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Activity of 2′,2′-difluorodeoxycytidine (Gemcitabine) against human tumor colony forming units

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2′,2′-Difluorodeoxycytidine (LY 188011, Gemcitabine) is a novel pyrimidine antimetabolite with promising activity in preclinical models for leukemia and solid tumors. Phase I clinical trials with the agent are ongoing. In order to better define types of tumors with clinical sensitivity to Gemcitabine (to help target phase II trials), we have studied the antitumor effects of this agent against a variety of freshly explanted human tumor specimens using an *in vitro* capillary soft agar cloning system. Final concentrations of 2.0–200 μg/ml were used for short-term (1 h) and continuous incubations experiments. Using a short-term incubation, 94/215 (44%) tumor specimens were evaluable for the determination of antitumor activity. The most common tumor types studied included colorectal, breast, non-small cell lung, ovarian cancer, kidney and melanoma. A concentration-dependent increase in the frequency of inhibited tumor specimens was noted (2 μg/ml: 6/94 specimens, 20 μg/ml: 13/94 specimens, 200 μg/ml: 33/94 specimens; $p < 0.0001$). A similar increase in tumor growth inhibition was found using a continuous incubation (2 μg/ml: 0/14 specimens, 20 μg/ml: 1/14 specimens, 200 μg/ml: 7/14 specimens; $p < 0.001$). We conclude that Gemcitabine is an active antitumor agent against tumor colony forming units from a variety of human malignancies if sufficiently high concentrations can be achieved. The agent should be evaluated for Phase II clinical activity against those tumor types.

Key words: 2′,2′-Difluorodeoxycytidine, Gemcitabine, growth modulation, tumor colony forming units.

Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine, LY 188011, dFdCyd) is a novel pyrimidine antimetabolite with considerable activity against preclinical leukemia models.[1,2] After intracellular phosphorylation by deoxycytidine kinase, and further not yet identified enzymes, dFdCTP is formed as the major nucleotide and leads to inhibition of DNA synthesis.[3,4] Gemcitabine is functionally similar to cytosine arabinoside but is more potent and has a wider spectrum of activity.[5] Antitumor effects have been observed against human tumor xenograft models of lung cancer, breast cancer, colon cancer, and head and neck cancer.[1,6]

Recently, a clinical phase I study with Gemcitabine has been completed using a weekly infusion schedule. In this study, two partial responses have been noted (colorectal cancer, adenocarcinoma of lung).[7]

We have studied the antitumor activity of gemcitabine against a variety of human malignancies *in vitro* using a capillary human tumor cloning system in order to help define types of tumors that warrant phase II clinical trial evaluation.

Materials and methods

Compounds

Gemcitabine (2′,2′-difluorodeoxycytidine, LY 188011, dFdCyd) was kindly provided by the Eli

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Lilly Company (Indianapolis, IN). Final concentrations ranged from 2.0 to 200 μg/ml and were prepared in distilled water.

Human tumor cloning system

After obtaining informed consent in accordance with federal and institutional guidelines, tumor specimens (primary tumors and metastases) were collected by sterile standard procedures as part of routine clinical measures. Biopsies of solid tumors were stored in McCoy's 5A medium containing 10% newborn calf serum, 10 mM HEPES, 90 U/ml penicillin and 90 μg/ml streptomycin (all Gibco, Grand Island, NY), for transport to the laboratory. Preservative-free heparin (10 U/ml, O'Neill, Johns and Feldman, St Louis, MO) was added immediately after collection of fluids to prevent coagulation. Solid specimens were minced and repeatedly passed through metal meshes with mesh widths of 40 μm (E.C. Apparatus, St Petersburg, FL) to obtain a single cell suspension. Effusions were centrifuged at 150 *g* for 5–7 min and passed through 25-gauge needles to obtain single cell suspensions when necessary. All specimens were suspended in McCoy's 5A medium (Gibco) containing 5% horse serum (HS), 10% fetal calf serum (FCS) (both Hyclone, Logan, UT), 2 mM sodium pyruvate, 2 mM glutamine, 90 U/ml penicillin, 90 μg/ml streptomycin and 35 μg/ml L-serine (all Gibco).

Soft agar tumor cloning experiments were performed in glass capillary tubes as described earlier.[8-10] Cells were plated at a density of 5×10^4/capillary in 100 μl glass capillaries in a mixture of 0.3% agar in CMRL medium 1066 (Irvine Scientific) containing 15% HS, 2% FCS, 5 mg% vitamin C (Gibco), 90 U/ml penicillin, 90 μg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM glutamine (all Gibco), 2 U/ml insulin (Iletin I; Eli Lilly), 2 μg/ml transferrin and 4 ng/ml hydrocortisone (both Sigma). Immediately prior to plating, HEPES (Gibco, 10 mM final concentration), asparagine (100 μg/ml final concentration) and sodium pyruvate (2 mM final concentration) were added. Each experiment included a control with orthosodiumvanadate (10^{-3} M, Sigma) to assure the presence of a good single-cell suspension (positive control).[11] Capillaries were incubated at 37°C. After 14 days, colonies were counted with an inverted microscope. An experiment was considered evaluable when the water control had ≥3 colonies/capillary and the positive control showed ≤30% colony formation compared to the solvent control. A decrease in tumor colony formation was considered significant if survival of colonies was ≤0.5-fold compared to the control.

Statistical analysis

Data were expressed as means and standard deviations of 4–6 capillaries per data point. Percent survival was calculated by expressing the average number of tumor colony forming units from Gemcitabine-treated cells in percent of the average number of tumor colony forming units from untreated controls. Statistical analyses were performed using the χ^2-test for linear trend.

Results

The effects of Gemcitabine (2′,2′-difluorodeoxycytidine) on tumor colony formation were studied in a total of 226 tumor specimens using short-term (1 h) exposure experiments. Eleven specimens were confirmed benign by pathology review and did not form colonies in controls. These specimens were excluded from further analyses. As summarized in Table 1, the largest tumor subgroups accrued were colorectal, breast, non-small cell lung, ovarian, kidney cancer and melanoma. Final concentrations of Gemcitabine ranged from 2.0 to 200 μg/ml. As shown in Table 2, the compound significantly inhibited the *in vitro* proliferation of tumor colony forming units. At 200 μg/ml, 33/94 specimens were inhibited as compared with 6/94 at 2 μg/ml ($p < 0.0001$). Colorectal cancer, melanoma and renal cancer were the most sensitive tumor types.

A total of 14/33 (40%) tumor specimens studied using a continuous incubation (14 days) with Gemcitabine were evaluable for response. Although the sample size is much smaller, *in vitro* antitumor activity was notable with 7/14 cancer specimens at 200 μg/ml as compared with 0/14 specimens at 2 μg/ml ($p < 0.001$; Table 3).

Fourteen tumor specimens were used for a head-to-head comparison of short- and long-term exposure to Gemcitabine. In both groups, tumor colony formation of 8/14 (57%) specimens was significantly inhibited. There was no evidence for increased tumor sensitivity with long-term exposure.

In order to determine whether deamination of Gemcitabine affects its *in vitro* activity against tumor

Table 1. Tumor types studied with Gemcitabine.

Tumor type	No. evaluable/no. attempted (%)
Colorectal	17/41 (41)
Breast	15/38 (39)
Lung, non-small cell	12/34 (35)
Ovary	14/31 (45)
Kidney	16/29 (55)
Melanoma	16/28 (57)
Other tumor types	4/14 (29)
Total	94/215 (44)

Table 2. Concentration-dependent inhibition of tumor colony forming units by Gemcitabine after short-term incubation (1 h)

Tumor type	No. specimens with inhibition[a]/no. specimens evaluable (μg/ml Gemcitabine)		
	2.0	20.0	200.0
Colorectal	2/17	3/17	9/17
Breast	0/15	0/15	3/15
Lung, non-small cell	0/12	0/12	4/12
Ovary	0/14	1/14	2/14
Kidney	1/16	4/16	6/16
Melanoma	3/16	5/16	7/16
Other	0/4	1/4	2/4
Total	6/94 (6%)	13/94 (14%)	33/94 (35%)[b]

[a] Colony survival $\leq 0.5 \times$ control.
[b] $p < 0.0001$; χ^2-test for linear trend.

Table 3. Concentration-dependent inhibition of tumor colony forming units by Gemcitabine after long-term incubation

Tumor type	No. specimens with inhibition[a]/no. specimens evaluable (μg/ml Gemcitabine)		
	2.0	20.0	200.0
Lung, non-small cell	0/5	1/5	3/5
Breast	0/3	0/3	2/3
Other	0/6	0/6	2/6
Total	0/14 (0%)	1/14 (7%)	7/14 (50%)[b]

[a] Colony survival $\leq 0.5 \times$ control.
[b] $p < 0.0001$; χ^2-test for linear trend.

colony forming units we have performed cloning experiments (1 h exposure) in the presence of tetrahydrouridine (100 μM). Of 21 evaluable tumor specimens, tumor colony formation was inhibited by Gemcitabine/tetrahydrouridine in five specimens (24%) and by Gemcitabine alone in four (19%).

Discussion

Gemcitabine (2',2'-difluorodeoxycytidine, LY 188011, dFdCyd) is a novel pyrimidine antimetabolite with functional similarity to cytosine-arabinoside. Like ara-C, intracellular phosphorylation to its triphosphate derivative (dFdCTP) is required for antitumor activity. Deamination of dFdCTP results in inactive metabolites.

In this study, we have tested the antitumor activity of Gemcitabine against a variety of freshly explanted tumor specimens using a soft agar cloning system. We have found a moderate activity after short-term incubation. The maximal effect was observed at the highest concentration studied with inhibition of 33/94 sensitive tumors. The most sensitive tumor entities were colorectal cancer, renal cell cancer and melanoma. *In vitro* sensitivity of colorectal cancer specimens is in agreement with the clinical response in a patient with Dukes D colorectal cancer as reported by Abbruzzese *et al.*[7] These investigators have also observed a partial response in a patient with adenocarcinoma of the lung. Modest activity of Gemcitabine against non-small cell lung cancer has been observed in our experiments with an *in vitro* response rate of 33%. This also correlates well with a preliminary report by Anderson *et al.* of partial responses in 7/29 patients (24%) with non-small cell lung cancer in a clinical phase II study.[12]

A potential limit to the predictive value of the *in vitro* antitumor activity of Gemcitabine may be that clinically achievable plasma concentrations may not be high enough to produce objective antitumor effects. In our experiments, some activity has been observed at 20.0 μg/ml. Clinically, peak plasma concentrations of 5.4–37.5 μg/ml have been observed after administration of 790 mg/m^2.[7] A number of preliminary reports on the clinical activity and toxicity of gemcitabine now indicate that higher doses may be feasible and, as predicted by our results, may yield increased objective response rates.[13–15] *In vitro* activity of Gemcitabine thus may help predict the clinical outcome in cancer patients as a function of drug concentrations achieved.

Although Gemcitabine is an antimetabolite and its activity may therefore be dependent on specific cell cycle phases, we did not find increased antitumor activity after long-term exposure when tumors were tested in head-to-head comparisons of long- and short-term incubation. The reasons for this observation are not clear but may potentially include deamination reactions including increased dCMP deaminase activity or decreased phosphorylation by nucleoside kinase. Both mechanisms would lead to decreased antitumor activity. Decreased dFdCyd phosphorylation seems to be unlikely since Gemcitabine has activity in tumor xenograft models.[1,6] In control experiments with tetrahydrouridine, a potent inhibitor of nucleoside deaminases, we did not find loss of antitumor activity of Gemcitabine.

In summary, we have found moderate *in vitro* activity of Gemcitabine against tumor colony forming units from a variety of human tumor types. Our results correlate with the clinically observed activity of this compound. Further investigations in clinical phase II studies in patients with colorectal cancer, non-small cell lung cancer, renal cell cancer and melanoma are warranted at high plasma concentrations in order to optimize the antitumor activity of Gemcitabine.

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