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Pilot phase I–II study on 5-aza-2′-deoxycytidine (Decitabine) in patients with metastatic lung cancer

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5-Aza-2′-deoxycytidine (5-AZA-CdR, Decitabine) is a nucleoside analog and an active drug for the therapy of acute leukemia. The incorporation of 5-AZA-CdR into DNA blocks DNA methylation and can result in the activation of specific genes, such as tumor suppressor genes. This novel mechanism of action of 5-AZA-CdR stimulated our interest in its potential for cancer therapy in patients with lung cancer. Using a colony assay we observed that 5-AZA-CdR showed a potent antineoplastic effect against two human lung carcinoma cell lines. The objective of this preliminary phase I–II study was to evaluate the toxicity and clinical efficacy of 5-AZA-CdR in patients with stage IV non-small cell lung carcinoma. There were 15 patients that entered the clinical study. For nine assessable patients that received 5-AZA-CdR by a single 8 h i.v. infusion of 200–660 mg/m^2 for one or more cycles, the median survival duration was 6.7 months, with three patients surviving more than 15 months. The steady-state plasma concentration of 5-AZA-CdR during the infusion was estimated in some patients and was in the same range that produced activation of a tumor suppressor gene in human lung tumor cell lines as reported by other investigators. The major side effect of 5-AZA-CdR was hematopoietic toxicity which required a 5–6 week recovery period before the next cycle of therapy. This study suggests that 5-AZA-CdR may have some clinical activity against metastatic lung carcinoma using this type of dose schedule.

Key words: 5-Aza-2′-deoxycytidine, clonogenic assay, hematopoietic toxicity, lung cancer, pharmacokinetics.

Introduction

Chemotherapy for metastatic non-small cell lung cancer (NSCLC) with conventional antitumor drugs has only limited effectiveness.[1] Even though various combinations of cytotoxic drugs can produce response rates of 20–40%, the survival time of most patients with NSCLC is less than 12 months.[1] There is an urgent need to develop new approaches for the chemotherapy of this disease.

5-Aza-2′-deoxycytidine (5-AZA-CdR, Decitabine) is an analog of deoxycytidine and an experimental antineoplastic agent.[2] Initial interest in 5-AZA-CdR originated from the observation that it was more active than cytosine arabinoside in the mouse model of L1210 leukemia.[3] Phase I–II clinical trials on 5-AZA-CdR in both childhood and adult leukemia showed that it was an active antileukemic agent.[4–7] In addition, this analog was demonstrated to produce responses in patients with myelodysplastic syndrome.[8]

5-AZA-CdR has a unique mechanism of action. After conversion to a nucleotide by phosphorylation, this analog is incorporated into DNA.[2] The presence of 5-AZA-CdR in DNA blocks DNA methylation. The hypomethylation of DNA produced by 5-AZA-CdR can result in gene activation and induction of differentiation of neoplastic cells.[9,10] 5-AZA-CdR was demonstrated to induce the *in vitro* differentiation of human leukemic cell lines[11,12] and leukemic blasts from patients.[13]

In pilot studies in patients with tumors, 5-AZA-CdR produced a modest response,[14,15] which may have been due to the sub-optimal dose schedule used. A renewed interest in this nucleoside analog as an antitumor agent has been generated by the recent reports of its activation of tumor suppressor genes. Herman *et al.*[16] reported that 5-AZA-CdR activated the tumor suppressor gene VHL in human renal carcinoma cell lines. Otterson *et al.*[17] and Merlo *et al.*[18] demonstrated that 5-AZA-CdR could activate the expression of p16/CDKN2 tumor suppressor gene in lung cancer cell lines and in some lung tumor surgical specimens. The p16/CDKN2 tumor suppressor gene inhibits the enzyme activity of cyclin-dependent kinases which mediate the phos-

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phorylation of the retinoblastoma (Rb) gene product. The hypophosphorylation of Rb is thought to prevent the entry of tumor cells into the S phase of the cell cycle.[19,20] The expression of p16 was reported to be low or absent in most primary tumors of the lung,[19] mesotheliomas,[20] breast,[21] colon[21] and bladder.[22] Also of interest is the report that 5-AZA-CdR can activate the expression of E-cadherin in primary tumors of breast and prostate.[23] E-cadherin is a cell adhesion molecule which suppresses tumor cell invasion and metastasis in experimental tumor models. In addition, the activation of the expression of estrogen receptor (ER) in ER-negative human breast carcinoma cell lines by 5-AZA-CdR suggests that it may be possible to use this agent to improve the response of hormonal therapy in patients with ER-negative breast cancer.[24]

Since we observed that 5-AZA-CdR showed significant antineoplastic activity against different human lung carcinoma cell lines and since this analog has a novel mechanism of action, we initiated a pilot clinical trial in patients with advanced metastatic NSCLC in order to evaluate its clinical antitumor activity and toxicity.

Methods

Cell culture

The human lung squamous carcinoma cell lines, SK-MES-1 and NCI-H520, were obtained from the American Tissue Culture Collection (Rockland, MD). The cells were grown in MEM medium containing non-essential amino acids and 10% fetal calf serum. For colony assay, the cells were trypsinized, counted with a Coulter Counter ZM and 100 cells were placed in 60 mm tissue culture dish. The following day the indicated concentration of 5-AZA-CdR was added to the dish for the time indicated. After 16–18 days, the colonies were stained with Giemsa and counted.

Clinical study

This clinical study was approved by the Ethics Committee of both hospitals of the investigators. Written informed consent was obtained in every case stating that the patient was informed of the investigational nature of this treatment regimen. Pretreatment evaluation included medical history, physical examination, complete blood count, serum biochemical analyses, chest roentgenogram and urine analysis. All patients had a diagnosis of stage IV NSCLC with assessable lesions and a performance status of 0–1 as defined by WHO prior to the start of treatment. All patients did not receive any prior chemotherapy. Complete blood count and biochemical tests were obtained weekly during this trial. Tests of measurable disease parameters were performed every 4–6 weeks. The first patient was entered into the study on 15 November 1990 and the last patient entered on 8 November 1991.

5-AZA-CdR (Decitabine) was obtained from Pharmachemie (Haarlem, The Netherlands) as 50 mg sterile powder containing 0.3 mmol of potassium phosphate buffer, pH 7.0. The 5-AZA-CdR was dissolved in 0.45% sodium chloride on the day of the infusion and used immediately. The solution in a plastic sac was placed between two ice packs and administered by continuous i.v. infusion for 6–8 h using an in-line filter (0.2 μm Sartorius Minisart). The patients that received a single infusion were hospitalized for about 24 h to receive the 5-AZA-CdR and then monitored on an outpatient basis. The interval between each cycle of 5-AZA-CdR therapy was 5–7 weeks.

Pharmacokinetics

Blood samples (1–3 ml) were obtained by i.v. aspiration in heparinized tubes on the opposite side of the site of the drug injection before and during the i.v. infusion of 5-AZA-CdR. After the addition the cytidine deaminase inhibitor, 8 μl of tetrahydrouridine (500 μg/ml), to the tube the samples were centrifuged at 2000 g for 15 min and stored at −70°C until assayed. The bioassay for 5-AZA-CdR was performed as described previously using the growth inhibition of L1210 leukemic cells as the end-point.[4,14] 5-AZA-CdR was also assayed by high performance liquid chromatography (HPLC) as described previously[25–27] using a reverse-phase C18 column. 5-Azacytidine was used as the internal standard.

Results

The *in vitro* antineoplastic activity of 5-AZA-CdR on two human lung squamous carcinoma cell lines was evaluated by a clonogenic assay (Table 1). For the lung SK-MES-1 tumor cells, 5-AZA-CdR at a concentration of 0.1 μg/ml produced a loss of clonogenicity of 47.3 and 70.0% for an 8 and 24 h exposure, respectively. Under the same conditions this analog produced a 21.7 and 87.3% loss of clonogenicity for

Table 1. Clonogenic assay of 5-AZA-CdR on human lung carcinoma cell lines

Cell line	5-AZA-CdR concentration (μg/ml)	Loss of clonogenicity (%)	
		8 h	24 h
SK-MES-1	0.1	47.3 ± 15.0[a]	70.0 ± 17.4
SK-MES-1	1.0	76.0 ± 9.2	96.0 ± 5.3
SK-MES-1	10	97.7 ± 2.5	98.0 ± 3.5
NCI-H520	0.1	21.7 ± 12.2	87.3 ± 13.0
NCI-H520	1.0	76.3 ± 7.2	99.0 ± 1.0
NCI-H520	10	96.0 ± 2.6	100

[a]Mean ± SD. $n = 3$.

an 8 and 24 h exposure, respectively, for the lung NCI-H520 tumor cells. For both cell lines a concentration of 5-AZA-CdR above 0.1 μg/ml produced a greater loss of clonogenicity at both exposure times.

Patient 1 was a 68-year-old male with a diagnosis of metastatic lung adenocarcinoma. He received a 6 h i.v. infusion of 5-AZA-CdR at 200 mg/m^2 for three consecutive days for a total dose of 600 mg/m^2. The hematopoietic toxicity for this patient is shown in Figure 1. This 5-AZA-CdR treatment produced a severe leukopenia (below 1000/μl) between days 14 and 30, and required hospitalization with supportive antibiotic therapy. It is interesting to note that the platelet count on day 28 reached a level that was much higher than the pretreatment value.

In an attempt to reduce the hematopoietic toxicity of 5-AZA-CdR, the of number of days of infusion was reduced from 3 to 2. Patient 2 was a 42-year-old female with a diagnosis of metastatic lung adenocarcinoma. She received a 6 h i.v. infusion of 5-AZA-CdR at 200 mg/m^2 for two consecutive days for a total dose of 400 mg/m^2. This treatment also produced a marked leukopenia (below 2000/μl) between days 14 and 34 (Figure 1), and required hospitalization with supportive antibiotic therapy.

All the additional patients entered into this study were administered only a single i.v. infusion of 8 h for each cycle in an attempt to further reduce the extent of 5-AZA-CdR-induced hematopoietic toxicity. Patients 3–7 received an initial dose of 200 mg/m^2, whereas patient 8 received a dose of 400 mg/m^2. Patients 9–15 received an initial dose of 600 mg/m^2. A summary of the responses to the 5-AZA-CdR treatments is shown in Table 2.

Patient 3, a 62-year-old male with a diagnosis of mixed squamous–adenocarcinoma, received two cycles of 5-AZA-CdR at a dose of 200 mg/m^2 per cycle and two cycles of 400 mg/m^2 per cycle. The interval between cycles was 6–7 weeks. He had stable disease for 8 months. For personal reasons he refused further treatment with 5-AZA-CdR.

Patient 4 received a single cycle of 5-AZA-CdR at a dose of 200 mg/m^2 and was not assessable due to death from cardiac arrest 1 month post-treatment, possibly due to myocardial infarction. His platelet count at the start of the therapy was 517 000/μl and increased to 1 260 000/μl on day 27 after therapy. Patient 5 received a single cycle of 5-AZA-CdR at a dose of 200 mg/m^2 and went off study due to disease progression.

Patient 6 received one cycle of 5-AZA-CdR at a dose of 200 mg/m^2 and two cycles of 400 mg/m^2. There was a 5 week interval between cycles. The disease was stable with no sign of progression for 3 months. His disease showed progression after the third cycle of treatment and he went off study. Patient 7 received one cycle of 5-AZA-CdR at a dose of 200 mg/m^2 and one cycle at a dose of 400 mg/m^2, and showed stable disease. Further analysis of the antitumor activity of 5-AZA-CdR was not assessable due to cardiopulmonary arrest following surgical amputation of his leg for reason of arterial insufficiency at 21 days after the second cycle.

Patient 8 received one cycle of 5-AZA-CdR at a dose of 400 mg/m^2 and was not assessable due to cardiopulomary arrest 20 days post-treatment. He had no history of heart disease.

Patient 9 received one cycle of 5-AZA-CdR at a dose of 660 mg/m^2 and four cycles at a dose of 495 mg/m^2 per cycle at intervals of 5–6 weeks between cycles. After the last cycle of 5-AZA-CdR she showed signs of disease progression and was removed from the study. Starting 6 months after the last cycle of 5-AZA-CdR she was administered three courses of vindesine therapy during a 15 month interval. This patient is currently off therapy with a reasonable good quality of life. She shows signs of very slow disease progression as evaluated by chest

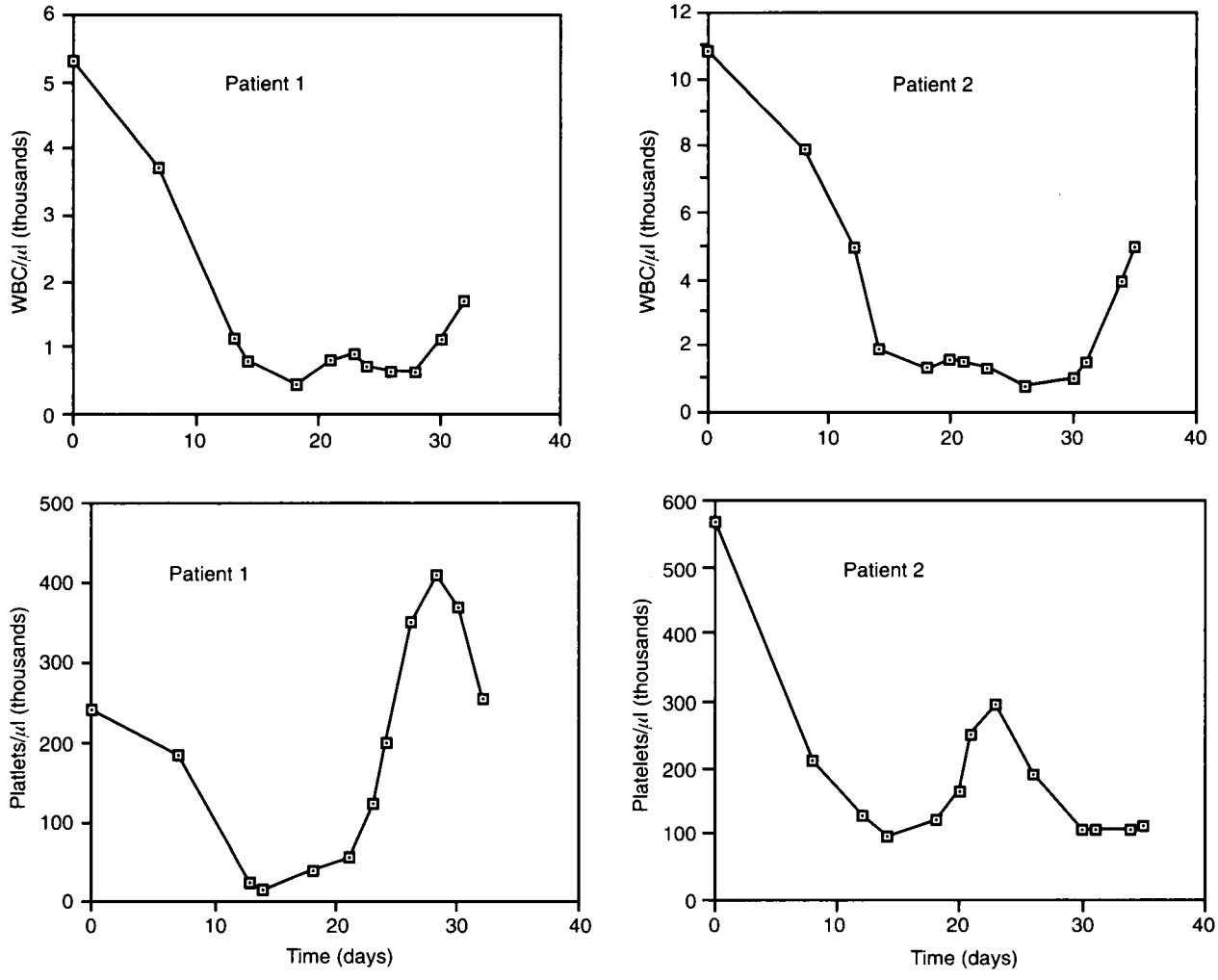

Figure 1. Hemogram following treatment with 5-AZA-CdR administered as a 6 h i.v. infusion (200 mg/m^2/day) on days 1, 2 and 3 (patient 1), and on days 1 and 2 (patient 2).

X-ray at 60+ months from the initial 5-AZA-CdR treatment.

Patients 10 and 11 received a single cycle of 5-AZA-CdR at a dose of 660 mg/m^2 and went off study due to disease progression. Patient 11 was not assessable due to cardiopulmonary arrest at 1 month post-treatment. His platelet count at the start of therapy was 501 000/μl and increased to 676 000/μl on day 27 after therapy.

Patient 12 received three cycles of 5-AZA-CdR at a dose of 660 mg/m^2 per cycle at intervals of 5–6 weeks between cycles. She had stable disease for 3 months and went off study due to progressive disease after the third cycle of treatment. Patients 13 and 15 received two cycles of 5-AZA-CdR at a dose of 660 mg/m^2 per cycle. Both patients showed slight disease progression after the first cycle and went off study due to significant disease progression after the second cycle of treatment. Patient 14 received only one cycle of 5-AZA-CdR at a dose of 660 mg/m^2. He had stable disease for 1 month, but died suddenly from thrombosis at 1 month post-treatment. The median survival duration of the nine assessable patients who received a single infusion of 5-AZA-CdR for 8 h for one or more cycles was 6.7 months.

The hemograms of patients 9, 12 and 13 are shown in Figure 2. All these patients showed an interval of leukopenia after therapy with 5-AZA-CdR followed by a significant increase in the white blood cell count between days 30 and 40. The platelet count at about day 30 for these patients was about 2- to 3-fold greater than the platelet count at the start of the 5-AZA-CdR treatment. The other patients showed similar hemograms. A moderate to marked thrombocytosis between days 25 and 30 was also observed in patients 3–5, 7, 10, 12 and 13.

Table 2. Response to i.v. infusion of 5-AZA-CdR in patients with NSCLC

Patient	Diagnosis	Age/sex	First cycle dose/ response	Second cycle dose/ response	Third cycle dose/ response	Fourth cycle dose/ response	Fifth cycle dose/ response	Survival time (months)
3	mixed	62/M	200/stable	200/stable	400/stable	400/stable		15.3
4	squam	54/M	200/prog	not eval				1.0
5	mixed	39/F	200/prog					1.5
6	adeno	62/M	200/stable	400/stable	400/prog			4.3
7	adeno	51/M	200/stable	400/stable	not eval			2.1
8	adeno	58/M	400/not eval					0.7
9	adeno	64/M	660/stable	495/stable	495/stable	495/stable	495/stable	68+
10	adeno	59/F	660/prog					3.3
11	squam	60/M	660/prog	not eval				1.0
12	adeno	51/F	660/stable	660/stable	660/prog			16.0
13	adeno	47/M	660/sl prog	660/prog				6.7
14	squam	60/M	660/stable					1.3
15	squam	62/F	660/sl prog	660/prog				9.3

Dose, mg/m^2 administered as 8 h i.v. infusion; interval between cycles 5–7 weeks.
M, male; F, female; prog, progressive disease; sl, slight; not eval, not evaluated; adeno, adenocarcinoma; squam, squamous cell carcinoma; mixed, squamous cell and adenocarcinoma.

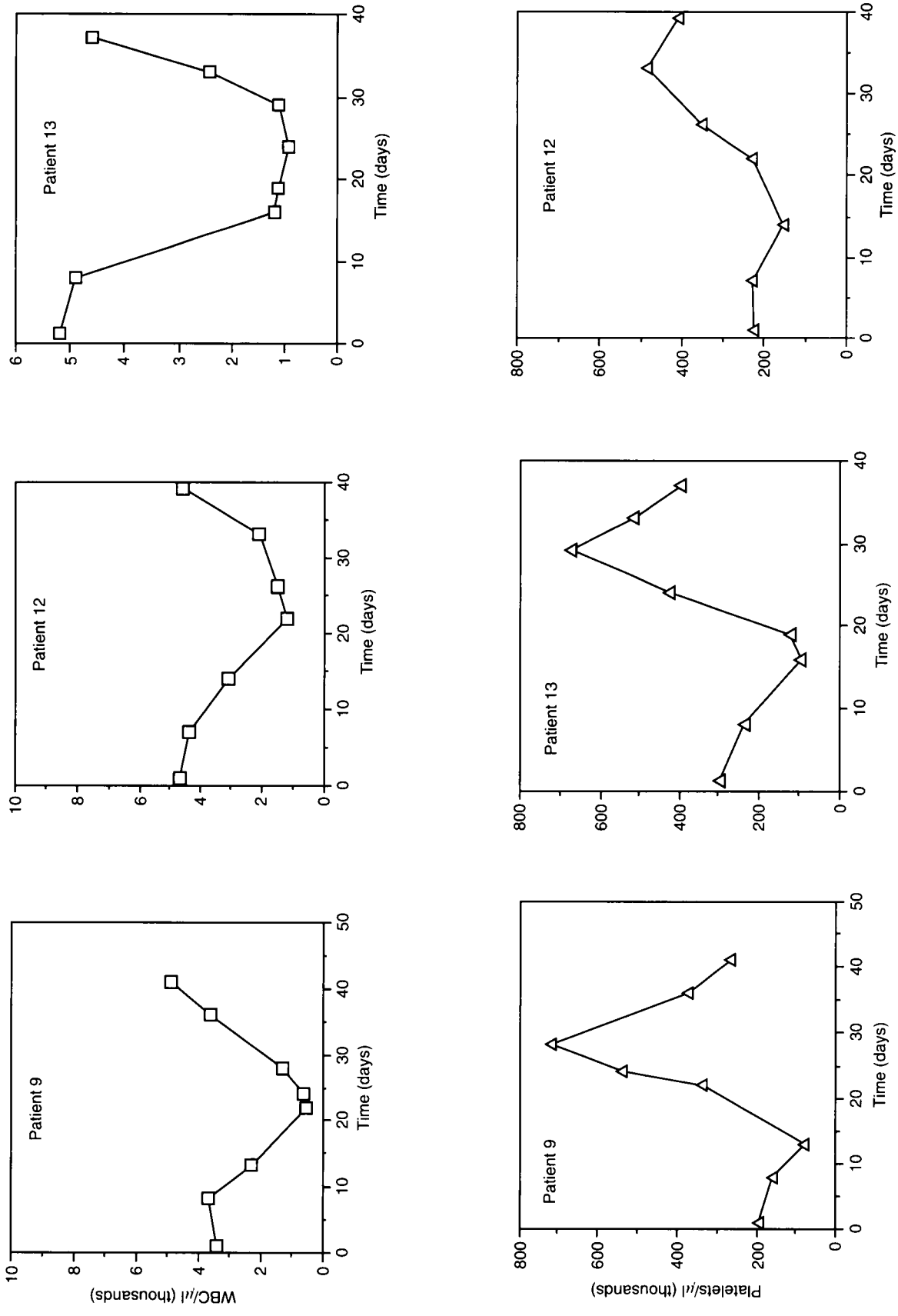

Figure 2. Hemogram following treatment with 5-AZA-CdR administered as a single 8 h i.v. infusion (660 mg/m^2) on day 1 of patients 9, 12 and 13.

A summary of the non-hematological toxicity is shown in Table 3. Nausea and vomiting of grade 2 (WHO) was observed in eight patients and grade 3 in one patient. For most of these patients the nausea and vomiting disappeared between 2 and 4 h after the termination of the 5-AZA-CdR infusion. Grade 2 infection was observed in two patients and grade 3 in one patient. Mucositis of grade 2 occurred in two patients.

The steady-state plasma concentration during the i.v. infusion of 5-AZA-CdR was determined in several patients using both a HPLC assay and a bioassay as shown in Table 4. The bioassay showed slightly lower concentrations of 5-AZA-CdR than the HPLC assay. During the 6 h infusion of 100 mg/m^2 the steady-state concentration of 5-AZA-CdR was estimated to 0.31–0.39 μg/ml. The range of concentrations of 5-AZA-CdR during the 660 mg/m^2 infusion was 0.41–16 μg/ml.

Discussion

In several phase I–II studies 5-AZA-CdR treatment was demonstrated to induce complete remissions in patients with leukemia.[3–7] The objective of this study was to determine if 5-AZA-CdR could produce a response in patients with NSCLC and to evaluate its toxicity. In support of a clinical trial in NSCLC patients is the preclinical data on human lung carcinoma cell lines which show that 5-AZA-CdR has a potent antitumor activity (Table 1). In addition, the novel mechanism of action of 5-AZA-CdR on the inhibition of DNA methylation which can lead to the

Table 3. Non-hematological toxicity of 5-AZA-CdR treatment

Toxicity	Grade (WHO)			Total[a](%)
	1	2	3	
Nausea and vomiting	3	8	1	12 (80)
Increased bronchial secretions		3		3 (20)
Infection		2	1	3 (20)
Mucositis		2		2 (13.3)
Asthenia	1	1	3	5 (33.3)
Diarrhea		1		1 (6.7)
Cardiac[b]			1	1 (6.7)
Vascular[b]			1	1 (6.7)
Hiccups	1			1 (6.7)
Inappetence		1	2	3 (20)
Dysuria		1		1 (6.7)
Myalgia		2		2 (13.3)

[a]Total number of patients = 15.
[b]Maybe unrelated to drug treatment.

Table 4. Pharmacokinetics of 5-AZA-CdR in patients with lung carcinoma

Patient	Dose (i.v. infusion)	Estimated plasma concentration of 5-AZA-CdR (μg/ml)	
		HPLC	Bioassay
1	100 mg/m^2 (6 h)	0.39	0.31 ± 0.02[b]
9	660 mg/m^2 (8 h)	0.59 ± 0.01[a]	0.58 ± 0.04
10	660 mg/m^2 (8 h)	1.16 ± 0.35	0.73 ± 0.17
11	660 mg/m^2 (8 h)	0.95 ± 0.12	0.41 ± 0.11
12	660 mg/m^2 (8 h)	0.88	0.74 ± 0.11
13	660 mg/m^2 (8 h)	0.87 ± 0.23	0.60 ± 0.06
14	660 mg/m^2 (8 h)	0.94	0.83 ± 0.46

[a]Mean ± SD. $n = 2$.
[b]Mean ± SD. $n = 3$ 6.
Estimated concentration is the steady-state level during i.v. infusion.

activation of tumor suppressor gene[16,17] and putative metastasis suppressor gene[23] has generated considerable interest in the clinical potential of this analog for tumor therapy.

Since the median survival of most patients with metastatic NSCLC following treatment with chemotherapy is less than 1 year,[1] we initiated a pilot clinical trial with 5-AZA-CdR. We chose a starting dose schedule of 200 mg/m^2 of 5-AZA-CdR administered as a 6 h i.v. infusion daily for 3 days. Since this dose schedule produced severe hematopoietic toxicity in patient 1 (Figure 1) we reduced the number of daily infusions. Prolonged leukopenia was also observed in patient 2 after receiving two daily 6 h infusions of 200 mg/m^2 of 5-AZA-CdR (Figure 2). Therefore, we administered only a single 8 h infusion of this analog for each cycle of treatment in the remaining patients.

Patient 3 received two cycles of 5-AZA-CdR at a dose of 200 mg/m^2 per cycle and three cycles of 400 mg/m^2 per cycle at intervals of 6–7 weeks between cycles. There was no significant progression of the disease in this patient for 8 months (Table 2). Patients 6 and 12 showed stable disease for 3 months after two cycles of 5-AZA-CdR treatment. Progressive disease occurred after the third cycle. The other patients showed disease progression after a single cycle of 5-AZA-CdR or were not assessable.

One problem that is encountered during the evaluation of the antitumor response of a differentiating agent, such as 5-AZA-CdR, is the very slow and gradual reduction of the tumor size following treatment. This phenomenon can be expected from the action of 5-AZA-CdR which by the induction of differentiation, possibly by activation of tumor suppressor genes,[16–18] produces a loss in the proliferative potential of the tumor stem cell. These tumor cells undergo terminal differentiation and eventual cell death due to senescence, which can be a slow process. The response can also be analyzed from the mechanism of action of 5-AZA-CdR on DNA methylation. After the first cell division only one strand of the DNA duplex is demethylated and following the second cell division the complementary DNA strand becomes demethylated.[9] Gene activation by 5-AZA-CdR occurs when both strands of DNA are demethylated.[9] Since this requires at least two cell divisions (a 4-fold increase in cell number), an initial increase in tumor size can occur during a short time interval immediately following the 5-AZA-CdR therapy.

This type of phenomenon was observed in some patients with acute leukemia who showed an increase in the blood leukemia blasts during the first week of therapy and a very large percentage of blasts in the bone marrow 4 weeks after 5-AZA-CdR treatment, but still went into complete remission without further therapy.[4,5] Since most of the conventional cytotoxic agents used in cancer therapy produce a rapid cytolytic effect on the tumor cells and rapid reduction in tumor size, the current conventional approach to evaluate the response is to quantitate the reduction in tumor size after treatment. For the reasons discussed above, the lack of tumor progression or stable disease following therapy with 5-AZA-CdR should probably be used as one of the parameters to evaluate the antitumor response.

The major toxicity produced by 5-AZA-CdR in this study was leukopenia (Figures 1 and 2). Hematological toxicity was also the major side effect produced by this analog in patients with leukemia.[4–8] Even in the presence of a marked leukopenia, only three of the 15 patients had a grade 2 or 3 (WHO) infection (Table 3). All the patients that received 8 h infusions of 5-AZA-CdR were followed on an outpatient basis between cycles of treatment. It is possible that following the 5-AZA-CdR treatment, the administration of hematopoietic growth factors, such as granulocyte colony stimulating factor, would decrease the risk of infection in patients.[28] Since 5-AZA-CdR is an S-phase-specific agent,[2] the resting hematopoietic stem cells would escape its inhibitory effects and be responsive to growth factors. A marked thrombocytosis was observed in some patients following the 5-AZA-CdR treatment. Since patients with ischemic heart disease may be at risk for myocardial infarction, the administration of aspirin during the interval of thrombocytosis may prevent the risk of coronary-artery thrombosis.[29]

The level of steady-state plasma concentration of 5-AZA-CdR during the infusions (Table 4) was as predicted by pharmacokinetic parameters of this analog obtained in previous studies.[4,14,27] 5-AZA-CdR has a short plasma half-life of 15–30 min due to its rapid inactivation by deamination by liver cytidine deaminase.[30] It is interesting to note that these plasma concentrations of 5-AZa-CdR are in the same range as the *in vitro* concentrations of this analog that activated the expresson of tumor suppressor gene p16 in three human NSCLC cell lines.[18]

In this study, four out of nine assessable patients showed stable disease for 6 months or more after the 5-AZA-CdR treatment (Table 2). If stable disease for 6 months is used as the parameter to define response, this would represent a response rate of about 44%. The median survival duration was 6.7 months. Three assessable patients had a survival

time greater than 15 months following 5-AZA-CdR therapy. These preliminary results are similar to those obtained with the difluoro-deoxycytidine analog, Gemcitabine, in patients with NSCLC.[31]

Of major interest is patient 9 who received one cycle of 5-AZA-CdR at a dose of 660 mg/m^2 per cycle and four cycles of 495 mg/m^2. This patient is still alive with signs of slow disease progression 68+ months post-treatment with 5-AZA-CdR. It should be pointed out that patient 9 received the most numbers of cycles of 5-AZA-CdR and has the longest survival duration (Table 2). After the fifth 5-AZA-CdR therapy, since there were some signs of tumor progression, this patient was not administered any additional 5-AZA-CdR treatments. This patient received three courses of vindesine during the post-treatment period. The 5+ year survival of patient 9 is surprising. It is possible that this patient has a very rare form of metastatic NSCLC that progresses very slowly. On the other hand, it is also possible that the five cycles of 5-AZA-CdR therapy were responsible for this interesting response. Can the pharmacology of 5-AZA-CdR^2 explain this result?

Since 5-AZA-CdR is a S-phase-specific agent one would expect from the cell kinetics of human tumors[33] that only a small fraction of the tumor stem cells (below 10%) were the targets during each 8 h cycle of chemotherapy. In addition, the 6–7 week interval between cycles of therapy could permit the replacement of the dying tumor stem cells by cellular proliferation. One possible explanation for the results observed in patient 9 is that repetitive treatments with a S phase agents such as 5-AZA-CdR eradicated the most rapidly proliferating tumor stem cells leaving only the survival of tumor stem cells with very low growth potential. Another possible explanation is that the 5-AZA-CdR therapy produced a bystander effect in which the activation of the expression of tumor suppressor genes by this analog in the S phase tumor stem cells resulted in the production of growth inhibitory proteins which penetrated the surrounding non-S phase tumor cells. The bystander effect has been reported for NSCLC cells both *in vitro* and *in vivo* after retroviral transduction with the wild-type p53 gene.[33–35] The existence of a bystander effect was proposed since the therapeutic effect of wild-type p53 gene replacement exceeded that expected from the fraction of cells transduced by the viral vector.

In order to evaluate the full clinical potential of 5-AZA-CdR, its optimal dose schedule will have to be determined since the antineoplastic activity of S-phase-specific agents is highly dependent on the schedule.[36] The dose-limiting toxicity of 5-AZA-CdR is myelosuppression. One approach to overcome this problem is with gene therapy utilizing a drug resistance gene to protect the hematopoietic progenitor cells.[37,38] We have demonstrated that transfer of the human cytidine deaminase gene into cells confers drug resistance to cytosine nucleoside analogs, such as 5-AZA-CdR.[39–41] The insertion of the cytidine deaminase gene into normal hematopoietic cells from patients has the potential to confer protection to the 5-AZA-CdR-induced myelosuppression and would permit one to shorten the interval between cycles of therapy which could lead an increase in the clinical response.

It is also possible that the maximal clinical antitumor activity of 5-AZA-CdR may be obtained when this analog is used in combination with other agents. In a preclinical study, we observed that 5-AZA-CdR in combination with another differentiating agent, all-*trans* retinoic acid, produced a synergistic loss of clonogenicity of human DLD-1 colon carcinoma cells.[42] This synergistic antitumor interaction was due to the demethylation by 5-AZA-CdR in the tumor cells of the retinoic acid receptor β gene,[43] a putative tumor suppressor gene.[44]

Finally, if tumor biopsies could be obtained before and after the 5-AZA-CdR treatment, it would be of interest in future clinical trails to determine if this analog activates the expression of one or more tumor suppressor genes and to correlate these data with the clinical response. Recently, a rapid PCR assay to detect the specific sites of 5-methyl-cytosine in genomic DNA was developed and should facilitate the genetic analysis of tumor samples from patients.[45]

Addendum

Patient 9 smoked 1.5 packs of cigarettes per day for 40 years and ceased smoking 6 years before the start of therapy.

Acknowledgments

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