Anti-Cancer Drugs 1995, **6**, Suppl 6, pp. 7–13

Preclinical characteristics of gemcitabine

William Plunkett, Peng Huang and Varsha Gandhi

Section of Cellular and Molecular Pharmacology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA.

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a nucleoside analogue of deoxycytidine in which two fluorine atoms have been inserted into the deoxyribofuranosyl ring. Once inside the cell gemcitabine is rapidly phosphorylated by deoxycytidine kinase, the rate-limiting enzyme for the formation of the active metabolites gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP). Gemcitabine diphosphate inhibits ribonucleotide reductase, which is responsible for producing the deoxynucleotides required for DNA synthesis and repair. The subsequent decrease in cellular deoxynucleotides (particularly dCTP) favours gemcitabine triphosphate in its competition with dCTP for incorporation into DNA. Reduction in cellular dCTP is an important self-potentiating mechanism resulting in increased gemcitabine nucleotide incorporation into DNA. Other self-potentiating mechanisms of gemcitabine include increased formation of active gemcitabine di- and triphosphates, and decreased elimination of gemcitabine nucleotides. After gemcitabine nucleotide is incorporated on the end of the elongating DNA strand, one more deoxynucleotide is added, and thereafter the DNA polymerases are unable to proceed. This action, termed "masked chain termination", appears to lock the drug into DNA because proof-reading exonucleases are unable to remove gemcitabine nucleotide from this penultimate position. Incorporation of gemcitabine triphosphate into DNA is strongly correlated with the inhibition of further DNA synthesis. Compared with ara-C, gemcitabine serves as a better transport substrate, is phosphorylated more efficiently, and is eliminated more slowly. These differences, together with self-potentiation, masked chain termination and the inhibition of ribonucleotide reductase, which are not seen with ara-C, may explain why gemcitabine is, and ara-C is not, active in solid tumours. This unique combination of metabolic properties and mechanistic characteristics suggests that gemcitabine is likely to be synergistic with other drugs that damage DNA, and also with other modalities such as radiation.

Introduction

Optimal design of therapeutic regimens employing anticancer drugs is aided greatly by an understanding of the pharmacokinetics of the agents involved. Furthermore, a knowledge of cellular pharmacology and the mechanisms by which metabolites exert their activities also provides a rationale on which to design strategies for combining agents or modalities. Nucleoside antimetabolites comprise one of the most effective classes of drugs for the treatment of cancer and viral diseases. As a rule, nucleoside analogues are active only after entry into the cell and phosphorylation to nucleotide derivatives, generally the corresponding triphosphates. The biological activity of most nucleoside analogues is due to their action in targeting DNA synthesis, an essential function for both cellular replication and for the repair of DNA damage that may be caused by other agents.

Arabinosylcytosine (cytarabine, ara-C) has long been the paradigm of nucleoside antimetabolites (Figure 1). It is an analogue of deoxycytidine which is metabolized by the same pathways and acts after incorporation into DNA. Ara-C is the single most effective agent in adult acute leukaemias and exhibits its activity in other leukaemias and lymphomas.[1,2] Unfortunately, numerous trials in solid tumours have indicated that its activity is confined to the haematological malignancies. These shortcomings have in part contributed to the synthesis and evaluation of different nucleoside analogues, in the hope that they may possess activity in solid tumours.

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC, Figure 1) is a new pyrimidine nucleoside analogue,

Studies from the authors' laboratories were supported in part by Grant DHP-1 from the American Cancer Society and Grants CA28596, CA32839 and CA57629 from the National Cancer Institute, Department of Health and Human Services.

Correspondence to W Plunkett
Section of Cellular and Molecular Pharmacology
University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030, USA
Tel: (+1) 713 792 3335; Fax: (+1) 713 794 4316

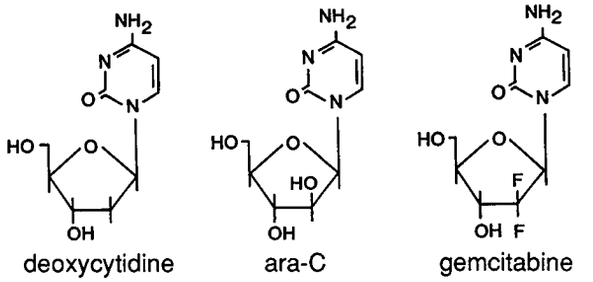

Figure 1. Chemical structures of deoxycytidine, arabinosylcytosine (ara-C) and gemcitabine (2',2'-difluorodeoxycytidine).

Table 1. Comparison of properties of gemcitabine and ara-C

Site of action	Gemcitabine	Ara-C
Activity against solid tumours	Effective	No activity
Transport substrate	3	1
Phosphorylation efficiency	6	1
Ribonucleotide reductase inhibition	Strong	None
Triphosphate elimination	Slow	Rapid
DNA termination	Masked	Terminal
Excision from DNA	Resistant	Yes
Self-potentiation	Multiple	None

which is also an antimetabolite of deoxycytidine.[3] The placement of geminal fluorine atoms on the 2'-carbon is the basis of the biological activity of gemcitabine as well as for the proprietary name of the drug. Unlike ara-C, gemcitabine displays multiple mechanisms of action which appear to interact to potentiate the overall cytotoxicity of the drug. These actions may contribute to the observed efficacy of gemcitabine in cell lines and experimental solid tumours. Encouragingly, clinical responses in phase II trials of gemcitabine have been seen in a wide spectrum of human solid tumours.[4,5] These results suggest that gemcitabine may have clinical potential alone and in combinations that surpass the activity of ara-C. This article will review the preclinical biological activity of gemcitabine, its cellular pharmacology, and its mechanisms of action, with a view to the goal of formulating a rationale for optimizing the use of gemcitabine in combinations with other drugs and with radiotherapy. Table 1 summarizes the biological, biochemical and molecular properties of gemcitabine and ara-C to facilitate comparisons.

Preclinical biological activity

The initial report[6] of the activity of gemcitabine and subsequent detailed publications[7–9] demonstrated potent cytotoxicity against human leukaemia cells and rodent fibroblasts. The activity of gemcitabine was reversed by deoxycytidine[7,8] and cells lacking deoxycytidine kinase[7] were not sensitive to the drug. This was consistent with the conclusion that gemcitabine is phosphorylated by deoxycytidine kinase and that accumulation of gemcitabine nucleotides is required for cytotoxicity. These studies were extended to experimental chemotherapy models which suggested a wide spectrum of activity in a variety of murine solid tumours as well as in leukaemia models.[8] Tumour response was schedule-dependent, with a staggered schedule of every third day for four doses being more effective than daily injections. Interestingly, ara-C was less effective at producing responses than was gemcitabine. Similar treatment schedules produced favourable responses against xenografts derived from human head and neck tumours[10] and sarcomas and ovarian tumours[11] in nude mice. Toxicological studies showed a decrease in haemoglobin as well as white blood cell count,[8] with compensatory increases in spleen size that normalized within 4 weeks.[12]

Cellular pharmacology

Gemcitabine appears to be a substrate for transport across the cell membrane.[7] Entry into cells was 65% more rapid than that of ara-C. Several nucleoside transporters have been identified by physiological characteristics, but detailed studies of gemcitabine transport have not yet been conducted. Another aspect of the cellular penetration of gemcitabine relates to its lipophilicity. The partitioning of gemcitabine between 1-octanol and aqueous buffer, a measure of lipophilicity, is approximately 5 times greater than that of ara-C. It is possible that this physical property may contribute to free diffusion of gemcitabine across the plasma membrane, but again more study is needed to evaluate this possibility.

Gemcitabine is an excellent substrate for deamination by cytidine deaminase.[9] The plentiful occurrence of this catabolic enzyme in large body organs provides a credible explanation for the rapid metabolic clearance of gemcitabine upon clinical infusion.[13,14] As is the case with the ara-C deamination

product (arabinosyluracil), 2',2'-difluorodeoxyuridine does not appear to have biological activity.[9]

Deoxycytidine kinase is the major enzyme responsible for phosphorylation of gemcitabine to the gemcitabine monophosphate (dFdCMP).[7] The fact that deoxycytidine spares the toxicity of gemcitabine is consistent with this finding.[7] Separation of cellular nucleoside kinases from the human leukaemia cell line K562 indicated that deoxycytidine kinase, but not adenosine kinase or deoxyguanosine kinase, was the major activity that phosphorylated gemcitabine.[15] In cells that lack deoxycytidine kinase activity, the cytotoxicity of gemcitabine is impaired.[7,16] Kinetic studies with highly purified enzyme preparations have demonstrated that the phosphorylation kinetics of gemcitabine by deoxycytidine kinase are similar to those of deoxycytidine.[17,18] Interestingly, gemcitabine appears to be a more efficient substrate for phosphorylation by deoxycytidine kinase than ara-C.[7,18] This is consistent with the observations that phosphorylation to the monophosphate is the rate-limiting step in accumulation of the respective 5'-triphosphates,[19–21] and that gemcitabine triphosphate accumulates to higher cellular concentrations than ara-C triphosphate.[7,15]

Gemcitabine monophosphate is a fair substrate for deamination by dCMP deaminase; its substrate efficiency is approximately 10% that of dCMP.[22,23] Subsequent metabolism or biochemical activities of the product, 2',2'-difluorodeoxyuridine 5'-monophosphate, have not been reported. Nevertheless, this appears to be an important pathway for the catabolism of gemcitabine nucleotides, because inhibition of dCMP deaminase with deoxytetrahydrouridine markedly affects the elimination kinetics of gemcitabine triphosphate.[22] Because gemcitabine triphosphate is itself an inhibitor of dCMP deaminase, this is seen as a mechanism by which nucleotides of gemcitabine affect their own metabolism, an action known as self-potentiation. The elimination of gemcitabine triphosphate is monophasic, with a half-life of 4–6 h in cells that have accumulated less than 100 μM of this metabolite. However, the elimination kinetics become biphasic with a prolonged terminal elimination time once gemcitabine triphosphate exceeds a cellular concentration of 100 μM.[7,9,22] This level is sufficient to inhibit the activity of dCMP deaminase.[22] The prolonged elimination of gemcitabine triphosphate has also been observed in circulating leukaemia cells during a phase I trial.[14] Because the residence time of gemcitabine triphosphate was correlated with the extent of inhibition of DNA synthesis in these cells, it is reasonable to propose that the prolonged retention of the triphosphate is a unique aspect of the cellular pharmacology of the drug which may contribute to its clinical activity.

Although the enzymes that successively phosphorylate gemcitabine monophosphate to the diphosphate and then phosphorylate the diphosphate to form the triphosphate have not been formally identified, it is assumed that this occurs by the sequential action of deoxycytidylate kinase and nucleoside diphosphate kinase, respectively. A better understanding of the regulatory properties of all the enzymes required for gemcitabine nucleotide formation will be important to optimize conditions for modulating gemcitabine metabolism.[15]

Mechanisms of action

The major biological activity of gemcitabine involves a selective interference with DNA synthesis. This was first seen in rodent fibroblasts[7] and human leukaemia cell lines.[9] Precursor incorporation studies demonstrated that a 2 h incubation with 15–25 nM gemcitabine was sufficient to inhibit [^{3}H]-thymidine incorporation by 50%, whereas [^{3}H]-uridine incorporation was not affected by up to 1 μM gemcitabine.[7,9,24] Subsequent studies in the same cells by the Dutch group showed similar results after a 4 h incubation, but that [^{3}H]-uridine incorporation was inhibited after incubation with 0.1 μM gemcitabine for 24 h.[25] Because cells lose clonogenic capacity[24] and show characteristics of apoptosis after incubation with these concentrations of gemcitabine for 2–4 h,[26,27] it is possible that the effects of gemcitabine on RNA metabolism after a 24 h incubation may reflect secondary effects of this treatment.

An inverse correlation exists between the cellular concentration of gemcitabine triphosphate and the ability of cells to incorporate [^{3}H]-thymidine during a pulse incubation.[7,9] In this respect, ara-C triphosphate was considerably more potent at inhibiting DNA synthesis than was gemcitabine triphosphate.[7] After washing cells into drug-free medium, metabolic elimination of gemcitabine triphosphate from the cells was associated with recovery of [^{3}H]-thymidine incorporation. Studies with structurally and metabolically related nucleosides suggested that effects of gemcitabine on deoxynucleotide pools and incorporation of gemcitabine nucleotides into DNA might provide biochemical and molecular explanations for the metabolic effects associated with cytotoxicity.

Inhibition of ribonucleotide reductase

The major source of deoxynucleotides which are required for DNA synthesis and repair of damaged DNA is ribonucleotide reductase. Cells incubated with gemcitabine show a loss of deoxynucleotides that is both time and concentration dependent.[9,28] The major effect seen in the CCRF-CEM lymphoblast line is on dCTP, which is quickly reduced to levels that seem inadequate to support DNA synthesis.[28] dATP is also substantially decreased in K562 cells which were derived from a patient with chronic myelogenous leukaemia.[9] In HT-29 human colon carcinoma cells, dATP was the most severely affected deoxynucleotide.[29] Further studies with additional cell lines will be necessary to establish whether or not deoxynucleotides are affected in a tissue-specific pattern. *In situ* assays of ribonucleotide reductase reflect more accurately the activity of this highly regulated enzyme in intact cells. Studies using these techniques, which measure the flux of ribonucleosides through ribonucleoside reductase and accumulation in deoxynucleotide pools and DNA, demonstrated that the reduction of ribonucleotides was substantially inhibited in CCRF-CEM cells by 0.1 and 1 μM gemcitabine.[28] In fact, gemcitabine was more effective at lowering deoxynucleotide pools than was the classic reductase inhibitor hydroxyurea. In contrast, ara-C had little effect on cellular deoxynucleotide pools or the flux of ribonucleotides through ribonucleotide reductase.

The diphosphate of gemcitabine appears to be the nucleotide which inhibits ribonucleotide reductase. There is a strong inverse correlation between accumulation of the diphosphate in intact cells and the loss of enzyme activity determined in an *in situ* assay. The triphosphate is much less inhibitory than the diphosphate in partially purified preparations of ribonucleotide reductase from human leukaemia cells.[28] The small inhibitory activity which was seen may be attributed to conversion of the triphosphate to the diphosphate, probably by a small contaminating activity of nucleoside diphosphate kinase. The diphosphate probably exerts its activity as an inhibitory alternative substrate, inhibiting the enzyme in a mechanism-based fashion.[28,30] The details of this activity remain to be elucidated. In contrast to gemcitabine, the nucleotides of ara-C do not appear to affect the activity of ribonucleotide reductase.[31]

The triphosphate of gemcitabine does not directly inhibit DNA polymerases. Rather, the apparent Km value for its incorporation is only slightly greater than that of the competing deoxynucleotide dCTP; therefore, it must be considered a relatively good substrate for incorporation into DNA.[24] Studies of cells incubated with radioactive gemcitabine demonstrate that gemcitabine nucleotide is mainly in internal sites in DNA recovered from whole cells.[24] Model systems demonstrated that gemcitabine triphosphate is utilized by a DNA polymerase and is incorporated into the extending daughter strand with kinetics that indicate a lower substrate efficiency than the natural substrate.

Masked DNA chain termination

Once gemcitabine nucleotide has been incorporated, the DNA polymerase incorporates a single additional deoxynucleotide. Subsequently, it would seem that the abnormal conformation of the drug greatly inhibits addition of the next deoxynucleotide. Thus, gemcitabine nucleotide appears to reside predominantly in the penultimate position relative to the 3'-end of the extending DNA chain.[24]

This unique pattern of incorporation may mask the gemcitabine nucleotide from "proof-reading" activities. It is now generally established that several mammalian DNA polymerases possess exonuclease activities which degrade one strand of the DNA duplex in the 3' to 5' direction. These exonucleases are thought to function as proof-reading enzymes that remove mismatched base pairs once the polymerizing portion of the DNA polymerase perceives such an error. This signal is probably transmitted as a change in the configuration of the template primer in the polymerization active site. Such a signal might also be generated by the steric shift produced once a nucleotide analogue has been incorporated in place of the deoxynucleotide. Assuming that the cytotoxic activity of the incorporated gemcitabine nucleotide is derived from its ability to inhibit further DNA synthesis once it has been incorporated into the growing DNA strand, and that the exonuclease activities associated with the DNA polymerases have the ability to remove the nucleotide analogue to permit the resumption of DNA synthesis, then the efficiency of analogue removal by the exonuclease would be an indicator of the relative resistance of the cell to inhibition by any particular nucleotide antimetabolite. As of yet, only DNA polymerase epsilon has been evaluated for the ability of its associated 3'→5' exonuclease to remove nucleotide analogues from DNA termini. Gemcitabine nucleotide was relatively resistant to excision from the 3'-terminus.[24] When gemcitabine nucleotide was placed one nucleotide from the 3'-terminus, the position in which it resides predominantly in model DNA syn-

thesis systems, it was essentially resistant to excision. This further indicated that the masked feature of its incorporation at the penultimate position in a growing DNA strand may play an important role in gemcitabine's action. In contrast, ara-C nucleotide was excised at roughly one-third the rate of terminal deoxynucleotides, and eventually all of the ara-C-terminated DNA was degraded.[24]

Because the triphosphate of each nucleotide analogue is the proximal active metabolite its residence time in the cell is likely to be directly related to the potency of its cytoxicity. Although heterogeneity in the elimination half-life is the hallmark of nucleotides among cell lines and fresh human leukaemia cells, there are obvious differences in the general ability of cells to retain the triphosphates of different nucleosides. For instance, ara-C triphosphate is eliminated from human leukaemia cells with a median half-life of 2–3 h with monophasic kinetics,[19] whereas the triphosphate of gemcitabine is eliminated relatively slowly from circulating AML blasts.[20,21] Interestingly, gemcitabine triphosphate exhibits a concentration-dependent elimination which is biphasic with an exceedingly long terminal half-life in leukaemia cells during therapy.[14,21] These properties have implications for the dose schedules upon which these drugs might be administered to achieve and maintain maximal concentration of the active triphosphates. For instance, ara-C might best be administered as continuous infusions or on a frequent intermittent schedule, whereas daily administration of gemcitabine could be adequate to maintain therapeutic levels of triphosphate in target cells.

Self-potentiation of gemcitabine activity

The mechanism(s) by which the metabolites of a drug enhance the cytotoxicity of the drug are termed self-potentiation. This is most commonly brought about by actions which either increase the accumulation of an active metabolite or which decrease its inactivation or elimination. As indicated by this name, it is expected that such actions should enhance the overall effects of the drug. No such activities have been identified for ara-C. On the other hand, the ability of gemcitabine diphosphate to inhibit ribonucleotide reductase comprises a strong case for self-potentiation. Inhibition of ribonucleotide reductase is important because the activity of deoxycytidine kinase is regulated by deoxynucleotides such as dCTP, and the phosphorylation of all the analogues is competitive with deoxycytidine. Because the cellular concentration of dCTP, and presumably deoxycytidine, is inversely proportional to kinase activity, inhibition of ribonucleotide reductase with the corresponding decrease in deoxynucleotides should increase the ability of a cell to phosphorylate the nucleotide antimetabolites. In addition, dCTP competes with the gemcitabine triphosphate for incorporation into DNA. Lower cellular concentrations of the natural substrate would facilitate greater incorporation of the analogue into DNA, an action that is strongly correlated with cytotoxicity.[24] Each of these actions—increased drug phosphorylation and a greater extent of incorporation into DNA—is likely to enhance the cytotoxicity of the drug.

Gemcitabine has additional self-potentiating actions.[22,32] At high cellular concentrations it appears that gemcitabine triphosphate inhibits the activity of CTP synthetase, the enzyme which converts UTP to CTP.[33] By mass action, this may also have an effect on cellular dCTP levels. Gemcitabine monophosphate is known to be a substrate for dCMP deaminase, which converts it to the corresponding uridine monophosphate analogue; this reaction essentially inactivates the drug. dCMP deaminase, however, requires dCTP as a cofactor, and when cellular dCTP levels are lowered, as is the case with gemcitabine inhibition of ribonucleotide reductase, gemcitabine monophosphate is not deaminated and remains as a substrate for phosphorylation to the active di- and triphosphates. Furthermore, gemcitabine triphosphate appears to compete with dCTP for activation of dCMP deaminase, and, at high cellular concentrations, may exert a second inhibitory effect on the activity of the enzyme.[22] In summary, gemcitabine diphosphate inhibits the formation of deoxycytidine and its nucleotides by ribonucleotide reductase, and gemcitabine triphosphate may also affect the level of cellular cytidine nucleotides. Because there would be less deoxycytidine to compete for deoxycytidine kinase activity, and lower dCTP, which would release feedback inhibition of the enzyme, more gemcitabine should be phosphorylated and accumulate as the active di- and triphosphates. The ratio of the cellular concentrations of gemcitabine triphosphate to dCTP would rise, facilitating additional incorporation of gemcitabine triphosphate into DNA with an increased cell kill. Lowering cellular dCTP levels would also reduce the inactivation of gemcitabine nucleotides by dCMP deaminase, and the activity of the enzyme would be further diminished at higher gemcitabine triphosphate concentrations. This should decrease the rate of gemcitabine triphosphate elimination, thus pro-

longing the inhibitory actions of the drug. Finally, the long residence time of gemcitabine nucleotides is likely to maintain inhibitory concentration of the triphosphate for times sufficient to permit cycling cells to continue in the cell cycle until they enter the sensitive S phase and are killed.

Future directions

Until recently ara-C was the drug of choice as an inhibitor of DNA repair in the design of therapeutic regimens. In general, the clinical results of such attempts at treating solid tumours have been disappointing. As detailed above, the newer nucleoside analogues exhibit properties which should make them more effective inhibitors than ara-C of both DNA replication and repair. Experimental evidence already exists for synergistic interactions of gemcitabine and radiation.[29] Combinations of gemcitabine and other chemotherapeutic agents which elicit a DNA repair response are being explored. We are only now recognizing the potential of these new antimetabolites such as gemcitabine as single agents and as modulators of the metabolism and activities of other established drugs and modalities such as radiation. Although it will be a major challenge to identify optimal doses, schedules and combinations, the present state of our knowledge of the pharmacokinetics, metabolism, and actions of gemcitabine will serve as a guide to therapeutic strategies which will be forthcoming in the future.

Summary

Gemcitabine is a novel nucleoside analogue requiring phosphorylation into the active metabolites gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP). The pharmacology and metabolism of gemcitabine are quite different from that of agents like ara-C and explain why gemcitabine is unique as a nucleoside analogue in having activity in solid tumours. Gemcitabine exhibits an extraordinary array of self-potentiating mechanisms that increase the concentrations and prolong the retention of its active nucleotides in tumour cells: (i) gemcitabine diphosphate lowers intracellular dCTP, which reduces the inhibition (by dCTP) of deoxycytidine kinase, the rate-limiting enzyme for the activation of gemcitabine; (ii) reduced levels of dCTP also result in an inactivation of dCMP deaminase, a principal enzyme involved in the elimination of gemcitabine; (iii) dCMP deaminase is also directly inhibited by gemcitabine triphosphate. The cytotoxic activity of gemcitabine is related to the incorporation of gemcitabine triphosphate into DNA, and the consequent inhibition of further DNA synthesis. Gemcitabine diphosphate inhibits ribonucleotide reductase, which is responsible for producing the deoxynucleotides required for DNA synthesis and repair. The reduction in cellular deoxynucleotides (particularly dCTP) favours gemcitabine triphosphate in its competition with dCTP for incorporation into DNA. After gemcitabine nucleotide is incorporated on the end of the daughter DNA strand, one more deoxynucleotide is allowed to pair before DNA chain polymerization is halted. This process, termed "masked chain termination", appears to reduce the ability of proof-reading exonucleases to remove gemcitabine nucleotide from the penultimate position. This mechanism, sustained because cells are generally unable to remove the drug from DNA, is probably effective at inhibiting both DNA replication and repair. Ribonucleotide reductase inhibition may also lead to therapeutic strategies for combining gemcitabine with other drugs and modalities which cause DNA damage.

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