Eur J Cancer Clin Oncol, Vol. 24, No. 12, pp. 1891–1895, 1988.
Printed in Great Britain

0277-5379/88$3.00 + 0.00

Cytotoxic Activity of 9-β-D-Arabinofuranosyl-2-fluoroadenine 5-Monophosphate (Fludarabine, NSC 312887) in a Human Tumor Cloning System

BERND LATHAN,* VOLKER DIEHL,* GARY M. CLARK† and DANIEL D. VON HOFF†

*First Department of Medicine, University of Cologne, 5000 Köln 41, Federal Republic of Germany and †Cancer Therapy and Research Center of South Texas and the Division of Medical Oncology, Department of Medicine, University of Texas, Health Science Center at San Antonio, Texas 78284, U.S.A.

Abstract—*A human tumor cloning system was utilized to screen for* in vitro *antitumor effects of the new purine antimetabolite 9-β-D-arabinofuranosyl-2-fluoroadenine 5-monophosphate. Two hundred and thirty-one specimens were evaluable for drug sensitivity information (i.e. ≥20 colonies on control plates). The overall* in vitro *response rates (defined as a ≤50% survival of tumor colony forming units) at two different concentrations of the new drug* (0.1; 1.0 μg/ml) *were between* 21 *and* 24%. *The new drug had significant antitumor activity (i.e. in more than* 35% *of specimens of those with at least five tested specimens) only against non-Hodgkin's lymphoma and breast cancer.*

INTRODUCTION

THE high antileukemic activity of the pyrimidine nucleoside, cytosine arabinoside, has stimulated the search for purine analogs with similar activity. One of the first analogs was adenine arabinoside. However, this compound was of limited value, due to the rapid deamination by adenosine deaminase to hypoxanthine arabinoside [1]. Several haloadenine nucleoside derivatives were then synthesized, which are resistant to inactivation by deamination [2, 3]. One of these derivatives, 9-β-D-arabinofuranosyl-2-fluoroadenine was found to be curative against murine leukemia L1210 and to be active *in vitro* against CCRF-CEM human lymphoblastoid cells [4–6]. The compound is thought to react after being phosphorylated and converted to its triphosphate nucleotide [7] by inhibiting both nucleoside reductase and the RNA polymerases with subsequent inhibition of DNA synthesis [8, 9]. However, the compound is rather insoluble and difficult to formulate for clinical use. Therefore 9-β-D-arabinosyl-2-fluoroadenine 5-monophosphate (fludarabine, NSC 312887), its water soluble monophosphate derivative, was synthesized [2, 5] and was found to be active against a variety of animal tumor lines, including L1210 and P388 leukemias as well as the solid tumors CD8F mammary carcinoma and the human LX-1 lung xenograft [2, 5]. In a phase I clinical trial with fludarabine in 13 patients with solid malignancies the maximal tolerated dose was 25 mg/m^2 as a single dose on each of five consecutive days, with the dose limiting toxicity being granulocytopenia and thrombocytopenia [10]. Following intravenous infusion over 30 min fludarabine was found to be rapidly dephosphorylated [10].

Since fludarabine is undergoing clinical phase II evaluation, it is desirable to know if the compound has activity against human tumors and to predict against which types of tumors it is active. We have utilized a human tumor cloning system [11, 12] to test fludarabine for *in vitro* cytotoxic activity against human tumor specimens of 31 different histologic origins.

MATERIALS AND METHODS

After informed consent the patients underwent thoracentesis, paracentesis, bone marrow aspiration, or surgery as part of routine diagnostic workups or therapeutic maneuvers.

Collection of cells

Effusions were collected in preservative-free heparinized vacuum bottles, centrifuged at 150 ***g*** for 10 min, and washed twice in McCoy's Medium 5a

Accepted 14 July 1988.
Please address all correspondence and reprint requests to: Bernd Lathan, M.D., First Department of Medicine, University of Cologne, Joseph-Stelzmann-Str. 9, D-5000 Köln 41, F.R.G.
Supported by the Anne Guttentag Memorial Research Fund and by the Deutsche Forschungsgemeinschaft (La 480/1-1).

with 10% heat inactivated fetal calf serum and 1% penicillin/streptomycin solution (all materials Grand Island Biological Company, Grand Island, NY). Bone marrow specimens were collected in heparinized syringes and processed in the same manner as were the effusions, except that after centrifugation only the buffy coat was removed and processed. Solid tumors removed by biopsy or at operation were immediately placed in McCoy's Medium 5a with 10% newborn calf serum plus 1% penicillin/streptomycin in the operating room and transported to the laboratory within 24 h, where they were mechanically dissociated by forcing through a No. 200 wire mesh gauze into McCoy's Medium 5a plus 10% fetal calf serum. They were then passed through progressively smaller needles and processed in the same manner as were the effusions.

In vitro *drug exposure*

Fludarabine was obtained from the Division of Cancer Treatment, National Cancer Institute. Stock solutions were prepared in sterile distilled water and stored at −70°C in aliquots sufficient for individual assays. Subsequent dilutions for incubations with cells were made with sterile distilled water. Two different concentrations of fludarabine (i.e. 0.1 and 1.0 μg/ml) were utilized.

For a 1 h drug exposure tumor cell suspensions were transferred into tubes and adjusted to a final concentration of 1.0×10^6 cells per ml in the presence of the appropriate drug dilution or control medium. Cells were incubated with or without drug for 1 h at 37°C in McCoy's Medium 5a plus 10% fetal calf serum and prepared for culture. For a continuous drug exposure cells were plated in the presence of the appropriate drug concentration in soft agar.

Assay for tumor colony forming units (TCFUs)

The culture system utilized in the study has been previously described [11–14]. In brief, cells to be tested were suspended in 0.3% agar in enriched CMRL Medium 1066 supplemented with 15% horse serum to yield a final cell concentration of 5×10^5 cells per ml. One milliliter of this mixture was pipetted into each of three 35-ml Petri dishes containing 1 ml 0.5% agar in enriched McCoy's Medium 5a but without conditioned medium.

Cultures were incubated at 37°C in 7% CO in humidified air. All assays were set up in triplicate. The plates were then screened for the presence of cell clusters on the day of plating with a Bausch and Lomb FAS II automatic scanner to assure the plating of single cells only. Colonies (≥60 μm in diameter) usually appeared in 10–15 days and the number of colonies on control and drug-treated plates was determined with an automatic scanner and was also in part checked by visual counting. At least 20 tumor colonies per control plate (above background) were required for a drug experiment to be considered evaluable for measurement of drug effects.

Positive control

To ensure the presence of an excellent single cell suspension, a positive control consisting of chromomycin A_3 (Sigma Chemical Company) at a concentration of 100 μg/ml was utilized. In order for an experiment to be considered evaluable, the chromomycin had to produce a ≤30% survival of TCFUs.

Data analysis

The results of the *in vitro* cloning assay were expressed as percentage of survival of TCFUs for fludarabine at a particular concentration and exposure time relative to the untreated control. This quantity was calculated as the ratio between the mean number of colonies surviving on triplicate plates and the mean number of colonies growing on triplicate control plates. A ≤50% survival of TCFUs was considered as definition of *in vitro* activity [15].

RESULTS

A total of 806 human tumors were placed in culture and had fludarabine tested against them. This represents 31 different types of cancer. Two hundred and thirty-one of the 806 specimens placed in culture (29%) formed sufficient colonies to be evaluable for drug sensitivity information (i.e. growth of ≥20 colonies on control plates) and are the database from which the following results were derived. The cloning efficiency in soft agar for the evaluable specimens varied between 0.37% and 0.004% with the median cloning efficiency of 0.014%.

The antitumor activity of fludarabine against individual tumor types is detailed for a 1 h exposure time in Table 1. Sixty-five evaluable specimens were tested at 0.1 μg/ml final concentration and 217 specimens were tested at 1.0 μg/ml final concentration. With 23% and 24% the overall response rates at these two concentrations were not different.

Of all tumor types with at least five evaluable specimens tested, the highest antitumor activity of fludarabine was found throughout the different concentrations against breast cancer and in specimens of tumors with unknown primary origin. Some minor *in vitro* cytotoxic activity was noticed for non-small cell lung cancer and ovarian cancer. The drug also had some activity against a variety of other tumor types with only a few specimens tested, among those interestingly one of two brain tumors. The new compound had no *in vitro* cytotoxic activity

Table 1. Summary of sensitivity of fludarabine results in the human tumor cloning system at 1 h exposure

Tumor type	No. of specimens with ≤50% survival of TFUs		No. of evaluable specimens	
	0.1 μg/ml		1.0 μg/ml	
Brain	—	—	1/2	(50%)
Breast	4/11	(36%)	12/40	(30%)
Cervix	0/1	(0%)	1/1	(100%)
Colon	1/2	(50%)	3/22	(14%)
Head + neck	0/2	(0%)	1/3	(33%)
Kidney	—	—	0/11	(0%)
Lung:				
small cell	—	—	2/4	(50%)
non-small cell	2/7	(29%)	8/23	(22%)
Melanoma	0/3	(0%)	2/7	(29%)
Mesothelioma	0/2	(0%)	0/10	(0%)
Ovary	6/22	(27%)	14/57	(25%)
Sarcoma	0/4	(0%)	3/9	(33%)
Unknown primary	2/5	(40%)	5/16	(31%)
Miscellaneous*	0/6	(0%)	0/12	(0%)
Total	15/65	(23%)	52/217	(24%)

*Bladder, corpus uteri, acute leukemia, Hodgkin's lymphoma, pancreas, stomach, testis, thymus.

against cancer of the kidney and against mesothelioma.

Twenty-eight evaluable specimens were also treated with a continuous exposure to fludarabine at 1.0 μg/ml. The cytotoxic activity of the drug is detailed for individual tumor types in Table 2.

Three of seven non-Hodgkin's lymphoma specimens were sensitive and all eight ovary cancer specimens were resistant to fludarabine. It might be noteworthy that the one acute leukemia specimen was also sensitive to fludarabine.

Table 2. Summary of sensitivity of fludarabine results in the human cloning system at continuous exposure to 1.0 μg/ml

Tumor type	No. of specimens with ≤50% survival of TCFUs
Breast	0/2 (0%)
Acute leukemia	1/1 (100%)
Lung:	
small cell	1/2 (50%)
non-small cell	1/3 (33%)
Non-Hodgkin's lymphoma	3/7 (43%)
Ovary	0/8 (0%)
Unknown primary	0/2 (0%)
Miscellaneous*	0/3 (0%)
Total	6/28 (21%)

*Colon, kidney, stomach.

DISCUSSION

In retrospective and prospective clinical trials the human tumor cloning system has shown some promise for predicting for response or lack of response in an individual patient's tumor [15]. Based on this experience the assay was also utilized for predicting cytotoxic activity of investigational drugs prior to their entrance into phase II clinical trials [15–17].

In previous studies a ≤30% survival of TCFUs *in vitro* was often utilized for predicting which patient would respond to a particular chemotherapeutic agent. In an earlier prospective clinical trial utilizing standard agents it was shown that a ≤50% survival of TCFUs has as reasonable a predictive value in terms of clinical response as a ≤30% survival of TCFUs [18]. Therefore, for the present study, as well as for a variety of previous studies with investigational new drugs in the human tumor cloning system, a ≤50% survival of TCFUs was utilized as a definition of *in vitro* activity [16, 19, 20]. It will make the system more sensitive for detecting antitumor activity but could also result in an overprediction of clinical activity [16].

Of 806 human tumors that were placed in culture, only 29% formed enough colonies to be evaluable for drug sensitivity data. This is well within the range of other studies with the cloning system, but could limit the results of the study. It was shown that the growth of tumor colonies in soft agar

by itself indicates a worse prognosis for patients survival compared to the lack of growth in soft agar [21]. Therefore it cannot be ruled out that the cytotoxic activity of fludarabine in tumors without sufficient growth in agar is different from that of well growing tumor specimens.

For *in vitro* studies of anticancer agents it is essential to choose appropriate drug concentrations. During a phase I clinical trial information about the pharmacokinetics of fludarabine in man was derived from study of seven patients [10]. Fludarabine was found to be rapidly dephosphorylated and no measurable levels of fludarabine were detected. These findings were in concordance with previously obtained data from mice and dogs [22]. The peak plasma level of the dephosphorylated drug (9-β-D-arabinofuranosyl-1,2-fluoroadenine) in man averaged 1 μg/ml 1 min after completion of a 30 min i.v. infusion of 18 mg/m^2 fludarabine.

Against individual tumor types with at least five evaluable specimens fludarabine had only minor activity against ovarian cancer and non-small cell lung cancer, and moderate *in vitro* activity (i.e. 30–36%) against breast cancer (all at 1 h exposure).

Interesting antitumor activity of the new drug was found against non-Hodgkin's lymphoma (i.e. 3/7 (43%) at continuous exposure). Due to poor growth of lymphoma specimens, there were unfortunately no data available for a 1 h exposure.

Some *in vitro* cytotoxic activity of fludarabine was found against tumors with unknown primary origin and it is unfortunate that we were unable to establish an exact diagnosis in these cases. The overall response rate of the new drug was equally low for 1 h exposure and for continuous exposure at 1.0 μg/ml. The situation was different for a 10.0 μg/ml concentration: 22 of 93 specimens (24%) were sensitive at 1 h compared to 11 of 28 (39%) at continuous exposure (data not shown).

Since the number of tested specimens at continuous exposure was small a schedule dependency of fludarabine's cytotoxic activity cannot be excluded.

Since the initiation of this study a variety of phase II clinical trials with fludarabine have been completed covering different types of tumors. In a recently published study at the Ohio State University an interesting antitumor activity of fludarabine was found in patients with non-Hodgkin's lymphoma (32% complete and partial responses [23]). Additional promising clinical antitumor activity was found against two other lymphoid malignancies, i.e. chronic lymphocytic leukemia (54% response rate) and mycosis fungoides (25% response rate) [24, 25].

In phase II clinical trials with patients suffering from renal cancer [26] and colorectal cancer [27] no responses were obtained, and one partial remission was seen out of eight patients with cancer of the breast [28].

The preliminary data of phase II clinical trials indicate some interesting activity of fludarabine against lymphoid malignancies. The number of specimens tested *in vitro* for individual tumor types was certainly small, but it seems to be noteworthy that the new drug also presented some *in vitro* cytotoxic activity against non-Hodgkin's lymphoma.

REFERENCES

1. LePage GA, Khaliq A, Gottlieb JA. Studies of 9-β-D-arabinofuranosyladenine in man. *Drug Metab Dispos* 1973, **1**, 756–759.
2. Montgomery JA. Has the well gone dry? The first Cain Memorial Award lecture. *Cancer Res* 1982, **42**, 3911–3917.
3. Montgomery JA. Studies on the biological activity of purine and pyrimidine analogs. *Med Res Rev* 1982, **2**, 271–308.
4. Montgomery JA, Clayton SD, Shortnay AT. An improved procedure for the preparation of 9-β-D-arabinofuranosyl-2-fluoroadenine. *J Heterocycl Chem* 1979, **16**, 157–160.
5. Brockman RW, Cheng Y-C, Schabel FM Jr, Montgomery JA. Metabolism and chemotherapeutic activity of 9-β-D-arabinofuranosyl-2-fluoroadenine against murine leukemia L1210 and evidence of its phosphorylation by deoxycytidine kinase. *Cancer Res* 1980, **40**, 3610–3615.
6. Plunkett W, Chubb S, Alexander L, Montgomery JA. Comparison of the toxicity and metabolism of 9-β-D-arabinofuranosyl-2-fluoroadenine and 9-β-D-arabinofuranosyladenine in human lymphoblastoid cells. *Cancer Res* 1980, **40**, 2349–2355.
7. Avramis VI, Plunkett W. 2-Fluoro-ATP: a toxic metabolite of 9-β-D-arabinofuranosyl-2-fluoroadenine. *Biochem Biophys Res Comm* 1983, **113**, 35–43.
8. Tseng WC, Derse D, Cheng YC, Brockman RW, Bennet LL Jr. *In vitro* biological activity of 9-β-D-arabinofuranosyl-2-fluoroadenine and the biochemical actions of its triphosphate on DNA polymerases and ribonucleotide reductase from HeLa cells. *Mol Pharmacol* 1982, **21**, 474–477.
9. White EL, Shaddix SD, Brockman RW, Bennet LL Jr. Comparison of the actions of 9-β-D-arabinofuranosyl-2-fluoroadenine and 9-β-D-arabinofuranosyladenine of target enzymes from mouse tumor cells. *Cancer Res* 1982, **42**, 2260–2264.
10. Hutton JJ, Von Hoff DD, Kuhn J, Phillips J, Hersch M, Clark G. Phase I clinical

investigation of 9-β-D-arabinofuranosyl-2-fluoroadenine 5-monophosphate (NSC 312887), a new purine antimetabolite. *Cancer Res* 1984, **44**, 4183–4186.
11. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977, **197**, 461–463.
12. Hamburger AW, Salmon SE. Primary bioassay of human myeloma stem cells. *J Clin Invest* 1977, **60**, 840–854.
13. Hamburger AW, Salmon SE, Kim MB *et al.* Direct cloning of human ovarian carcinoma cells in agar. *Cancer Res* 1978, **38**, 3438–3444.
14. Von Hoff DD, Casper J, Bradley E *et al.* Direct cloning of human neuroblastoma cells in soft agar culture. *Cancer Res* 1980, **40**, 3591–3597.
15. Hanauske AR, Hanauske U, Von Hoff DD. The human tumor cloning assay in cancer research and therapy. *Curr Probl Cancer* 1985, **9**, 1–66.
16. Lathan B, Von Hoff DD, Clark GM. Comparison of *in vitro* prediction and clinical outcome for two anthracene derivates: mitoxantrone and bisantrene. *Human Tumor Cloning* 1984, 607–617.
17. Shoemaker RH, Wolpert-DeFilippes MK, Kern DH *et al.* Application of a human tumor colony-forming assay to new drug screening. *Cancer Res* 1985, **45**, 2145–2153.
18. Von Hoff DD, Clark GM, Stogill BJ *et al.* Prospective clinical trial of a human tumor cloning system. *Cancer Res* 1983, **43**, 1926–1931.
19. Lathan B, Von Hoff DD, Elslager E. Use of a human tumor cloning system to evaluate analogs of methotrexate and mitoxantrone. *Cancer Treat Rep* 1984, **68**, 733–738.
20. Lathan B, Von Hoff DD. Cytotoxic activity of echinomycin in a human tumor cloning system. *Cancer Drug Del* 1984, **1**, 191–198.
21. Bertoncello I, Bradley TR, Campbell JJ *et al.* Limitations of the clonal agar assay for the assessment of primary human ovarian tumor biopsies. *Br J Cancer* 1982, **45**, 803–811.
22. Noker PE, Duncan GF, El Dareer SM, Hill DL. Disposition of 9-β-D-arabinofuranosyl-2-fluoroadenine 5-phosphate in mice and dogs. *Cancer Treat Rep* 1983, **67**, 445–446.
23. Leiby JM, Snider KM, Kraut EH, Metz EN, Malspeis L, Grever MR. Phase II trial of 9-β-D-arabinofuranosyl-2-fluoradenine 5′-monophosphate in non-Hodgkin's lymphoma: prospective comparison of response with deoxycytidine kinase activity. *Cancer Res* 1987, **47**, 2719–2722.
24. Grever MR, Coltman CA, Files JC *et al.* Fludarabine monophosphate in chronic lymphomcytic leukemia. A Southwest Onclogy Group study. *Blood* 1986, **68**, 223a.
25. Von Hoff, personal communication.
26. Balducci L, Blumenstein B, Von Hoff DD *et al.* Evaluation of fludarabine phosphate in renal cell carcinoma. A Southwest Oncology Group study. *Cancer Treat Rep* 1987, **71**, 543–544.
27. Harvey WH, Fleming T, Von Hoff DD, Katterhagen JG, Coltman CA Jr. Phase II study of fludarabine in previously untreated patients with colorectal carcinoma: a Southwest Oncology Group study. *Cancer Treat Rep* (in press).
28. Mittelman RA, Ahmed T, Charuvanki V, Friedland M, Arlin Z. Phase II trial of 2-fluro-ara-AMP (fludarabine phosphate) in patients with advanced breast cancer. *Proc AACR* 1985, **26**, 170.