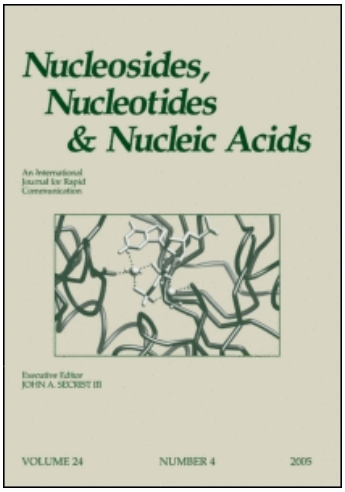

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GEMCITABINE: ACTIONS AND INTERACTIONS

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ABSTRACT. Gemcitabine is a nucleoside analog that acts by multiple mechanisms. Incorporation of the triphosphate and subsequent inhibition of DNA replication and repair appears to be the major mode of action. Ribonucleotide reductase is inhibited by gemcitabine diphosphate, an activity that leads to metabolic self-potentiating effects.

In recent years, nucleoside analogs have had a major impact on the therapy of viral and malignant diseases. Gemcitabine was conceptualized and synthesized by Larry W. Hertel at Lilly Research Laboratories.[1] Its potential for development into an anticancer drug became evident from studies directed by the late Gerald B. Grindey.[2,3] Subsequent laboratory investigations have revealed the cellular metabolism and pharmacokinetics of gemcitabine, as well as aspects of the pharmacodynamics of the drug.[4] Clinical studies have supported its approval for use in the treatment of pancreatic carcinoma, and continued investigations in other diseases[5].

The biologic activity of gemcitabine is derived from the substitution of the hydrogens of carbon-2' in deoxycytidine with geminal fluorines (Fig. 1). Because this is the primary focus of discrimination for both ribonucleoside diphosphate reductase and DNA polymerases, it was predicted that these enzymes would be targets for the drug. In fact, as a series of investigations have demonstrated, the inhibitory actions of gemcitabine are directed almost entirely at DNA metabolism. As reviewed below, the molecular mechanisms of gemcitabine nucleotides that have been demonstrated to act against specific enzymes of DNA replication and repair are adequate to account for the cytotoxicity of gemcitabine.

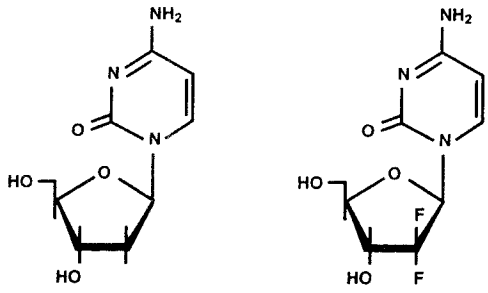

Fig. 1. Structures of deoxycytidine and gemcitabine

Early studies clearly demonstrated the perturbation of the cellular pools of deoxynucleoside triphosphates (dNTPs) in human leukemia cell lines treated with gemcitabine.[6-8] Substantial decreases in dNTPs were produced after a 2-hr incubation with 0.01 μM gemcitabine. There was little dose dependency in an incubation of this duration. The major effect was on cellular dCTP concentrations, although dATP and dGTP were also decreased. This paralleled the effects of the reductase inhibitor hydroxyurea, but was in contrast to ara-C, which is a potent DNA synthesis inhibitor that has no effect on ribonucleotide reductase. Studies in a human colon carcinoma[9] cell line showed similar perturbations, except that dATP was most effected. These findings suggest the need for direct comparisons of effects of gemcitabine on specific dNTPs among human cell lines.

Investigations of partially purified human ribonucleoside diphosphate reductase demonstrated that gemcitabine diphosphate was more inhibitory than the triphosphate.[8] CDP reduction was inhibited 50% at 3 μM gemcitabine diphosphate. Mechanistic studies with highly purified enzyme from *E. coli* indicated that gemcitabine diphosphate inhibited CDP reductase activity rapidly and apparently irreversibly.[10] Thus, it seems that gemcitabine diphosphate is a potent mechanism-based inhibitor of ribonucleoside diphosphate reductase, an action that demonstrates its properties as an cytidine analog.

As detailed below, the contribution of this activity to gemcitabine metabolism and biological activity seems to be the key to the self-potentiating activities of the drug.

The catalytic activity and the substrate specificity of ribonucleotide reductase is closely regulated by the concentrations of both ribonucleoside and deoxyribonucleoside triphosphates. Because these regulatory loops are lost in the isolated enzyme, assays of reductase activity in intact cells are likely to provide a clearer picture of the effects of metabolic inhibitors such as gemcitabine diphosphate. To determine the activity of ribonucleotide reductase in intact leukemia cells, exponentially growing cells were incubated with individual ribonucleosides that were radioactively labeled.[8] At 15-min intervals, the radioactivity associated with the corresponding deoxynucleotide and also in DNA was quantitated. This functional assay of the flux of ribonucleosides through reductase in human lymphoblastoid cells demonstrated that CDP reduction was most sensitive to gemcitabine inhibition, followed in order by UDP > GDP > ADP. Studies of CDP reduction demonstrated an inverse relationship between the rise in the cellular concentration of gemcitabine diphosphate over 2 hr and the decrease in the ability of the cells to make deoxycytidine nucleotides. CDP reductase activity was inhibited 50% when the cellular concentration of gemcitabine diphosphate was 0.3 μmole/L. This value, the functional equivalent of an IC_{50} determination, indicates a potent inhibitory action of gemcitabine diphosphate on this enzyme in whole cells. Nevertheless, the failure to restore DNA synthesis in gemcitabine-treated cells by repletion of dNTP pools upon incubation with exogenous deoxynucleosides lends greater importance to the direct action caused by incorporation of the drug into DNA.[11]

Studies with radioactive precursors of DNA, RNA, and protein synthesis demonstrated that the major effect of gemcitabine was directed at DNA.[6, 12] Separation on density gradients of nucleic acids from human lymphoblastoid cells incubated for several hours with tritiated gemcitabine demonstrated that virtually all of the radioactivity separated with DNA.[12] Longer incubations resulted in a minor fraction of radioactivity being associated with RNA, but neither the chemical identity of

the incorporated nucleotide nor its biological consequences have been established.[13] Degradation of the DNA followed by identification of the terminal and internal nucleotides demonstrated that more than 90% of the gemcitabine monophosphate had been located internally in DNA.[12] This indicated that DNA with gemcitabine monophosphate incorporated at the 3'-terminus can be extended by DNA polymerases.

Model systems of DNA synthesis confirmed that the triphosphate of gemcitabine could be incorporated into extending primer strands by both human DNA polymerases α and ε.[12] This incorporation was competitive with dCTP, with each DNA polymerase expressing a 20-fold preference for the normal nucleotide. Because these nucleotide compete for insertion into DNA by DNA polymerases, alteration of the ratio of dCTP to gemcitabine triphosphate by inhibition of ribonucleotide reductase by gemcitabine diphosphate increases the probability that gemcitabine triphosphate would be incorporated into DNA. Although gemcitabine incorporation did not give rise to strict termination of strand elongation, other studies demonstrated that once gemcitabine monophosphate was inserted into the 3'-terminus of DNA, it was extremely difficult for either DNA polymerase α or DNA polymerase ε to add a second gemcitabine nucleotide.[14] Thus, it is likely that both the ratio of dCTP to gemcitabine triphosphate in the cell and the G:C richness of particular segments of DNA will be key determinants in the ability of the drug to become incorporated and potentially terminate further DNA strand elongation.

It was unique, however, to find that after incorporation of the gemcitabine monophosphate, one more deoxynucleotide was inserted before the DNA polymerase stopped.[12,14] This pattern was distinct from that of arabinosyl compounds which halt polymerase progression at the analogue insertion site. This may have important implications for potential resistance mechanisms. For instance, the 3'->5' exonuclease activities associated with DNA polymerases are the only known means of removing carbohydrate analogs such as gemcitabine and ara-C from DNA.[12] Kinetic studies demonstrated that incorporated ara-C monophosphate could be removed from the

terminus of DNA by the 3'->5' proofreading exonuclease activity of human DNA polymerase ε at 37% the rate of dCMP removal. However, 3'-terminal gemcitabine monophosphate was removed at only a fraction of the dCMP excision rate by either the human enzymes[12] or that of *E coli*.[15] When placed in the penultimate position, excision of gemcitabine monophosphate proceeded at barely 2% of control. Thus the incorporation of gemcitabine nucleotide, which causes DNA polymerases to stop after one additional nucleotide has been inserted, may serve to cap the analog in DNA and make it more resistant to removal. This takes on increased significance given the strong correlation between incorporation of gemcitabine into DNA and loss of clonogenic survival of human lymphoblasts.[12] Cell death exhibits the characteristics of apoptosis, the stimulatory mechanisms of which are under investigation.[16-18]

It is clear from the variety of metabolic interactions in which gemcitabine participates that the unique actions of its nucleotides on cellular metabolic regulatory processes serve to enhance the overall inhibitory activity of this analog on cell viability. These interactions, which are termed self-potentiation, and are not evidenced to such an extent in other anticancer drugs.[4,11,15]

The sites of action of gemcitabine nucleotides and the pathways of metabolic self-potentiation are summarized in Fig. 2. Gemcitabine diphosphate (dFdCDP) is an inhibitory alternative substrate for ribonucleotide reductase (pathway 1), which supplies deoxynucleotides required for both DNA replication and DNA repair. The subsequent decrease in cellular deoxynucleotides, particularly dCTP, is critically important because gemcitabine triphosphate (dFdCTP) competes directly with dCTP for incorporation into DNA by DNA polymerases (pathway 2). Thus, the decrease in the cellular dCTP concentration is an important self-potentiating mechanism that would increase gemcitabine nucleotide incorporation into DNA. The fact that gemcitabine nucleotide incorporation into DNA is strongly correlated with loss of cell viability suggests that this is a major mechanism by which gemcitabine causes cell death. Molecular studies have shown that after gemcitabine nucleotide is incorporated on the end of the

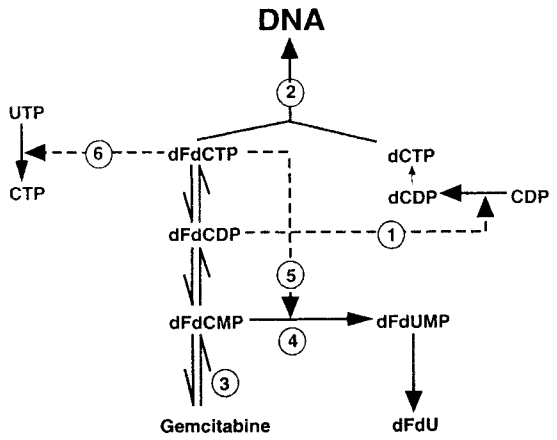

Figure 2. Self-potentiating actions of gemcitabine nucleotides.[11] The numbered reaction pathways are each discussed in the text. Dashed lines indicate inhibitor reactions. Abbreviations: dFdU and dFdUMP, 2',2'-difluorodeoxyuridine and its 5'-monophosphate.

elongating DNA strand, one more deoxynucleotide is incorporated, and that thereafter, the DNA polymerases are not able to proceed effectively.[12] This action apparently locks the drug into DNA, because other studies have demonstrated that proof-reading enzymes are unable to remove gemcitabine nucleotide from this penultimate position.[12,14,15] Presumably, the presence of gemcitabine nucleotide in this penultimate position distorts the growing DNA chain such that it is no longer an efficient substrate for DNA polymerases.

Phosphorylation of gemcitabine by deoxycytidine kinase is inhibited by deoxycytidine and by dCTP[21,22] (reaction 3); when the cellular dCTP and consequently the cellular deoxycytidine levels are lowered, the rate of gemcitabine phosphorylation is increased. This results in greater dFdCTP accumulation in the cell as a substrate for incorporation into DNA. In addition, because the cellular concentration of the diphosphate and the triphosphate generally are maintained at a fixed ratio, this would result in higher levels of dFdCDP that would maintain inhibition of ribonucleoside diphosphate reductase. In contrast to its inhibitory effect in

regulating deoxycytidine kinase, dCTP is a co-factor required for the activity of dCMP deaminase (reaction 4), the rate-limiting enzyme for elimination of gemcitabine nucleotides from the cell.[11,23] When the cellular dCTP level declines as a result of inhibition of ribonucleotide reductase by dFdCDP, dCMP deaminase activity also decreases. This action, which results in a lower rate of gemcitabine nucleotide removal from the cell, is likely t contribute to the retention of the active nucleotides in tumor cells. Furthermore, dFdCTP is itself capable of inhibiting dCMP deaminase (reaction 5), indicating an additional mechanism that contributes to the prolonged retention of gemcitabine nucleotides. It is possible to pharmacologically probe the importance of the dCMP deaminase-mediated reaction for gemcitabine nucleotide retention by inhibiting the enzyme with 3,4,5,6-tetrahydro-2'-deoxyuridine. At low cellular gemcitabine triphosphate concentrations elimination was monophasic with a half-life of 3.6 hr. When dCMP deaminase was inhibited by treatment with 3,4,5,6-tetrahydro-2'-deoxyuridine, dFdCTP elimination kinetics changed to biphasic with a terminal half-life of 19 hr.[11] This mechanism for maintaining active nucleotide analogue concentrations has been extended to clinical material; studies of gemcitabine triphosphate metabolism in human leukemia cells during clinical trials have also demonstrated a concentration dependent prolonged retention of dFdCTP.[24] Finally, at high cellular concentrations, dFdCTP inhibits CTP synthetase (reaction 6), blocking the synthesis of CTP, and by mass action, that of dCTP as well.[25] Presumably, this action halts the supply of cytidine diphosphate as a substrate for ribonucleotide reductase, and in so doing, further stresses the normal pathways of dCTP formation.

Thus, an extraordinary array of self-potentiating mechanisms increase the concentrations and prolong the retention of the active nucleotides of gemcitabine in tumor cells. Inhibition of ribonucleotide reductase by gemcitabine diphosphate lowers dCTP levels and facilitates incorporation of the drug into DNA. The cytotoxic action of gemcitabine is related to the incorporation of the gemcitabine triphosphate into DNA, and the consequent inhibition of further DNA synthesis. This mechanism,

which is sustained because cells are generally unable to remove the drug from DNA, is probably effective at inhibiting both DNA replication and repair. Thus, it is likely that gemcitabine will synergize with drugs which damage DNA and also with radiation. A full understanding will be achieved only after we gain a knowledge of the surveillance mechanisms that allow cells to perceive the inability to re-establish DNA synthesis and to contend with unrepairable DNA damage, which subsequently activate a signaling pathway that results in cell death by apoptosis.

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