**Cellular pharmacology of gemcitabine**

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Gemcitabine (2',2'-difluoro 2'-deoxycytidine, dFdC) is the most important cytidine analogue developed since cytosine arabinoside (Ara-C). The evidence of its potent antitumor activity in a wide spectrum of *in vitro* and *in vivo* tumor models has been successfully confirmed in the clinical setting. Despite structural and pharmacological similarities to Ara-C, gemcitabine displays distinctive features of cellular pharmacology, metabolism and mechanism of action. Following influx through the cell membrane via nucleoside transporters, gemcitabine undergoes complex intracellular conversion to the nucleotides gemcitabine diphosphate (dFdCDP) and triphosphate (dFdCTP), responsible for its cytotoxic actions. The cytotoxic activity of gemcitabine may be the result of several actions on DNA synthesis. dFdCTP competes with deoxycytidine triphosphate (dCTP) as an inhibitor of DNA polymerase. dFdCDP is a potent inhibitor of ribonucleoside reductase, resulting in depletion of deoxynucleotide pools necessary for DNA synthesis and, thereby, potentiating the effects of dFdCTP.

dFdCTP is incorporated into DNA and after the incorporation of one more nucleotide leads to DNA strand termination. This extra nucleotide may be important in hindering the dFdCTP from DNA repair enzymes, as incorporation of dFdCTP into DNA appears to be resistant to the normal mechanisms of DNA repair. Gemcitabine can be effectively inactivated mainly by the action of deoxycytidine deaminase to 2,2'-difluorodeoxyuridine. Also, 5'-nucleotidase opposes the action of nucleoside kinases by catalysing the conversion of nucleotides back to nucleosides. Additional sites of action and self-potentiating effects have been described. Evidence that up- or down-regulation of the multiple membrane transporters, target enzymes, enzymes involved in the metabolism of gemcitabine and alterations in the apoptotic pathways may confer sensitivity/resistance to this drug, has been provided in experimental models and more recently also in the clinical setting. Synergism between gemcitabine and several other antineoplastic agents has been demonstrated in experimental models based on specific pharmacodynamic interactions. Knowledge of gemcitabine cellular pharmacology and its molecular mechanisms of resistance and drug interaction may thus be pivotal to a more rational clinical use of this drug in combination regimens and in tailored therapy.

**Key words:** cellular pharmacology, gemcitabine, mechanisms of action, metabolism

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**Introduction**

Gemcitabine (2',2'-difluoro 2'-deoxycytidine, dFdC) is an analogue of cytosine arabinoside (Ara-C) from which it differs structurally due to its fluorine substituents on position 2' of the furanose ring (Figure 1) [1]. It has been the most important cytidine analogue to be developed since Ara-C, displaying distinctive pharmacological properties (Figure 2) and a wide spectrum of antitumor activity. Originally investigated as an antiviral agent, it was then developed as an anticancer drug on the basis of its impressive *in vitro* and *in vivo* antitumoral activity. The evidence of the efficacy of gemcitabine to inhibit the growth of human neoplasms was obtained in a broad range of solid and hematological cancer cell lines, as well as in *in vivo* murine solid tumors and human tumor xenografts in nude mice. Consequently gemcitabine was extensively studied in a variety of tumors in which significant clinical activity has been reported. Today gemcitabine is indicated as a single agent in the treatment of patients with metastatic pancreatic cancer [2] and in combination chemotherapy in non-small cell lung cancer [3], bladder cancer [4] and breast cancer [5]. Gemcitabine has also been successfully used in other tumors such as ovarian cancer, mesothelioma and head and neck cancers.

Like Ara-C, gemcitabine is a prodrug which requires cellular uptake and intracellular phosphorylation. Inside the cell, gemcitabine is phosphorylated to gemcitabine monophosphate (dFdCMP) by deoxycytidine kinase (dCK), which is then converted to gemcitabine di- and triphosphate (dFdCDP and dFdCTP, respectively). These are the active drug metabolites [6].

In contrast to Ara-C, gemcitabine has multiple intracellular targets. Its antiproliferative activity is believed to be dependent mainly on several inhibitory actions of DNA synthesis [6–8].

dFdCTP is an inhibitor of DNA polymerase [8] and is also incorporated into DNA [9]. After incorporation of only one additional nucleotide by DNA polymerase into the DNA chain, it leads to termination of chain elongation [9]. The non-terminal position of dFdCTP in the DNA chain prevents
detection and repair by DNA repair enzymes (masked chain termination) [10, 11]. These molecular events are critical to gemcitabine-induced apoptosis. A metabolite of dFdC, presumably dFdCTP, is also known to incorporate into RNA [12]. The effect of this RNA incorporation on cell function is, however, still unclear.

These effects are enhanced by a number of unique additional pharmacodynamic activities of gemcitabine di- and triphosphate, which prolong maintenance of high intracellular concentrations of gemcitabine metabolites and increase the probability of successful incorporation of gemcitabine into nucleic acids, mainly DNA, by reducing competing natural metabolites (self-potentiation). For example, in contrast to Ara-C, dFdCDP potently inhibits ribonucleotide reductase (RR) [13, 14], resulting in a decrease of competing deoxyribonucleotide pools necessary for DNA synthesis [13–15]. Other reported activities of gemcitabine metabolites include the inhibition of cytidine triphosphate synthetase (CTP synthetase) [16] and the inhibition of deoxycytidylate deaminase (dCMP deaminase) by dFdCTP [17].

It has recently been shown that gemcitabine can poison topoisomerase I, suggesting that induction of topoisomerase I-mediated DNA break formation can contribute to the cytotoxicity of this drug [18].

Changes in drug intracellular metabolite levels and activities of drug transporters, target enzymes and enzymes involved in drug metabolism can cause variable sensitivity to gemcitabine in normal and tumor cells (reviewed in Bergman et al. [19]). Data on the mechanisms of gemcitabine resistance have been obtained mostly in in vitro tumor models. Clinical pharmacogenomic studies have, however, confirmed the role of some of these molecular determinants in drug response of individual cancer patients [20–22].

Moreover, specific pharmacodynamic drug interactions constitute the basis of synergism between gemcitabine and other antineoplastic agents [23]. Knowledge of gemcitabine cellular pharmacology, molecular mechanisms of resistance and drug interactions may thus provide the rational basis for an optimal clinical use of this drug in combination regimens and tailored therapy.

This review provides an overview of gemcitabine cellular pharmacology issues related to its clinical use. Other relevant more detailed reviews on this topic have been published [19, 24, 25].

cellular pharmacology

transport

As for Ara-C and other nucleoside analogues, transport of gemcitabine across the cell membrane occurs via multiple active nucleoside transporters (NTs) located in the cell plasma membrane [26].

Human NTs (hNTs) are carriers either of the sodium-dependent (concentrative) type (CNT) or of the sodium-independent (equilibrative) type (ENT) [27, 28]. Both these types are involved in the uptake of gemcitabine [26, 29]. In particular, gemcitabine is a substrate, albeit at different affinity, for five of the hNTs, two of the equilibrative type (hENT1, hENT2) and three of the concentrative type (hCNT1, hCNT2, hCNT3) [26, 29, 30]. The presence of nucleoside transport activity is considered a prerequisite for cell growth inhibition and clinical efficacy of gemcitabine. Most of gemcitabine uptake...
is mediated by hENT1 and hENT1-deficient cells are in fact highly resistant to this nucleoside analogue [26]. Sensitivity to nucleoside analogues, including gemcitabine, in vitro and in the clinical setting has been shown to be correlated with the expression of this transporter [31–33].

Also, inhibition of nucleoside transport across the cell membrane results in resistance to gemcitabine [34]. Finally, there is recent evidence that nucleoside transport deficiency is an important predictive factor for gemcitabine response in the clinical setting [20].

**metabolism**

Like Ara-C, gemcitabine is phosphorylated intracellularly by dCK to produce dFdCMP, which is then converted to its active diphosphate and triphosphate metabolites (dFdCDP and dFdCTP) [6].

Kinetic studies have shown that dCK has a Km value of 4.6 μM for this drug compared to 1.5 μM for deoxycytidine, which makes dCK accept gemcitabine as a good substrate, although with a 3-fold lower affinity than that of deoxycytidine itself [35]. In addition, dCK has a higher affinity for gemcitabine than for Ara-C (Km=3.6 and 8.8 μM, respectively) and the Vmax/Km ratio is more favorable [6].

Gemcitabine is also phosphorylated by thymidine kinase 2 (TK2), a mitochondrial enzyme which phosphorylates natural nucleosides. The substrate specificity of this enzyme for gemcitabine is, however, only 5–10% of that for deoxycytidine [36].

Thus, phosphorylation of gemcitabine by dCK is the rate-limiting step for further phosphorylation to active metabolites and is essential to its cytotoxic activity. dCK deficiency is also a frequently described form of in vitro and in vivo acquired and intrinsic resistance to gemcitabine (reviewed in Bergman et al. [19]).

These data suggest a key role of dCK as a predictive determinant for gemcitabine efficacy in the clinical setting [37], although both retrospective and prospective studies on the relationship between pretreatment measurements of dCK expression and gemcitabine treatment outcome are still lacking. Instead, the role of TK2 in either gemcitabine host toxicity or antitumor activity is at present unclear since published studies are not univocal on this topic (reviewed in Bergman et al. [19]).

Gemcitabine is inactivated mainly by deoxycytidine deaminase (dCDA) mediated conversion to difluorodeoxyuridine (dFdU) [17]. Gemcitabine has a 50% lower affinity for this enzyme than does deoxycytidine (Km: 95.7 and 46.7 μM, respectively) [35]. Deamination occurs at the cellular level in vitro and is the major form of metabolic clearance in vivo due to the activity of dCDA in large body organs [38].

Deamination of dFdCMP to 2’-3’-difluorodeoxyuridine monophosphate (dFdUMP) by the action of dCMP-deaminase and subsequently to dFdU represents another inactivation pathway of gemcitabine [17]. Since gemcitabine and dFdU are not substrates for pyrimidine nucleoside phosphorylases, the drug is not further degraded and excreted out of the cell [17].

The role of dCDA in the development of gemcitabine resistance is not yet clear. Some in vitro studies have reported a relationship between increased dCDA activity and resistance to gemcitabine in cells transfected with this gene [39]. Human lung carcinoma cell lines made resistant to anthracyclines display collateral sensitivity to gemcitabine associated with decreased dCDA activity [40]. However, in human tumor cell lines and human tumor xenografts, natural sensitivity/ intrinsic resistance to gemcitabine is not related to dCDA activity [41, 42]. Although up-regulation of dCDA does not play a major role in intrinsic resistance to gemcitabine, up-regulation of this enzyme may play a key role in acquired resistance to this drug. A significantly higher rate of early disease progression and shorter overall survival has been observed in patients with advanced pancreatic carcinoma whose mononuclear cells had higher dCDA expression and activity levels, compared to patients with lower dCDA expression and activity levels [21].

Also 5’-nucleotidase (5’-NT) opposes the action of nucleoside kinase by catalyzing the conversion of nucleotides back to nucleosides. Several cytosolic 5’-NT are potentially important in degradation of nucleoside monophosphates. However, the substrate specificities have been defined for only few enzyme forms. While the kinetic properties of the human cytosolic 5’-NT II (cN-II) make it unlikely to contribute to the pyrimidine dephosphorylation [43], the overexpression of 5’-NT I (cN-I) confers resistance to several pyrimidine analogs [44]. In the gemcitabine-resistant leukemia K562 cell line, resistance was associated with increased 5’-NT activity [45]. Also, 5’-NT I gene transfection in HEK293 tumor cells conferred marked resistance to gemcitabine as compared to parental cells [44].

An important determinant of sensitivity to gemcitabine is the intracellular retention of dFdCTP. Clonogenic assays have demonstrated loss of viability after incubation with increasing gemcitabine concentrations for only 4 h [9]. This indicates that active nucleotide metabolites of gemcitabine, an S phase specific agent [7], are retained intracellularly for prolonged periods (several hours) necessary for most of the cell population to pass through the cell cycle. Cellular pharmacokinetic studies demonstrated that dFdCTP has a biphasic elimination from leukemic cells with an α half-life of 3.9 h and β half-life of 16 h while Ara-C triphosphate has monophasic elimination with an α half-life of only 0.7 h [6]. Long retention of this active metabolite is associated with increased sensitivity to gemcitabine as reported in in vitro and in vivo tumor models [15, 42]. The prolonged retention of gemcitabine nucleotides is thus potentially important for the cellular and clinical effects of this drug. A concentration dependence of dFdCTP cellular accumulation and elimination has been observed in vitro [17] and ex vivo during clinical investigations [38, 46, 47]. These findings have relevant implications for the use of different gemcitabine dose administration schedules and rates in the clinical setting.

**sites of action**

As with Ara-C, the main mechanism of action of gemcitabine is a potent inhibition of DNA synthesis [6, 7, 9]. The killing effects of gemcitabine are however not confined to the S phase of the cell cycle and the drug is equally effective against confluent cells and cells in log-phase growth [48]. Important differences exist
between Ara-C and gemcitabine concerning the mode of incorporation into DNA, existence of additional sites of action and consequent kinetic properties of cell growth inhibition and spectrum of activity [49].

The potent cytotoxic activity of gemcitabine is the result of additional actions on DNA synthesis: dFdCDP competes with deoxycytidine triphosphate (dCTP) as a weak inhibitor of DNA polymerase [8]. dFdCDP is incorporated into DNA and, after the incorporation of one more nucleotide, leads to DNA polymerization termination [9] and single strand breakage [50]. This ‘extra’ nucleotide may be important in hiding the dFdCTP from DNA repair enzymes, because incorporation of gemcitabine into DNA appears to be resistant to the normal mechanisms of DNA repair [11].

These effects on DNA synthesis represent the main action of gemcitabine, and emerging evidence demonstrates that incorporation of dFdCTP into DNA is critical for gemcitabine-induced apoptosis [51].

dFdCTP is also incorporated into RNA depending on the cell line. Incorporation of gemcitabine into RNA is time- and concentration-dependent, leading to inhibition of RNA synthesis [12]. In human tumor cell lines displaying different degrees of resistance to gemcitabine, sensitivity to this drug was related to differences in RNA incorporation [52]. The role of gemcitabine incorporation into RNA in inducing cytotoxicity is, however, still unclear.

Several metabolites of gemcitabine can inhibit various enzymes, leading to self-potentiation of gemcitabine action.

The inhibition of RR by dFdCDP is the most important one of these self-potentiating mechanisms. RR inhibition leads to depletion of dCTP, a potent feedback inhibitor of dCK, leading to a more efficient phosphorylation of gemcitabine [13]. On the contrary, an increase of dCTP pools due to increased RR activity will decrease phosphorylation of gemcitabine and reduce its activity [53]. In keeping with these observations, it has been recently reported that overexpression of RR subunit 1 (RRM1) is associated with gemcitabine resistance in non-small cell lung cancer cell lines, demonstrating that this subunit represents a critical determinant for antitumor activity of gemcitabine [54]. The key role for RRM1 in acquired gemcitabine resistance has also been confirmed in an in vivo tumor colon carcinoma model [55]. A clinical study suggests that RRM1 RNA expression is a crucial predictive marker of survival in advanced non small cell lung cancer patients treated with a gemcitabine and cisplatin combination chemotherapy regimen [22].

Moreover, since dFdCTP competes with dCTP, a decrease in dCTP pools increases incorporation of dFdCTP into DNA [13]. In addition, dCMP-deaminase is directly inhibited by dFdCTP at high concentrations, with consequent decrease of gemcitabine catabolism [17]. Since CTP may compete with dFdCTP for incorporation into RNA, the inhibition of CTP-synthetase, which may maintain CTP pools during gemcitabine exposure, by high cellular concentrations of dFdCTP leads to CTP pool depletion [17], possibly by increasing incorporation of dFdCTP into RNA and limiting RNA synthesis.

Theoretically, dFdUMP may serve as either a substrate or an inhibitor of thymidylate synthase (TS). The possible inhibition of TS by gemcitabine exposure could result in a depletion of thymidine monophosphate (TMP) pools, as suggested by Ruiz Van Haperen et al. [56]. Whether dFdUMP is an inhibitor of or a substrate for TS is, however, a question requiring more extensive research.

The gemcitabine self-potentiation mechanisms contribute also to the reported biphasic and slow elimination of dFdCTP [6].

Recent data demonstrate that incorporation of gemcitabine into DNA, like Ara-C, can trap topoisomerase I cleavage complexes [18, 57]. However, in contrast to Ara-C, structural conformation of gemcitabine enhances the stability of topoisomerase I cleavage complexes [57]. The stability of topoisomerase I cleavage complexes can generate DNA-strand breaks by interference with advancing replication or transcription forks. Poisoning topoisomerase I suggests that gemcitabine incorporation may increase the frequency of collisions between the topoisomerase I cleavage complexes and replication and transcription complexes, leading to the accumulation of strand breaks and thus cell death [18]. In view of these data, gemcitabine could exert its antiproliferative effects not only through the inhibition of the DNA polymerases, but also by poisoning cellular topoisomerase I.

As yet little is known about the capability of DNA repair mechanisms to reverse gemcitabine-induced DNA damage. It has been shown that the 3’→ 5’ exonucleases of the Klenow fragment cannot excise gemcitabine from DNA [9]. However, extensive research on the complex DNA repair mechanisms involved at the cellular level has not been performed.

It is still unclear by which downstream molecular pathway gemcitabine incorporation into DNA leads to cell death. Apoptosis-regulating genes, such as p53, bcl-2, bcl-xL, BAX, regulate cancer cell sensitivity/resistance to gemcitabine [58, 59]. In some solid human tumor cell lines (e.g. non-small cell lung cancer, pancreatic cancer) gemcitabine induces apoptosis by activation of various caspases (e.g. caspase 8 and caspase 3), key components of the apoptotic pathways [60, 61]. This suggests an important role of caspase activation in gemcitabine sensitivity/resistance.

conclusions

Gemcitabine has acquired a relevant role in the treatment of several solid tumors. Despite its success, the fact that the antitumor activity and toxicity of gemcitabine vary from patient to patient and the development of drug resistance remain important causes of low response rates and lack of efficacy in relapsed tumors as well as unpredictable severe toxic effects. These phenomena are believed to be related to variations in drug intracellular metabolite levels and activities of drug transporters, drug metabolizing enzymes, target enzymes and enzymes involved in programmed cell death between different normal and tumor cell types and individuals (reviewed in Bergman et al. [19]. Knowledge of gemcitabine intracellular pharmacology is thus potentially relevant to a more comprehensive understanding of the mechanisms of sensitivity/resistance and toxicity to this drug, and will assist in the development of therapeutic strategies aimed at improving treatment efficacy while reducing drug toxicity.

Recently, clinical pharmacogenomic and pharmacogenetic studies have provided initial confirmation of the role of
differential expression and polymorphisms of genes whose protein products are involved in gemcitabine transport, metabolism and action in drug response and toxicity [20–22, 62].

As for other antimetabolites, it has been shown that knowledge of gemcitabine cellular pharmacology and its interaction with other anticancer agents may also be pivotal to a more rational use of this drug in combination regimens [23].

Since gemcitabine lacks cross-resistance with other anticancer agents, it represents an ideal candidate drug for combination chemotherapy. Consequently it is widely used within empirical drug combination regimens in the clinic. Synergism with other antineoplastic agents (e.g. cisplatin, taxanes, camptothecins, anthracyclines, antifolates, trastuzumab) has been demonstrated in vivo and in vitro models thus contributing to the development of rational drug combinations possibly characterized by improved efficacy over single constituent drugs in clinical trials.

disclosures

Dr Mini has reported that he is currently conducting research sponsored by Eli Lilly.

acknowledgements

The work was supported in part by grants to E.M. from the Ministero dell’Istruzione, dell’Università e della Ricerca (Rome, Italy, PRIN 2005) and from the Cassa di Risparmio Firenze (Florence, Italy, 2005) and in part by a grant to T.M. from the Ministero dell’Istruzione, dell’Università e della Ricerca (Rome, Italy, PRIN 2005).

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