Saiki *et al. Cancer Drug Resist* 2020;3:819-31
**DOI:** 10.20517/cdr.2020.39

Cancer Drug Resistance

Review

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# Attempts to remodel the pathways of gemcitabine metabolism: Recent approaches to overcoming tumours with acquired chemoresistance

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**How to cite this article**: Saiki Y, Hirota S, Horii A. Attempts to remodel the pathways of gemcitabine metabolism: recent approaches to overcoming tumours with acquired chemoresistance. *Cancer Drug Resist* 2020;3:819-31. http://dx.doi.org/10.20517/cdr.2020.39

**Received**: 3 Jun 2020 **First Decision**: 2 Jul 2020 **Revised**: 19 Jul 2020 **Accepted**: 8 Sep 2020 **Available online**: 12 Oct 2020

**Academic Editor**: Nils Brünner **Copy Editor**: Cai-Hong Wang **Production Editor**: Jing Yu

## Abstract

Gemcitabine is a cytidine analogue frequently used in the treatment of various cancers. However, the development of chemoresistance limits its effectiveness. Gemcitabine resistance is regulated by various factors, including aberrant genetic and epigenetic controls, metabolism of gemcitabine, the microenvironment, epithelial-to-mesenchymal transition, and acquisition of cancer stem cell properties. In many situations, results using cell lines offer valuable lessons leading to the first steps of important findings. In this review, we mainly discuss the factors involved in gemcitabine metabolism in association with chemoresistance, including nucleoside transporters, deoxycytidine kinase, cytidine deaminase, and ATP-binding cassette transporters, and outline new perspectives for enhancing the efficacy of gemcitabine to overcome acquired chemoresistance.

**Keywords**: Gemcitabine, chemoresistance, deoxycytidine kinase, human equilibrative nucleoside transporter 1, cytidine deaminase, ATP-binding cassette transporters, metabolism

## INTRODUCTION

Gemcitabine [2',2'-difluoro-2'-deoxycytidine (dFdC)], was first described by Eli Lilly and Company in 1986[1] and is the most important deoxycytidine nucleoside analogue with fluorine substituents at the 2'

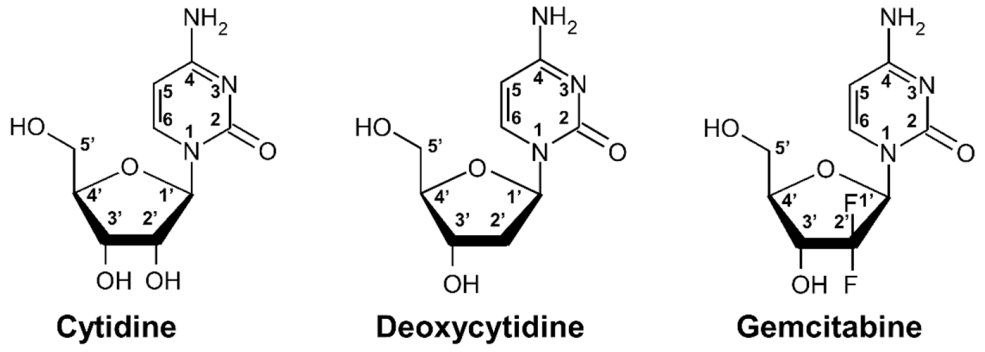


**Figure 1.** Structures of cytidine, deoxycytidine and gemcitabine

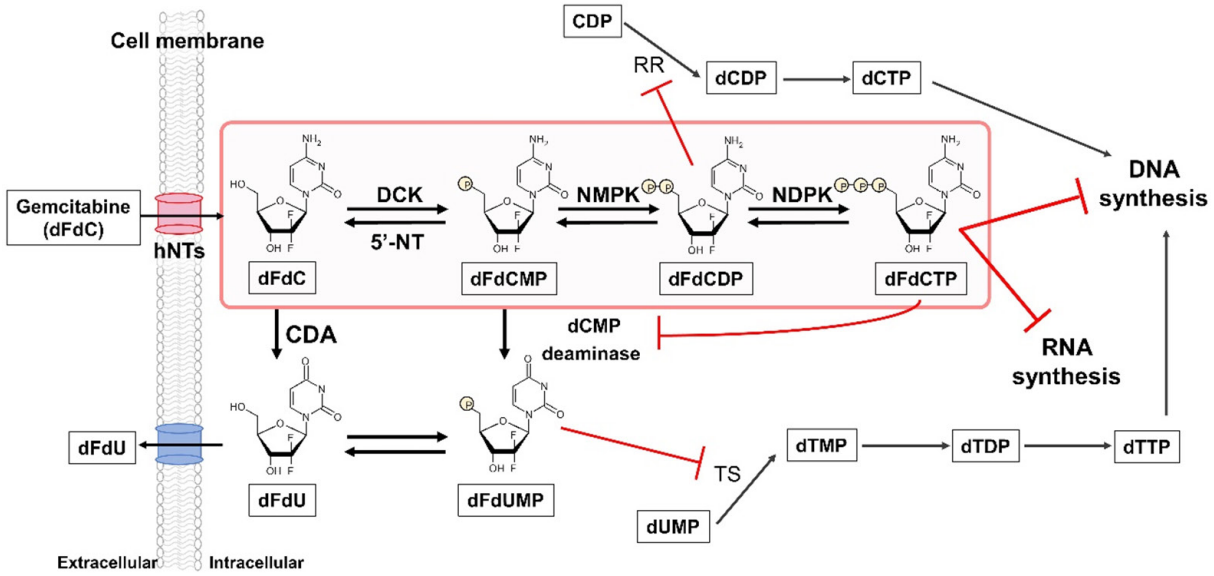


**Figure 2.** Metabolism and action of gemcitabine [difluoro 2'-deoxycytidine (dFdC)]. dFdC is transported into the cell through nucleoside transporters (hNTs), then stepwise phosphorylated by deoxycytidine kinase (DCK), nucleoside monophosphate kinase (NMPK), and nucleoside diphosphate kinase (NDPK), to form active triphosphate metabolite (dFdCTP). This molecule then inhibits DNA and RNA synthesis. Diphosphate metabolite (dFdCDP) inhibits ribonucleotide reductase (RR), an enzyme that catalyses the conversion of ribonucleotide (CDP) to deoxyribonucleotide (dCDP). The majority of dFdC is inactivated mainly by cytidine deaminase (CDA) mediated conversion to difluorodeoxyuridine (dFdU) and then excreted through the ABC transporter. Deamination of dFdCMP to dFdUMP by deoxycytidylate deaminase (dCMP deaminase) and subsequent dephosphorylation forms dFdU; this is another inactivation pathway of dFdC. dFdUMP inhibits thymidylate synthase (TS), resulting in the depletion of the dTMP pool. dFdCTP inhibits dCMP deaminase

position of the pentose ring [Figure 1][2]. Its metabolic pathway is illustrated in Figure 2. This molecule is hydrophilic, and can be transported into cells by nucleoside transporters (hNTs), including both sodium-dependent concentrative nucleoside transporters (hCNTs) and sodium-independent equilibrative nucleoside transporters (hENTs). hCNTs mediate unidirectional transportation of nucleosides. hENT1 can uptake gemcitabine with high affinity but low capacity, whereas hENT2 can uptake gemcitabine with low affinity but high capacity. The intracellular uptake of gemcitabine is mainly mediated by hENT1 in cancer cells. In hepatocytes, the uptake of gemcitabine is mainly mediated by low affinity hENT2[3,4].

Gemcitabine is a prodrug which requires intracellular phosphorylation for activation. Inside the cell, gemcitabine is phosphorylated to its monophosphate form (dFdCMP) by deoxycytidine kinase (DCK) and is then further phosphorylated to its diphosphate (dFdCDP) and then triphosphate forms (dFdCTP), as

shown in Figure 2. The resulting dFdCTP is incorporated into DNA and then the DNA strand synthesis is terminated after incorporation of another nucleotide, hiding dFdCTP from DNA repair enzymes[5]. dFdCTP is also incorporated into RNA[6,7], and sensitivity to gemcitabine is related to differences in RNA incorporation[8]. RNA incorporation of gemcitabine may play an important role in its activity. dFdCDP is an effective inhibitor of ribonucleoside-diphosphate reductase, an enzyme that transforms CDP into dCDP; this results in a decrease of the dCTP pool. Deamination of dFdCMP by dCMP-deaminase forms dFdUMP. Thymidylate synthase, which plays a key role in the synthesis of thymidine monophosphate (TMP)[9], is another target for gemcitabine, via dFdUMP. The natural substrate of TS, 2'-deoxyuridine monophosphate (dUMP), resembles dFdUMP, and it inhibits TS resulting in a depletion of the TMP pool.

Evidence for the usefulness of gemcitabine as a potent anti-tumour reagent has been reported; it is used either alone, or in combination with other agents for patients with pancreatic ductal adenocarcinoma (PDAC)[10] and several other human cancers, such as non-small cell lung cancer, breast cancer, ovarian cancer, and bladder cancer[11] (approved by FDA). However, acquisition of chemoresistance against gemcitabine significantly limits its effectiveness. Chemoresistance can be divided into two categories, intrinsic and acquired, in the course of drug treatment[12]. Activities of drug transporters and metabolizing enzymes have been considered to be strongly involved in the chemoresistance to gemcitabine. Epithelial-to-mesenchymal transition (EMT) is not only related to a phenotypic change in the tumour cells; it also contributes to gemcitabine resistance[13]. Based on gene expression profiles of pancreatic cancer cell lines, gemcitabine-resistant cells contain many features consistent with EMT[14]. Exosomes have shown to be involved in gemcitabine resistance by delivering miRNAs. Exosomal miR-106b from cancer-associated fibroblasts[15] and miR-210 from cancer stem cells[16] both promote gemcitabine resistance. However, these areas are beyond the focus of this review, and we will discuss the challenges of remodelling the gemcitabine metabolizing pathway to overcome acquired chemoresistance against gemcitabine.

## IMPROVEMENT OF GEMCITABINE UPTAKE

The membrane permeability of gemcitabine is poor in human cells. It is mediated by five distinct hNTs with different affinities; two equilibrative-type (hENT1, hENT2) and three concentrative-type transporters (hCNT1, hCNT2, hCNT3)[17-19]. Among these, hENT1 functions as the major gemcitabine transporter; *in vitro* experiments have demonstrated that increased expression of hENT1 is the critical factor for sensitivity to gemcitabine[20]. Restriction of intracellular uptake of gemcitabine by suppressed expression of hENT1 is one of the established mechanisms of drug resistance[19,21]. The majority of studies on patients with resected pancreatic cancer have suggested that high expression of this hENT1 may be predictive of improved survival in patients treated with gemcitabine[22-24]. Disrupted expression of hENT2 on the plasma membrane causes impaired uptake of gemcitabine, resulting in acquired chemoresistance of pancreatic cancer cells[25].

Currently, several approaches to enhancing the efficacy of gemcitabine uptake or to bypass the hNTs have been introduced. hCNT1 is frequently diminished in pancreatic cancer cells compared with normal pancreatic ductal epithelial cells[26], so drug inhibition or degradation of hCNT1 can increase the transportation of gemcitabine, and thus improve its efficacy[27]. A recent study indicated that mucin 4 (MUC4) suppresses hCNT1 expression and that inhibition of MUC4 enhances gemcitabine sensitivity[28].

NEO6002 is a gemcitabine modified cardiolipin [Figure 3A]. This molecule entes the cell independently of hNT, and exerts higher activity, with lower toxic adverse side effects in mouse tumour xenograft model[29]. Another lipophilic prodrug, gemcitabine-elaidic acid conjugate CP-4126 [Figure 3A], also known as CO-101, is transported into the cells independently of hENT1 and has been demonstrated to be effective *in vitro* and in various human cancer models[30]. However, a long-term survival analysis found that the survival rate of patients using CP-4126 was not superior to gemcitabine in patients with low expression of hENT1 in

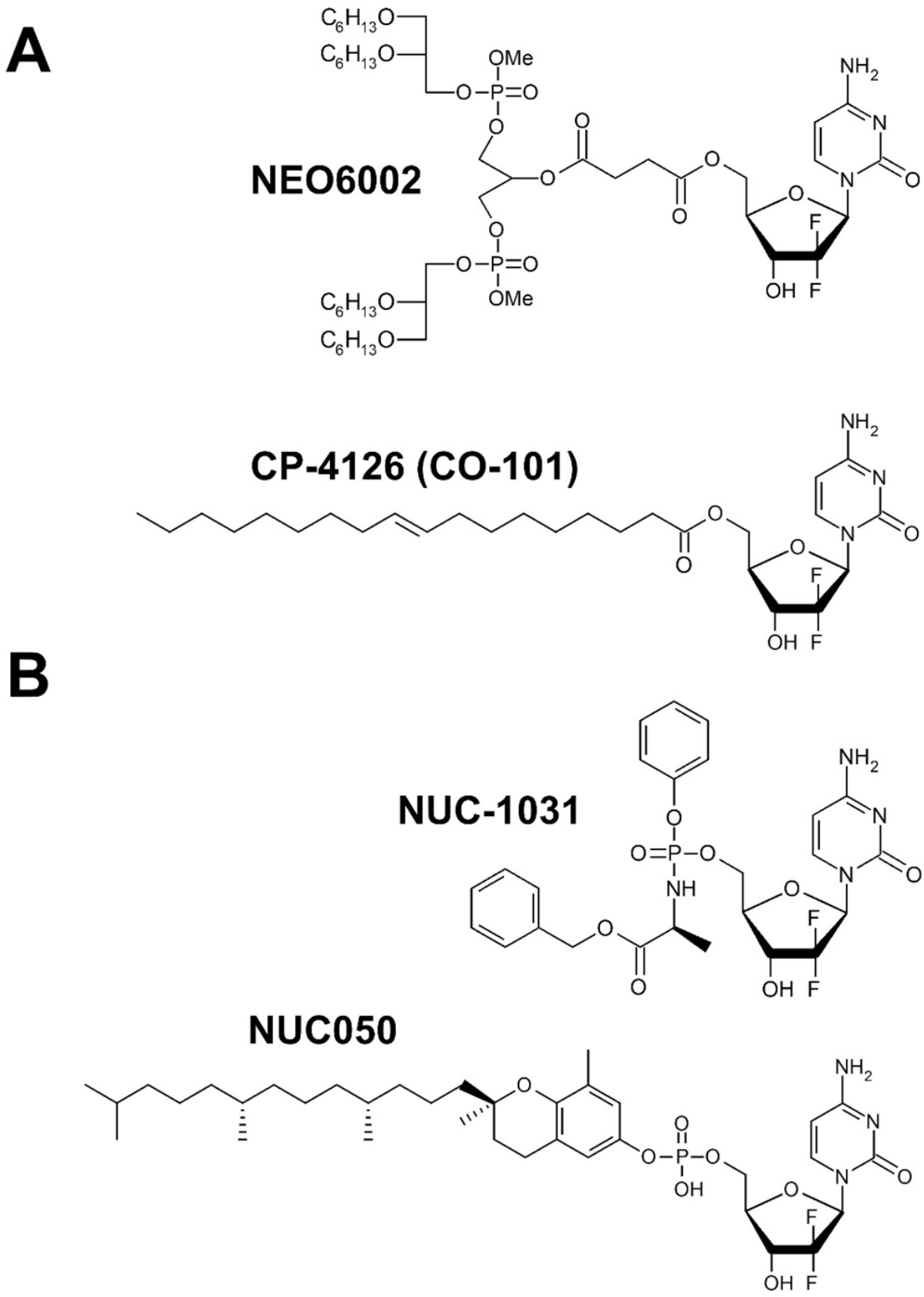


**Figure 3.** Structures of NEO6002 and CP4126, the gemcitabine-modified compounds that can bypass hNT-mediated introduction inside the cell (A); structures of NUC-1031 and NUC050, gemcitabine-modified compounds that can bypass the deoxycytidine kinase-mediated activation pathway of gemcitabine (B)

**Table 1. Summary of clinical trials**

| Responsible party | Condition or disease | Intervention/ treatment | Baseline participants (analyzed participants) | Age mean years (SD) | PFS median months (95%CI) | CR + PR % (95%CI[b]) | OS median months (95%CI) | NCT Number | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| Clovis Oncology, Inc. | Metastatic Pancreatic Adenocarcinoma (hENT Low) | CO-101 | 182 (114) | 62.5 (9.62) | - | - | 5.7 (4.7-7.6) | 01124786 | [31] |
| | | Gemcitabine | 185 (118) | 60.5 (11.19) | - | - | 6.1 (5.2-7.7) | | |
| National Cancer Institute (NCI) | Non-Small Cell Lung Cancer | FdCyd + THU | 25 (25) | 59.92 (9.95) | 2.3 (1.6-3.9) | 0.0 (0.0-13.7) | N/A | 00978250 | * |
| | Breast Cancer | | 30 (29) | 56.04 (10.07) | 3.7 (1.8-5.3) | 6.9 (0.8-22.8) | | | |
| | Bladder Cancer | | 18 (18) | 67.77 (8.93) | 3.6 (1.7-8.0) | 5.6 (0.1-27.3) | | | |
| | Head and Neck Cancer | | 22 (21) | 55.17 (11.70) | 1.7 (1.7-4.5) | 0.0 (0.0-16.1) | | | |
| Imperial College London | Several types of cancers | NUC-1031 | 68 (49) | 56.3 (range 20-83) | 4.0 (range 1-25) | 10 | N/A | 01621854 | [84] |

*https://www.clinicaltrials.gov/ct2/show/results/NCT00978250. FdCyd: 5-Fluoro-2'-Deoxycytidine; THU: Tetrahydrouridine; PFS: progression-free survival; OS: overall survival; CR: complete response; PR: Partial response; CI: Confidence interval; SD: Standard deviation

metastatic PDAC (NCT01124786) [Table 1][31]. This study was performed using an antibody against hENT (clone SP120), but recent report by Raffenne *et al.*[32] using another antibody for hENT1 showed different results. They used a clone 10D7G2 and demonstrated that hENT tumour expression was significantly associated with better DFS and OS in PDAC patients. Thus, the usefulness of CP-4126 should be re-evaluated.

Recently, nanoparticles loaded with gemcitabine have been developed. Nanoparticle encapsulation allows chemotherapeutic drugs to pass easily without being affected by cell surface NTs. GEM-HSA-NP is a gemcitabine-loaded albumin nanoparticle; using patient-derived xenograft models, this nanoparticle has been shown to be more effective than gemcitabine in inhibition of tumour growth, irrespective of expression levels of hENTs[33]. Squalenoyl-gemcitabine bioconjugate (SQdFdC) is self-assembled into a stable nanoparticle[34]. This particle passively diffuses into cancer cells, mainly accumulated within the cellular membrane including those of endoplasmic reticulum. Subsequently, it is released gradually into the cytoplasm and cleaved into dFdC[35]. This is an original transporter-independent pathway, and SQdFdC can overcome the acquired resistance in a transporter-deficient human leukemic cell line, *in vivo*[35]. Chitkara *et al.*[36] made gemcitabine conjugated to poly (ethylene glycol)-block-poly (2-methyl-2-carboxyl-propylene carbonate) (PEG-PCC) which could self-assemble into micelles of 23.6 nm. These micelles were shown to afford protection to gemcitabine from plasma metabolism. Wonganan *et al.*[37] created PLGA-b-PEG-OH nanoparticles incorporated with gemcitabine. They delivered gemcitabine effectively into hCNT-decreased tumour cells and were significantly more cytotoxic than free gemcitabine. These nanoparticles are summarized in Table 2.

The above mentioned strategies are promising delivery systems to address transporter-deficient resistant cancer in the clinical setting.

## REGULATION OF CDA EXPRESSION AND CDA INHIBITORS

Cytidine deaminase (CDA) is a ubiquitously expressed enzyme that catalyses cytidine and deoxycytidine into uridine and deoxyuridine, respectively. This enzyme participates in the pyrimidine salvage pathway that maintains the nucleotide pool balance for DNA and RNA synthesis. The great majority

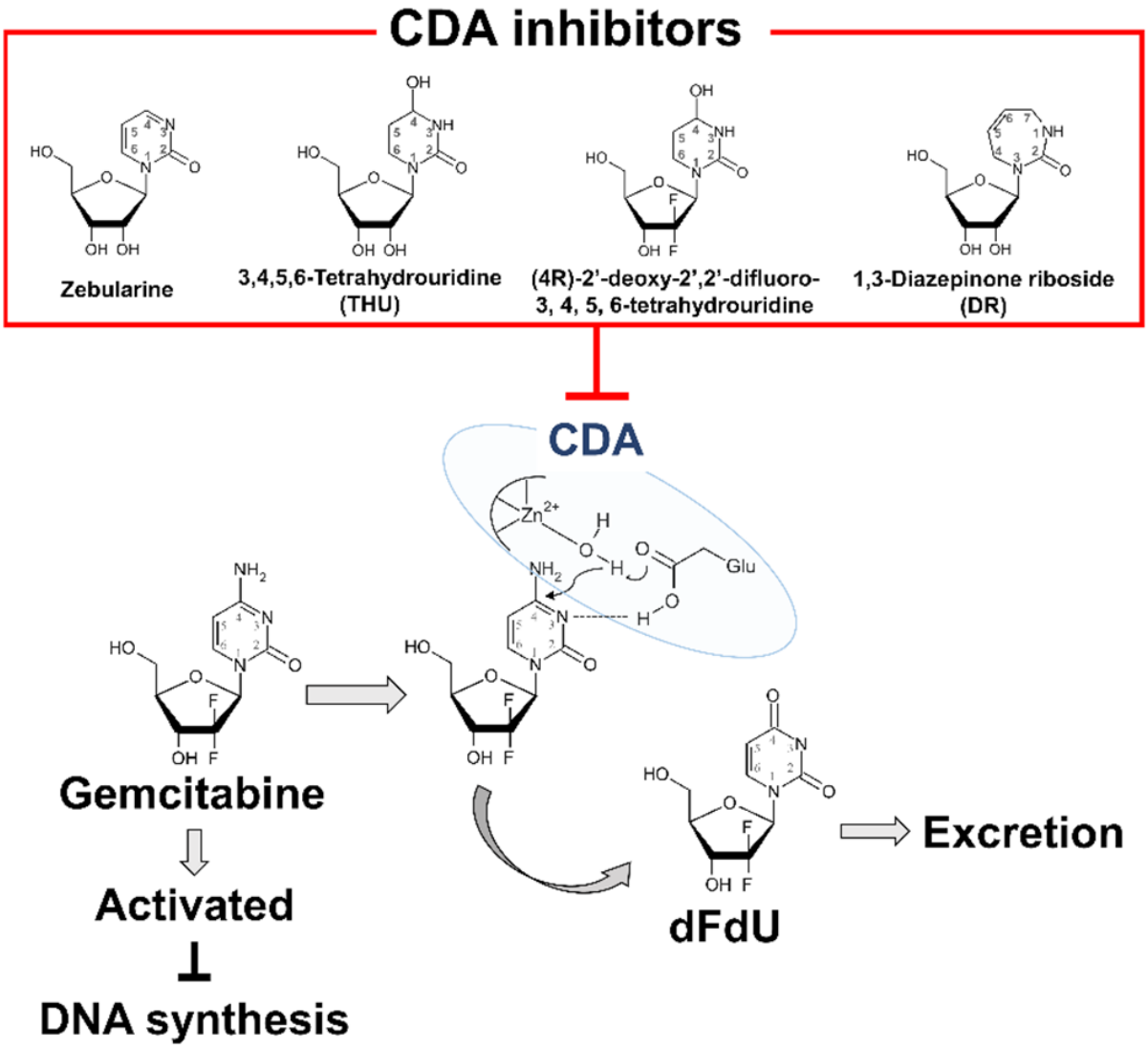


**Figure 4.** CDA-mediated processing of gemcitabine for excretion, and CDA inhibitors Zebularine, 3,4,5,6-Tetrahydrouridine (THU), 1,3-Diazepinone riboside (DR), and (4R)-2'-deoxy-2',2'-difluoro-3 4, 5, 6-tetrahydrouridine. CDA: cytidine deaminase

in 1967 using an affinity capture method with CDA as bait[58]. The inhibitory action of THU is based on its C4 hydroxyl group in the pyrimidine ring. Since the bioavailability of THU is weak[59], a new fluorinated version of this drug termed (4R)-2'-deoxy-2',2'w-difluoro-3 4, 5, 6-tetrahydrouridine [Figure 4] has been developed with better oral bioavailability[60]. DR was discovered in 1981; it cannot interact with CDA through the water/zinc complex. Its inhibitory activity instead results from an electrostatic interaction utilizing π electrons of the DR ring and the benzene ring of the F137 of CDA, the catalytic site of the enzyme[61]. However, no results of DR effectiveness have yet been reported even in cultured cells.

As mentioned before, CDA high-expressing tumours are theoretically more resistant to cytidine-based therapies, including gemcitabine. With this assumption, several studies combining various chemotherapies and CDA inhibitors have been conducted to date. A Phase II clinical trial (ClinicalTraials.gov: NCT00978250, see Table 1), combining treatment with 5-fluoro-2'-deoxcytidine and THU, has just been completed; all 93 patients eligible for the study were assessed as PFS, including patients with advanced non-small cell lung cancer, breast cancer, bladder cancer, or head and neck cancer (https://www.clinicaltrials.gov/ct2/show/results/NCT00978250). Weizman *et al.*[62] suggested that tumour infiltrating macrophages

for treatments. The identification of receptor overexpression in cancer cells will lead to the development of nanomedicines to improve the selectivity to the cancer cells and reduce off-target toxicities of gemcitabine. Further studies are needed for gemcitabine-based treatment to be included in personalized medicine tailored for numerous molecular therapeutic targets in multiple pathogenic pathways.

## DECLARATIONS

### Acknowledgments

We are grateful to Barbara Lee Smith Pierce (retired Adjunct Professor, University of Maryland University College) for her editorial work in the preparation of the manuscript.

### Authors' contributions

Made substantial contributions to conception of the review: Saiki Y, Horii A
Wrote the first draft of the manuscript: Saiki Y
Prepared tables and figures: Saiki Y, Hirota S
Brushed up the manuscript, tables, and figures: Saiki Y, Hirota S, Horii A

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.



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