methotrexate-QDs were significantly more cytotoxic than free methotrexate in methotrexate-resistant KB cells	Methotrexate-QDs were effectively taken up by methotrexate-resistant KB cells, and the nanoconjugates were shown to evade efflux	71
	Cytarabine	Transmembrane efflux pumps, decreased hENT1 expression, dCK deficiency, increase of CDA expression	Squalenoyl–cytarabine conjugate–based NPs can overcome cytarabine resistance in L1210R cells both in vitro and in vivo	The NPs may have increased the intracellular level of cytarabine and reduced intracellular cytarabine deamination	207
Natural products	Docetaxel	Overexpression of P-gp/MDR1, increased expression of BCRP	Docetaxel loaded in PLGA-D-α-tocopheryl polyethylene glycol 1000 succinate/Poloxamer 235 nanoparticles was significantly more cytotoxic to docetaxel-resistant human breast adenocarcinoma MCF-7/TXT cells than docetaxel solution in culture and in a mouse model	NPs increased the uptake and reduced the efflux of docetaxel in MCF-7/TXT cells	74
	Doxorubicin	Overexpression of ABC transporters such as P-gp, alterations of drug target, modulation of programed cell death pathways	Doxorubicin encapsulated in mesoporous silica nanoparticles can overcome MCF-7/MDR1 cell resistance to doxorubicin	The mesoporous silica nanoparticles reduced doxorubicin efflux	79
Hormones and hormone antagonists	Tamoxifen	Altered ER expression, especially on the plasma membrane; altered expression of microRNAs; signaling pathways that regulate EMT in the tumor microenvironment	Tamoxifen-incorporated MnSOD siRNA nanoparticles were prepared. Tamoxifen-resistance of breast cancer cells was reversed when the antagonistic MnSOD activity was silenced by the MnSOD siRNA nanoparticles both in vitro and in vivo	The siRNA inhibited MnSOD activity	85
Miscellaneous agents	Canthaplatin, a prodrug of cisplatin	Reduced drug uptake, DNA repair	Canthaplatin and a PP2A inhibitor (LB) were encapsulated into PEG-b-PLGA micellar nanoparticles, which can overcome tumor resistance to cisplatin both in vitro and in vivo	Nanoparticles increased the uptake of canthaplatin by tumor cells. PP2A inhibitor suppressed DNA repair	87

Abbreviations: MGMT, O^6-methylguanine methyltransferase; MMR, mismatch repair; LLL, trileucine; TfR, transferrin receptor; QDs, quantum dots; hENT1, human equilibrative nucleoside transporter 1; dCK, deoxycytidine kinase; CDA, cytidine deaminase; ABC, ATP-binding cassette; P-gp, P-glycoprotein; EMT, epithelial–mesenchymal transition; ER, estrogen receptor; BCRP, breast cancer resistance protein; PLGA, poly(lactic-co-glycolic acid); MnSOD, manganese superoxide dismutase; NPs, nanoparticles; PP2A, protein phosphatase 2A.

Alkylating agents

Alkylating agents are a major class of cancer chemotherapeutic drugs. Clinical chemoresistance is a common complication in alkylating agent treatment of malignant tumors.[56,57] Studies have shown that nanomedicines can overcome tumor chemoresistance to alkylating agents.[58–60] For example, temozolomide, a commonly used alkylating agent, is considered the gold standard for the treatment of glioblastoma.[61,62] However, growing resistance to temozolomide remains a major clinical challenge. The DNA repair enzyme MGMT plays a critical role in primary resistance to alkylating agents such as tomozolomide.[20] In addition, overexpression of epidermal growth factor receptor (EGFR) and Galectin-1 by tumor cells also significantly contributes to temozolomide resistance.[63,64] Messaoudi et al developed chitosan-grafted lipid nanocapsules to deliver both anti-EGFR and anti-Galectin-1 siRNA to tumor cells, which represents a promising strategy to overcome temozolomide resistance.[60,65,66] Patil et al synthesized multifunctional temozolomide nanoconjugates (6.5–14.8 nm) using poly(β-L-malic acid), which contained trileucine (LLL) and antibody to transferrin receptor.[58] It was found that temozolomide-resistant cells were sensitive to the temozolomide nanoconjugates,[58] clearly demonstrating the feasibility of overcoming tumor cell resistance to the alkylating agent temozolomide by formulating it into nanomedicine.

Antimetabolites

Antimetabolites are widely used cancer chemotherapeutic agents, mainly including purine analogs, pyrimidine analogs, and antifolate agents. There is evidence that chemoresistance to nucleoside analogs such as gemcitabine, cytarabine, and fluorouracil can be overcome by using nanomedicines.[67–70] The application of nanomedicine in overcoming gemcitabine resistance will be discussed in detail later.

Methotrexate, an antifolate agent, is indicated for the treatment of rheumatic disorders and malignant tumors. However, cancer cell resistance to methotrexate limits its applications. Johar-Ahar et al conjugated methotrexate to quantum dots (QDs) and showed that methotrexate–QDs were significantly more cytotoxic than free methotrexate in methotrexate-resistant KB cells (ie, IC_{50} values, 12.0 vs 105.0 μg/mL).[71]

Natural products

Nanomedicines have shown promise in combating chemoresistance to natural products such as paclitaxel, docetaxel, and vincristine.[72–75] For example, Yuan et al reported that paclitaxel-incorporated poly(D,L-lactic-co-glycolic) acid (PLGA)-Tween 80 nanoparticles can reverse multidrug resistance to paclitaxel.[72] Tang et al showed that docetaxel loaded in PLGA-D-α-tocopheryl polyethylene glycol (PEG) 1000 succinate/Poloxamer 235 nanoparticles was significantly more cytotoxic to docetaxel-resistant human breast adenocarcinoma MCF-7/TXT cells than Taxotere®, a commercial docetaxel solution, in culture and in a mouse model.[74]

DOX, an anthracycline antibiotic, is widely used in solid tumor therapy. However, tumor cell resistance to DOX reduces its therapeutic efficacy.[76] Many studies exploited the feasibility of using nanomedicines to reverse DOX resistance.[76–81] For example, Wang et al showed that DOX encapsulated in mesoporous silica nanoparticles (MSNPs) can overcome MCF-7/MDR1 cell resistance to DOX.[79] Unsoy et al synthesized chitosan-coated magnetic nanoparticles for targeted delivery of DOX. DOX-loaded nanoparticles were efficiently taken up by DOX-resistant MCF-7 breast cancer cells (MCF-7/1 μM, MCF-7/S) and were more cytotoxic than free DOX in DOX-resistant MCF-7 cells.[81] Yu et al also designed DOX nanoparticles that exhibited higher cytotoxic than free DOX in DOX-resistant MCF-7/ADR cells (ie, 54.4% viability vs 66.8% for free DOX).[80]

Hormones and hormone antagonists

In this class of chemotherapeutic agents, many researchers have reported the use of nanomedicines to overcome tamoxifen chemoresistance. Aromatase inhibitors are used to treat hormone receptor-positive, locally advanced, or metastatic breast cancer. Letrozole is a potent non-steroidal aromatase inhibitor that is indicated to treat hormone-responsive breast cancer after surgery, but some patients develop resistance to letrozole during treatment.[82,83] Nair et al developed hyaluronic acid-bound letrozole nanoparticles (HA-Letr-NPs) and showed that the HA-Letr-NPs can restore the sensitivity of tumors to letrozole in the LTLT-Ca letrozole-resistant breast tumor model.[84] Cho et al developed tamoxifen-incorporated manganese superoxide dismutase (MnSOD) siRNA nanoparticles that have an siRNA/poly(amidoamine) dendriplex core and an acid-sensitive polyketal shell and showed that the tamoxifen resistance of breast cancer cells was reversed when the antagonistic MnSOD activity was silenced by the MnSOD siRNA nanoparticles both in vitro and in vivo.[85]

Miscellaneous agents

Tumor resistance to platinum analogs is very common, and the clinical efficacy of platinum analogs is limited by intrinsic and acquired resistance.[86] There is an increasing interest in developing new platinum anticancer agents, but new platinum agents have been very slow to enter clinics. There is evidence that nanomedicine may offer an effective alternative to overcome the resistance. For example, Zhou et al designed canthaplatin and PP2A inhibitor (LB) encapsulated PEG-b-PLGA micelles (ie, polymeric micelles) and showed that the micelles can overcome tumor resistance to cisplatin.[87] In addition, micelles prepared with PCL-b-PABPA-b-POEGMEA (ie, polycaprolactone, PCL; 3-((tert-butoxycarbonyl)amino) propyl acrylate, ABPA; oligo(ethylene glycol) methyl ether acrylate, OEGMEA) and incorporated with curcumin and platinum were shown to be able to overcome tumor cell resistance to platinum.[88]

Limitations in using nanomedicine to overcome cancer cell chemoresistance

It is exciting that formulating cancer chemotherapeutic agents into nanomedicines can help overcome cancer cell chemoresistance. However, it is worth noting that to successfully overcome cancer chemoresistance, having nanomedicine formulations that can kill resistant cancer cells is often not sufficient. In vivo, the reticuloendothelial system (RES) and other physiological barriers can significantly impede the efficient delivery of nanomedicines to tumor cells, or even tumor tissues.[89,90] Opsonization of nanomedicines by nonspecific adsorption of plasma proteins, such as opsonins, facilitates their phagocytosis and clearance from the circulation by RES.[90,91] Many approaches have been utilized to limit the clearance of nanomedicines, either by delaying of RES clearance or by altering the surface properties of nanomedicines.[92,93] The physiological barriers are another obstacle that needs to be overcome for nanomedicines to effectively overcome cancer chemoresistance in vivo.[89] The accessibility of nanomedicines to solid tumors is determined by various mechanisms, such as the efficiency of the blood and lymphatic networks in tumor tissues, the permeability of the vascular barriers in tumors, and the constitution of the tumor stroma.[89,94] Several strategies have been explored to enhance the delivery of nanomedicines into tumor tissues such as normalizing the tumor vasculature or reducing tumor desmoplasia.[89]

Nucleoside analogs and gemcitabine

Nucleoside analogs are structurally similar antimetabolites that have a broad range of actions and are clinically effective in both solid tumors and hematological malignancies.[95] These agents may interact with DNA or RNA by inhibiting the ability of cancer cells to synthesize precursors of nucleic acids required to ensure sustained growth or by directly interfering with DNA or RNA synthesis. Nucleoside analogs have a generalized structure consisting of a purine or pyrimidine base linked to a deoxyribose sugar. Examples of purine nucleosides and related inhibitors include cladribine, fludarabine, and clofarabine; and examples of pyrimidine nucleoside analogs include the deoxycytidine analogs gemcitabine, fluorouracil, cytarabine, capecitabine.[10]

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is a deoxycytidine analog with antitumor activity against a wide variety of solid tumors such as pancreatic, non-small-cell lung cancer (NSCLC), breast cancer, and ovarian cancer, alone or in combination with other chemotherapeutic agents.[27–29,96] Moreover, gemcitabine is indicated in several hematological disorders such as acute leukemia.[97] Furthermore, gemcitabine enhances the cytotoxicity of cisplatin by increasing the formation of cytotoxic platinum-DNA adducts and is also a potent radiosensitizer used in radiation therapy.[98] However, drug resistance to gemcitabine often limits its efficacy in clinics,[30] and overcoming gemcitabine chemoresistance remains a challenge.[99]

Gemcitabine and its mechanisms of action

As a hydrophilic nucleoside analog, cellular uptake of gemcitabine is mediated by nucleoside transporters such as the human equilibrative nucleoside transporter 1 (hENT1).[100] Once taken up into cells, gemcitabine is phosphorylated by dCK to gemcitabine monophosphate (dFdCMP), gemcitabine diphosphate (dFdCDP), and gemcitabine triphosphate (dFdCTP).[101] The active metabolite, dFdCTP, can terminate DNA elongation by incorporating into DNA, finally leading to cell death.[99,102,103] In addition, dFdCDP can inhibit RR by binding to the large subunit (RRM1).[104,105] RRs catalyze the conversion of nucleoside 5′-diphosphates (ie, NDPs) to their corresponding deoxynucleotides (ie, dNDPs), which are phosphorylated to dNTPs for DNA synthesis.[105,106] Inhibition of RRs will reduce the dNTP pool, allowing dFdCTP to more effectively compete with dNTPs and inhibit DNA replication and repair.[104–107]

Mechanisms of gemcitabine resistance

The mechanisms of resistance to gemcitabine are in many aspects different from those of the other classes of cancer chemotherapeutic agents. Multiple factors, including decreased expression of nucleoside transporters,[108,109] changes in the expression of gemcitabine-activating or degradation enzymes and target molecules,[33,110–112] some signaling molecules (eg, NF-κB, P53) affecting cells that are resistant to apoptosis,[113,114] and the expression of efflux transporters commonly resulted in MDR,[115,116] have been reported to cause gemcitabine resistance.

Nucleoside transporters

Gemcitabine is a hydrophilic compound and cannot readily diffuse across cell membrane. Therefore, it requires nucleoside transporters to enter cells.[117,118] The concentrative nucleoside transporters (hCNTs) and hENTs are implicated in tumor cell uptake of gemcitabine.[118,119] Among these transporters, hENT1 is a major transporter involved in gemcitabine cellular uptake.[120] In fact, hENT1 has been reported as a vital predictive marker of tumor response to gemcitabine-based therapy.[32,121,122] Clinical data showed that cancer patients with a decreased tumor expression of hENT1 have a significantly lower survival rate after gemcitabine treatment than those with tumors that express a higher level of hENT1.[119,121–124] In culture, tumor cells lacking hENT1 expression become resistant to gemcitabine-mediated cytotoxicity.[108] For example, in the hENT1-deficient CCRF CEM-AraC-8C cells, the IC_{50} value of gemcitabine was reported to be 471-fold greater than that in the parent CCRF-CEM cells.[68]

dCK

Once gemcitabine enters cells, dCK is the rate-limiting enzyme responsible for the conversion of gemcitabine to its active metabolites.[125,126] Studies have indicated that in vitro and in vivo, dCK deficiency is related to gemcitabine resistance to pancreatic cancer, sarcoma, lymphoma, and leukemia.[127–131] For example, Ohmine et al showed that the attenuation of gemcitabine phosphorylation is likely a key process for the acquisition of resistance by the RPK9 human pancreatic adenocarcinoma (PDAC) cells.[101] Lower expression of dCK was shown to be associated with shorter overall survival in pancreatic cancer patients who received gemcitabine as an adjuvant therapy.[132] It is suggested that dCK expression in both protein and mRNA levels may serve as a biomarker to predict tumor cell sensitivity to nucleoside analogs such as gemcitabine.[133]

CDA

CDA, a key enzyme involved in gemcitabine metabolism, was identified in the early 1990s.[134] Deamination is the main mechanism by which gemcitabine is inactivated, and it is estimated that 90% of gemcitabine is inactivated to difluorodeoxyuridine by CDA intracellularly and extracellularly.[96] In vitro, macrophage-induced CDA upregulation in human Panc-1 pancreatic tumor cell line has been shown to confer gemcitabine resistance.[111] Data from ample studies have indicated that CDA polymorphisms alter CDA enzyme activity and the pharmacokinetics of gemcitabine,[135–138] and three functional polymorphisms of CDA (rs2072671, CDA 79A $>$ C; rs60369023, CDA 208G $>$ A; and rs1048977, CDA 435C $>$ T) could predict the clinical outcomes of gemcitabine-based tumor chemotherapy.[33,135,136]

RR or RNR

As mentioned above, RRs catalyze the conversion of NDPs to their corresponding dNDPs for DNA synthesis.[139,140] In mammals, RR is a heterodimeric tetramer consisting of two large subunits (RRM1) and two small subunits (RRM2 and RRM2B).[141,142] RRM1 catalyzes the rate-limiting step in the production of dNTPs and is an essential enzyme for DNA replication and repair.[139,143] The catalysis activity of RRs requires the binuclear iron center and a tyrosyl-free radical located in RRM2.[142] RRM2B was identified as a critical p53-inducible RR subunit that can be regulated by p53 and p73 genes/proteins.[144,145]

Gemcitabine self-potentiates its own effect by directly inhibiting RRM1. Therefore, upregulation of RRM1 can lead to gemcitabine resistance.[110,146] Conversely, RRM1 knockdown in the resistant MIA PaCa-2 pancreatic cancer cell line completely restored gemcitabine sensitivity.[147] The relationship between RRM2 mRNA expression and response to gemcitabine in clinical setting has been investigated in various cancers. For example, the response rate to gemcitabine is significantly higher in pancreatic cancer patients with low RRM2 mRNA expression in biopsy specimens.[148] Similarly, high RRM2 expression was found to be correlated with poor clinical outcome in patients with lower-risk prostate cancer.[112]

Other mechanisms of resistance to gemcitabine

The aforementioned are the main mechanisms of gemcitabine resistance. There are other factors associated with gemcitabine resistance as well. For example, excision repair cross-complementing protein 1 can repair gemcitabine-induced strand breaks, and its overexpression is well documented in poor gemcitabine responders.[149,150] Kozinn et al reported that microRNAs 1290, 138, let-7i, and let-7b are involved in gemcitabine resistance in bladder carcinoma cell lines.[151] In addition, a tumor microenvironment that favors cancer progression and metastasis can elicit drug resistance.[152] For example, Xu et al showed that sonic hedgehog (SHH) signaling in tumor microenvironment protects PDAC cells against gemcitabine-induced apoptosis and that overexpression of SHH in PDAC cells enhances drug resistance.[153] Other factors that contribute to gemcitabine resistance include NF-κB,[114] heat shock proteins, and the presence of highly resistant tumor stem cells.[154,155]

Overcoming gemcitabine resistance using nanomedicines

Nanomedicines have unique advantages in overcoming tumor cell resistance to gemcitabine, and various types of gemcitabine nanomedicine formulations, such as micelles,[156] liposomes,[157] supramolecular vesicular aggregates, and nanovesicles,[158–160] have been shown to circumvent gemcitabine resistance. The general mechanisms by which gemcitabine nanomedicines overcome gemcitabine chemoresistance are discussed below.

Reducing RR expression

Accumulating evidence indicates that increased expression of RRM1 is associated with a poor response of cancer patients to gemcitabine.[161,162] Higher levels of RRM1 were detected in tumors of various patients who respond poorly to gemcitabine.[139,143,163–165] For example, in gemcitabine-treated advanced NSCLC patients, those with RRM1-positive tumors were shown to have worse overall survival and disease control than those with RRM1-negative tumors.[163] Similarly, in patients with advanced nasopharyngeal carcinoma (NPC) and treated with gemcitabine-based regimens, high RRM1 expression is correlated with shorter progression-free survival, compared to patients with RRM1-negative expression.[161] In a recent study, RRM1 siRNA was used to downregulate RRM1 expression in tumor cells, and it was shown that pre-exposure of A549 lung cancer cells to RRM1 siRNA nanoconstructs significantly decreased the IC_{50} value of gemcitabine in the tumor cells compared to gemcitabine alone.[166] Previously, we have also shown in a mouse model with TC-1 mouse lung cancer cells that overexpress RRM1 (ie, TC-1-GR cells) that treatment with RRM1 siRNA-polyethylenimine (PEI) nanocomplexes (122±5 nm) significantly increased the effect of gemcitabine against the tumors, compared to treatment with control siRNA-PEI nanocomplexes.[167]

Increasing cellular uptake of gemcitabine

As mentioned above, gemcitabine depends on nucleoside transporters to enter cells.[117,168] Therefore, reduced expression of nucleoside transporters on tumor cells causes tumor cell resistance to gemcitabine.[123,169] For example, it was reported that high hENT1 expression in resected specimen of patients with PDAC who received postoperative gemcitabine therapy is correlated with increased overall survival,[170–173] whereas low hENT1 expression was linked to gemcitabine resistance and shorter overall survival.[109,123,169] In addition, low levels of hENT1 expression were also detected in tumors in gallbladder adenocarcinoma patients who respond poorly to gemcitabine.[122] Nanomedicine formulations of gemcitabine can overcome gemcitabine resistance caused by reduced expression of nucleoside transporters by delivering gemcitabine into tumor cells independent of the transporters. For example, we have data showing that our previously developed stearoyl gemcitabine solid lipid nanoparticles (ie, 4-(N)-GemC18-SLNs) enter tumor cells by clathrin-mediated endocytosis.[174] Hung et al showed that a nanoparticle formulation of gemcitabine has significantly smaller IC_{50} values, compared to free gemcitabine, in ovarian cancer cells that express low levels of hCNT1,[175] indicating that the nanoparticle formulation can bypass nucleoside transporter defects.

Reducing deamination of gemcitabine

Stromal and cellular CDAs convert gemcitabine to an inactive metabolite.[96,176] Preclinical and clinical studies have suggested that upregulation of CDAs increases gemcitabine resistance, while CDA deficient is associated with increased gemcitabine activity.[33,96] To protect gemcitabine from rapid deamination, many attempts have been made by chemically modifying gemcitabine.[177–180] For example, it was shown that conjugation of a fatty acid, such as stearic acid, to gemcitabine at the 4-N position decreases the sensitivity of the later to deaminase.[181,182] In addition, gemcitabine–fatty acid conjugates formulated into nanoparticles also become less sensitive to deamination, as they are no longer good substrates of CDAs.[182] Meng et al developed a MSNP for co-delivery of gemcitabine and paclitaxel to take advantage of paclitaxel's ability to inhibit CDA expression to increase tumor response to gemcitabine.[183]

Enhancing distribution and/or accumulation of gemcitabine in tumor tissues

The enhanced permeability and retention (EPR) effect discovered by Matsumura and Maeda has been exploited for passive targeting of anticancer drugs into tumors.[184,185] The discovery of EPR effect is of great significance to the design of antitumor nanomedicines.[186–188] Nanomedicine formulations of gemcitabine can take advantage of the EPR effect to increase gemcitabine content within tumor tissues upon intravenous injection.[189–192] Having more gemcitabine distributed into tumor tissues will provide them the chance to kill tumor cells or inhibit tumor cell growth.

Examples of using nanomedicine formulations of gemcitabine to overcome tumor cell resistance to gemcitabine

In their effort in finding a solution to overcome gemcitabine resistance, scientists showed that nanomedicine formulations of gemcitabine have promising potentials.[68,174,180,193] Examples of overcoming tumor cell resistance to gemcitabine using nanomedicines are shown in Table 2.

Polymeric nanoparticles

Recognizing that defective hCNT1 contributes to gemcitabine chemoresistance in ovarian cancer, Hung et al created PLGA-b-PEG-OH nanoparticles incorporated with gemcitabine. The gemcitabine–PLGA-b-PEG-OH nanoparticles effectively delivered gemcitabine into hCNT1-decreased ES-2 and TOV-21G tumor cells and were significantly more cytotoxic to those cells than free gemcitabine.[175] Papa et al reported gemcitabine-loaded PLGA nanoparticles (PLGem) and evaluated their cytotoxicity to aggressive human Panc-1 cells, which are well-known to exhibit gemcitabine resistance.[194] The PLGem was significantly more cytotoxic than free gemcitabine to Panc-1 cells.[194]

In another approach, gemcitabine nanoparticles were prepared by loading GemC18, a stearic acid amide derivative of gemcitabine, in PEG–poly(D,L-lactic) acid (PEG–PLA) polymeric micelles or by GemC18 self-assembling.[195] Both of the nanomedicines effectively reduced the viability of gemcitabine-resistant AsPC-1 cells in culture (IC_{50} values, 58.88 and 46.34 μM, respectively), whereas the molar equivalent free gemcitabine did not show any significant cytotoxicity to AsPC-1 cells. The GemC18 self-assembled nanoparticles showed greater in vitro cellular uptake and cytotoxicity than the GemC18-PEG-PLA polymeric micellar nanoparticles (ie, drug uptake in Panc-1 cells, 37.55%±2.21% for GemC18 self-assembled nanoparticles, 28.60%±1.85% for GemC18-PEG-PLA polymeric micelles, and 30.11%±1.98% for GemC18 in solution).[195]

Gemcitabine nanoparticles were also prepared based on a gemcitabine–squalene conjugate (SQ-dFdC or SQ-Gem), which displayed a stronger antiproliferative and cytotoxic activity than gemcitabine. After orthotopic Panc-1 tumor-bearing mice were treated two times at a 4-day interval with either gemcitabine (20 mg/kg) or SQ-Gem (20 mg/kg), SQ-Gem was more effective than gemcitabine in inhibiting the tumor growth.[178] The SQ-dFdC nanoparticles were also shown to overcome gemcitabine resistance in murine and human leukemia cells (ie, L1210-10K and CEM/AraC-8C, respectively).[180]

Liposomes

Xu et al developed pH-sensitive liposomes (PSLs) with a high content of gemcitabine. The cytotoxicity values of the various gemcitabine-PSLs developed were evaluated in the gemcitabine-resistant MIA PaCa-2 cell line.[67] Gemcitabine-PSLs with drug loading of 0.5% and 4.5% had similar IC_{50} values (ie, 1.1±0.1 versus 0.7±0.1 μM), but both were significantly smaller than that of free gemcitabine in solution and gemcitabine in non-PSLs. In an animal model, the gemcitabine-PSLs were significantly more effective than free gemcitabine in controlling the growth of gemcitabine-resistant pancreatic cancer cells.[67] Papa et al also reported gemcitabine-encapsulated nanoliposomes (GemPo), which were shown to be more cytotoxic than free gemcitabine in gemcitabine-resistant MDA-MB-231 breast cancer cells and sensitive 4T1 cells.[196]

Overcoming tumor cell resistance to gemcitabine using stearoyl gemcitabine-incorporated solid lipid nanoparticles

In our effort to improve the antitumor activity of gemcitabine, we previously developed a stearoyl gemcitabine nanoparticle formulation by incorporating 4-(N)-stearoyl gemcitabine (ie, 4-(N)-GemC18) into solid lipid nanoparticles engineered from lecithin/glycerol monostearate-in-water emulsions.[181] In animal models (ie, C57BL/6 mice with TC-1 mouse lung cancer cells, nude mice with BxPC-3 human pancreatic cancer cells), the 4-(N)-GemC18-SLNs were significantly more effective than the molar equivalent of free gemcitabine or 4-(N)-GemC18 in a Tween 20 solution in inhibiting tumor growth.[181] More importantly, we discovered that the 4-(N)-GemC18-SLNs can overcome tumor cell resistance to gemcitabine.[68] For example, 4-(N)-GemC18-SLNs were 15-fold more cytotoxic than gemcitabine HCl in the hENT1-deficient CCRF CEM-AraC-8C cells, and ~8-fold more cytotoxic in the $dCK^{-/-}$ CCRF CEM-AraC-8D cells.[68] In the gemcitabine resistant human Panc-1 tumor cells that overexpress RRM2, 4-(N)-GemC18-SLNs were >17-fold more cytotoxic than gemcitabine HCl.[68] In the RRM1-overexpresssing, gemcitabine-resistant TC-1-GR cells, the IC_{50} value of 4-(N)-GemC18-SLNs was only about 5% of that of gemcitabine HCl.[68] Importantly, although both 4-(N)-GemC18-SLNs and free gemcitabine HCl can significantly inhibit the growth of the highly gemcitabine-sensitive TC-1 tumor cells in a mouse model, only 4-(N)-GemC18-SLNs, but not free gemcitbaine HCl, can significantly inhibit the growth of the gemcitabine-resistant TC-1-GR tumors.[68] When elucidating the mechanisms underlying the 4-(N)-GemC18-SLNs'

Table 2 Examples of overcoming tumor cell resistance to gemcitabine using nanomedicines

Nanocarriers	Main excipients/ingredients	Payload type	Outcome	Reference
Nanoparticles	PLGA	Gemcitabine	Nanoparticles showed higher cytotoxicity than free gemcitabine in gemcitabine resistant Panc-1 cells	194
	Chitosan	HER2 antibody, gemcitabine	Nanoparticles showed higher cytotoxicity than gemcitabine and targeting effect in MiaPaca-2 cells	208
	BSA	Gemcitabine	Nanoparticles showed higher cytotoxicity compared to free gemcitabine in Panc-1 cells. In addition, nanoparticles more effectively reduced the tumor volume in comparison with free gemcitabine	209
	Soy lecithin, GMS, Tween 20	4-(N)-GemC18	GemC18-SLNs showed more cytotoxic than free gemcitabine HCl in the hENT1-deficient CCRF CEM-AraC-8C cells and the dCK$^{-/-}$ CCRF CEM-AraC-8D cells. GemC18-SLNs can also overcome gemcitabine resistance caused by RRM1 overexpression and RRM2 overexpression	68, 174, 182
	PLGA-b-PEG-OH	Gemcitabine, cisplatin	Nanoparticles increased cell sensitivity to gemcitabine compared to free gemcitabine in hCNT1-expressing cells	175
	Stearic acid	4-(N)-GemC18	Nanoparticles effectively reduced the viability of gemcitabine-resistant AsPC-1 cells in culture	195
	Squalene	Gemcitabine–squalene conjugate (SQ-dFdC)	SQdFdC self-assembled into nanoparticles, which were shown to overcome gemcitabine resistance in murine leukemia cells (ie, L1210 10K), human leukemia cells (ie, CCRF CEM-AraC-8C), and human pancreatic cancer cells (ie, Panc-1)	178, 180
	Lipid bilayer-MSNP (LB-MSNP)	Paclitaxel/gemcitabine	MSNP can suppress CDA expression, likely due to paclitaxel	183
Nanoassemblies (NAs)	Ethanol, aqueous dextrose solution	Squalenoyl conjugate of 5′-monophosphate-gemcitabine (SQdFdC-MP)	SQdFdC-MP NAs overcome gemcitabine resistance in Panc-1 and in L1210 10K cells caused by the downregulation of dCK	210
Liposomes	DPPC, Chol, DSPE-PEG2000	Gemcitabine	Liposomes were shown to inhibit the growth of a gemcitabine-resistant MDA-MB-231 breast cancer cell line	196
	DOPE, CHEMS, DSPE-mPEG2000	Gemcitabine	The pH-sensitive liposomes (PSL) were significantly more cytotoxic to gemcitabine resistant MIA PaCa-2 cells than gemcitabine	211
Micelles	PEG-PLA, stearic acid	4-(N)-GemC18	Polymeric micelles can effectively reduce the viability of gemcitabine-resistant AsPC-1 cells in culture	195
	PEG-PCC	Gemcitabine	Micelles significantly inhibited tumor growth in MiaPaCa-2 tumor cell-bearing mice	212
	Stearoyl-PEG	4-(N)-GemC18	Micelles can overcome gemcitabine resistance by inhibiting the expression of RRM1	193

Abbreviations: NPs, nanoparticles; SLNs, solid lipid nanoparticles; Gem, gemcitabine; BSA, bovine serum albumin; HER2, human epidermal growth factor receptor-2; PLGA, poly(lactic-co-glycolic) acid; PEG, polyethylene glycol; GMS, glyceryl monostearate; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phos-phocholinemonohydrate; Chol, cholesterol; DSPE, distearoyl phosphatidyl ethanolamine; DSPE-mPEG 2000, distearylphosphatidylethanolamine-N-(carbonyl methoxypolyethylene glycol-2000); PG, L-α-phosphatidyl-D,L-glycerol sodium salt; PEG-PCC, poly(ethyleneglycol)-block-poly(2-methyl-2-carboxyl-propylenecarbonate); PEG–PLA, poly(ethylene glycol)–poly(D,L-lactide); 4-(N)-GemC18, 4-(N)-stearoyl gemcitabine.

ability to overcome gemcitabine resistance, we discovered that the unique composition of the 4-(N)-GemC18-SLNs and the way by which the gemcitabine in the 4-(N)-GemC18-SLNs enters tumor cells are likely responsibe for their ability to more effectively kill tumor cells, especially tumor cells that are otherwise resistant to gemcitabine.[174] We concluded that for gemcitabine to effectively kill tumor cells, especially those resistant to gemcitaine, entering tumor cells is important, but not enough.[174]

The unique composition of the 4-(N)-GemC18-SLNs is critical for their ability to kill tumor cells resistant to gemcitabine

The conclusion that the unique composition of the 4-(N)-GemC18-SLNs is critical for their ability to overcome gemcitabine resistance is supported by the following findings: 1) a 3′-(O)-GemC18 ester synthesized by conjugating gemcitabine in the 3′-O position with stearic acid, when incorporated into the same solid lipid nanoparticles engineered from lecithin/glyceroyl monostearate-in-water emulsions, was not significantly more effective than free gemcitabine in controlling the growth of the gemcitabine-resistant TC-1-GR tumor cells in culture and in a mouse model; 2) the same 4-(N)-GemC18 amide when incorporated into polymeric PLGA nanoparticles was not more effective than gemcitabine in inhibiting the growth of the gemcitabine-resistant TC-1-GR cells; and 3) 4-(N)-GemC8, another amide gemcitabine derivative synthesized by conjugating gemcitaine in its 4-N position with a medium chain fatty acid, caprylic acid (C8), incorporated into the same solid lipid nanoparticles engineered from lecithin/glyceroyl monostearate-in-water emulsions was not more cytotoxic than free gemcitabine against the gemcitabine-resistant TC-1-GR cells.[174] Therefore, it seems that the amide nature of the 4-(N)-GemC18, the long chain fatty acid (ie, stearic acid) derivative nature of the 4-(N)-GemC18, and the solid lipid nanoparticles in which the 4-(N)-GemC18 is incorporated in are all critical for the resultant 4-(N)-GemC18-SLNs to overcome tumor cell resistance to gemcitabine.

It is noted that the 4-(N)-GemC18 needs to be incorporated into the solid lipid nanoparticles, as our results showed that 4-(N)-GemC18 alone and the physical mixture of it and blank solid lipid nanoparticles were not as cytotoxic as 4-(N)-GemC18-SLNs against the gemcitabine-resistant TC-1-GR cells.[174] Furthermore, we showed that our 4-(N)-GemC18-SLNs, but not free gemcitabine, nor 4-(N)-GemC18, significantly downregulate RRM1 expression in the gemcitabine-resistant TC-1-GR cells in culture and in a mouse model and increased the level of dFdCTP in TC-1-GR cells in culture.

The pathway by which the 4-(N)-GemC18 in the 4-(N)-GemC18-SLNs enters tumor cells is critical for its ability to kill tumor cells resistant to gemcitabine

The exact mechanisms why our 4-(N)-GemC18-SLNs can more effectively kill the TC-1-GR tumors that overexpress RRM1 than other gemcitabine formulations, including free gemcitabine, free 4-(N)-GemC18, 3′-(O)-GemC18-SLNs, 4-(N)-GemC8-SLNs, and 4-(N)-GemC18-PLGA-nanoparticles remains unknown, but we hypothesize that it is very likely related to the pathway by which 4-(N)-GemC18-SLNs deliver the gemcitabine into tumor cells. Free gemcitabine depends on nucleoside transporters such as hENT1 to enter tumor cells, and free 4-(N)-GemC18 enters cells by passive diffusion due to its high lipophilicity. Our 4-(N)-GemC18-SLNs, however, enter cells by clathrin-mediated endocytosis.[174] Alkalization of lysosomes (ie, increasing pH) did not affect the uptake and intracellular degradation of 4-(N)-GemC18 when it was taken up as free 4-(N)-GemC18 in solution. However, when cells were incubated with the 4-(N)-GemC18-SLNs, alkalization of lysosomal pH significantly inhibited the intracellular degradation of 4-(N)-GemC18.[174] Lysosomal acidification is required for the activation of many enzymes in lysosomes, indicating that the acidic lysosomal environment and thus many pre-enzymes in lysosomes activated in acidic environment are important for the degradation of the solid lipid nanoparticles, the release of the 4-(N)-GemC18 from the nanoparticles, and the hydrolysis of 4-(N)-GemC18 to free gemcitabine, when 4-(N)-GemC18 was brought into cells by the endocytosis of the 4-(N)-GemC18-SLNs.[174] The 3′-(O)-GemC18-SLNs were not as effective as 4-(N)-GemC18-SLNs because the ester bond in the 3′-(O)-GemC18 was readily hydrolyzed even before the 3′-(O)-GemC18-SLNs were endocytized.[174] Similarly, the 4-(N)-GemC8-SLNs were not as effective as 4-(N)-GemC18-SLNs because the 4-(N)-GemC8 was readily released or leaked from the 4-(N)-GemC8-SLNs before the nanoparticles were endocytized.[174]

Proposed mechanism by which 4-(N)-GemC18-SLNs overcome tumor cells resistant to gemcitabine

Based on the findings mentioned above, we hypothesized that the 4-(N)-GemC18-SLNs take advantage of the salvage nucleotide synthesis pathway and "channel" the 4-(N)-GemC18 into a "natural" pathway that has evolved for cells to efficiently reuse bases and nucleosides from within or outside cells. When cells take up the apoptotic bodies or foreign pathogens by endocytosis, the nucleic acids are enzymatically degraded into nucleosides and bases for reuse.[197] As shown in Figure 1, it is likely that after our 4-(N)-GemC18-SLNs enter tumor cells by clathrin-mediated endocytosis, enzymes in lysosomes catalyze the degradation of the solid lipid nanoparticles, and the degradation facilitates the release of 4-(N)-GemC18 from the nanoparticles. Lysosomal enzymes such as cathepsin B catalyze the hydrolysis of 4-(N)-GemC18 to free gemcitabine, a nucleoside analog. Gemcitaine released into the lysosomes can then be exported out of the lysosomes to the cytoplasm by nucleoside transporters, such as the lysosome-specific hENT3,[198] into the proper intracellular compartment for efficient phosphorylation to its active metabolites, dFdCDP and dFdCTP. In contrast, when free 4-(N)-GemC18 diffuses into tumor cells by passive diffusion, it may be hydrolyzed to release gemcitabine intracellularly, but not in the proper intracellular compartment for efficient phosphorylation, due to its high lipophilicity. Exposing gemcitabine in the "wrong" compartment in cells will likely subject it to deamination by CDA, considering that nucleotides normally do not enter cells in the form of long chain fatty acid conjugates. Free gemcitabine enters tumor cells with the help of nucleoside transporters, but it is subjected to extensive deamination intracellularly and extracellularly before being phosphorylated (Figure 1).[174] The small amount of dFdCTP generated may be sufficient to inhibit tumor cells that are sensitive to gemcitabine, but not against tumor cells that developed various resistant mechanisms (eg, overexpression of RRM1). Of course, more experiments will have to be carried out to generate data to fully support the hypothesized mechanism, but designing nanomedicine formulations of anticancer drugs that mimic or take advantage of a natural pathway, such as the nucleotide salvage pathway in the case of nucleoside analogs such as gemcitabine, likely represents a desirable strategy to improve the activity of the drugs and to overcome chemoresistance.

Finally, it is worth noting that the mechanism mentioned above was largely based on cell culture data.[174] In a mouse model with tumor cells that are resistant to gemcitabine due to the overexpression of RRM1, our 4-(N)-GemC18-SLNs significantly inhibited the tumor growth, although the molar equivalent dose of free gemcitabine did not show any significant antitumor effect.[68] Moreover, we have also engineered 3′-(O)-GemC18-SLNs by incorporating 3′-(O)-GemC18 into the same solid lipid nanoparticles; 3′-(O)-GemC18 was synthesized by conjugating stearic acid to gemcitabine in the 3′-O position to form an ester, which is more sensitive to hydrolysis than 4-(N)-GemC18. Therefore, 3′-(O)-GemC18-SLNs

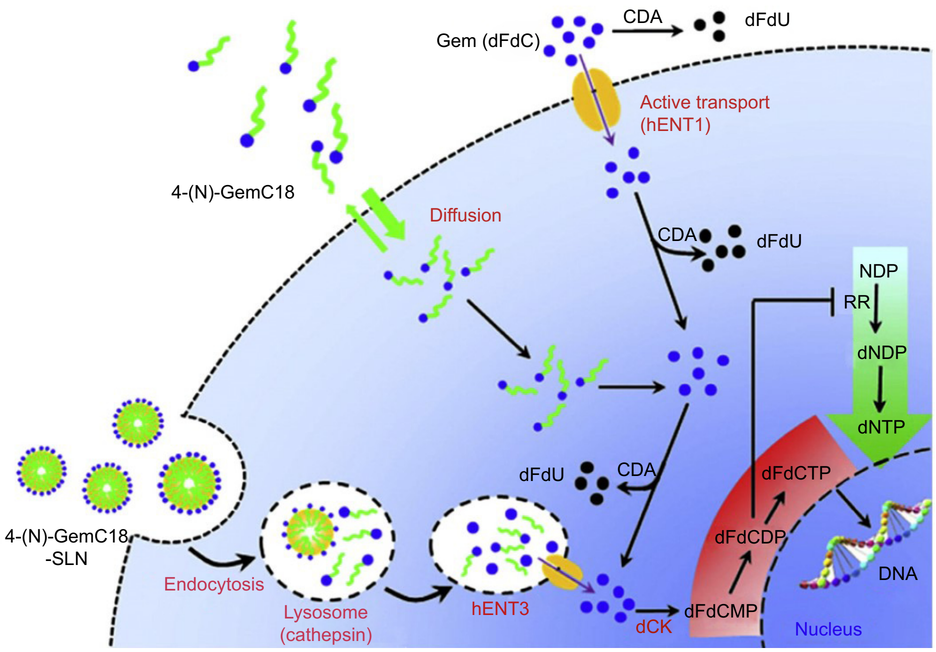

Figure 1 A schematic of the proposed mechanism by which 4-(N)-GemC18-SLNs overcome tumor cell resistance to gemcitabine.
Note: Reprinted from *J Control Release*. 169(1–2). Wonganan P, Lansakara PD, Zhu S, et al. Just getting into cells is not enough: mechanisms underlying 4-(N)-stearoyl gemcitabine solid lipid nanoparticle's ability to overcome gemcitabine resistance caused by RRM1 overexpression.17–27. Copyright 2013, with permission from Elsevier.[174]
Abbreviations: CDA, (deoxy)cytidine deaminase; dCK, deoxycytidine kinase; dFdC, gemcitabine; dFdCMP, gemcitabine monophosphate; dFdCDP, gemcitabine diphosphate; dFdCTP, gemcitabine triphosphate; dNDP, deoxyribonucleoside diphosphate; dNTP, deoxyribonucleoside triphosphate; hENT, human equilibrative nucleoside transporter; NDP, ribonucleoside diphosphates; RR, ribonucleotide reductase.

cannot as effectively as 4-(N)-GemC18-SLNs kill the RRM1-overexpressing tumor cells in culture and in vivo.[174] The data from our in vivo studies in a mouse model indicated that the mechanism we proposed above is also applicable in vivo. In other words, in the tumor-bearing mouse model we tested, some of our 4-(N)-GemC18-SLNs should have reached the tumor cells after intravenous injection and entered the endo-lysosomes of the tumor cells by endocytosis.

It is not easy for nanoparticles to evade uptake by the RES and overcome other physiological barriers to successfully reach tumor cells. For nanoparticles that reach tumor tissues intact, besides the aforementioned endocytosis by tumor cells, there are other potential mechanisms for the chemotherapeutic agents carried by the nanoparticles to enter tumor cells. One obvious mechanism is that the chemotherapeutic agents are released from the nanoparticles in the tumor tissues, diffuse to tumor cell surface, and then enter tumor cells by passive diffusion or transporter-mediated uptake. There are strategies to facilitate the release of chemotherapeutic agents from nanoparticles within tumor tissues. For example, secretory phospholipase A2 (sPLA2) are enzymes overexpressed in various tumors.[199–201] Liposomes that are responsible for sPLA2 (SPRL) were engineered to facilitate liposomal degradation and drug release in tumor tissues.[202–204] In a study comparing the uptakes and cytotoxicities of the SPRL encapsulated with DOX and sterically stabilized liposomes encapsulated with DOX, Moch et al suggested that the efficacy of the sPLA2 liposomes are mediated by cell-dependent mechanisms.[203] Recently, Hofmann et al provided evidence supporting the existence of drug delivery into cells without cellular uptake of the nanoparticles through a new "kiss-and-run" mechanism between (polymeric) nanoparticles and the cell membrane.[205]

Conclusion

Despite recent advances in targeted therapies and immunotherapies, chemotherapy using cytotoxic agents remains an indispensable modality in cancer treatment. Formulating cancer chemotherapeutic agents into nanomedicines represents an attractive approach to modify their pharmacokinetics, efficacy, and toxicity profiles. Moreover, cancer cells often develop resistance to chemotherapeutic agents prior to or during treatment, and there is encouraging evidence that formulating cancer chemotherapeutic agents into nanomedicines may also represent a viable approach to overcome cancer cell chemoresistance. However, better nanomedicine formulations of chemotherapeutical agents are often the results of rational mechanism-based design, and occasionally by accident.

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Disclosure

The authors report no conflicts of interest in this work.

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