BIOLOGICAL EVALUATION OF STABILITY OF 5-AZACYTIDINE

LI H. LI,* HAROLD H. BUSKIRK, CONSTANCE L. BLOWERS, GARY L. NEIL and LADISLAV J. HANKA

Research Laboratories, The Upjohn Company
Kalamazoo, Michigan 49001, U.S.A.

(Received for publication July 1, 1983)

5-Azacytidine (5-AzaCR) is a pyrimidine nucleoside antimetabolite and has been shown to be effective against acute myelogenous leukemia[1,2]. The major dose-limiting toxicity has been severe nausea and vomiting[3,4] which can be lessened by administration of the drug in divided doses[1,4] or by slow iv infusion[5~7]. 5-AzaCR has been reported to be somewhat chemically unstable[8,9] and metabolized extensively[9~13]. Therefore, it presents a concern regarding the clinical use of this drug.

The stability of 5-AzaCR was evaluated through three biological assays, namely, inhibition of L1210 cell growth in culture, *Escherichia coli* growth in synthetic medium, and L1210 leukemia *in vivo*. The residual activity of the drug was determined after the drug had been stored under various conditions for a given period of time.

[4-^{14}C]-5-AzaCR (4 mCi/mmol) was obtained through the Chemical and Drug Procurement Section, Chemotherapy, National Cancer Institute, from Monsanto Research Corporation, Dayton Laboratories, Dayton, Ohio. 5-AzaCR, supplied by Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin), was dissolved in sterile H_2O or buffer at 2.25 to 2.5 mg/ml and stored under the conditions described throughout this report. The basal medium for growing mouse leukemia L1210 cells was RPMI 1634 (Associated Biomedic Systems, Buffalo, New York) plus 5% fetal calf serum. The detailed procedure for determining the drug effects on a 3-day L1210 cell growth has been described elsewhere[14].

For *in vivo* antitumor testing, standardized protocols of the Drug Research and Development Program of the National Cancer Institute were followed[15]. Male BDF_1 (C57BL/6 ♀ × DBA/2 ♂) mice were used in the experiments. Mice were 6~8 weeks of age and weighed 18~25 g. Groups of 6~10 mice were housed in plastic cages and were given pelleted food and water *ad libitum*. L1210 leukemia was maintained by continuous ip passage in syngeneic DBA/2 female mice. The experiment was initiated by injecting ip 10^5 ascites L1210 cells into each BDF_1 mouse. One day later, a single dose (25 mg/kg) of 5-AzaCR, stored at room temperature for 0 to 24 hours, was injected ip in 0.2 ml volume into each mouse. The drug effect was measured as median survival time.

The microorganism used was *E. coli* ATCC 26, which was cultivated in the minimal synthetic medium. The conditions of the disc-plate assay have been described previously by HANKA[16] and NEIL *et al.*[17]. Briefly, Petri plates were prepared with the assay organism inoculated into the minimal synthetic agar further supplemented with 50 μg/ml of tetrahydrouridine. The freshly prepared standard solutions of the drug and the samples to be tested were applied to paper discs (12.7 mm diameter) and placed on the seeded Petri plates. These were cooled for 2 hours at +4°C and then incubated overnight at 32°C. After that, the zones of inhibition were recorded. A standard curve was constructed using the values from freshly made solutions and the values of all other samples were read off this curve.

The residual drug activity was determined after 5-AzaCR was dissolved in sterile distilled water

Table 1. Effect of temperature on the stability of 5-azacytidine (5-AzaCR).*

Storage temperature (°C)	Assay	% Drug activity remaining					
		1 day	3 days	7 days	14 days	21 days	Over 1 month
−10~−5	*Escherichia coli*	100	100	100	100	100	60
	L1210	100	100	100	100	100	60
25	*E. coli*	88	50	31	20	5	—
	L1210	86	60	32	22	8	—

* Concentration of 5-AzaCR=2.25 mg/ml in water.

and stored in freezer ($-10\sim-5$°C) or at room temperature (25 ± 1°C). The results (Table 1) indicate that although about 90% activity remained after a 1-day storage at room temperature, the loss of activity was proportional to storage time. Only 20% activity remained after a 2-week storage. However, no loss of activity was detected with both *in vitro* assays when the drug was stored in the freezer for at least 3 weeks. The correlation of these two *in vitro* assays was excellent.

The effect of temperature was further evaluated with RINGER's lactate solution used by LOMEN *et al.*[7]. The results indicate no loss of drug activity when the drug was stored in this vehicle at room temperature or in the freezer for at least 4 hours (data not presented).

The stability of 5-AzaCR was also evaluated through the *in vivo* testing for its antitumor activity. When the drug solution (2.5 mg/ml of H_2O) was stored at room temperature for up to 24 hours and then injected into animals which were inoculated with leukemia L1210 cells 1 day earlier, no significant loss of antitumor activity was detected (Table 2).

Overall, our results suggest that 5-AzaCR is reasonably stable for use with several clinical protocols. They are in general agreement with the stability data generated by physical chemical means. Although there was a rapid shift in UV absorption when 5-AzaCR was dissolved in an aqueous solution[1,8,9] which might reflect the reversible formation of *N*-formylribosylguanylurea (the initial hydrolytic product of 5-AzaCR), BEISLER found that about 63% of 5-AzaCR was retained when the aqueous drug solution was stored at 25°C for 24 hours[18]. He has postulated that *N*-formylribosylguanylurea might act as a depot form of 5-AzaCR and the product itself might not contribute significantly in terms of overall antitumor activity. Even though his hypothesis requires substantiation, our results clearly indicated that the majority of biological activity of this drug remained during the initial step of transformation either chemically or metabolically.

The subsequent reaction would be the degradation of *N*-formylribosylguanylurea which did not possess significant biological activity[8,10]. A rapid degradation of 5-AzaCR to this product would result in loss of biological activity. Our results clearly indicate that this was not the case,

Table 2. *In vivo* assay of stability of 5-AzaCR.

Storage time* (hours)	Mean survival time** (days±SD)	T/C*** (%)
Control (no drug)	11.2±1.2	100
0	15.6±1.0	139
3	15.1±1.2	135
6	15.3±0.9	137
24	15.2±1.0	136

* 5-AzaCR was dissolved in sterile water at 2.5 mg/ml and stored at room temperature.

** A single administration ip of drug at 25 mg/kg one day after mouse receiving ip injection of 10^5 ascities L1210 cells.

*** $T/C(\%) = \frac{\text{mean survival time (days) of drug-treated group}}{\text{mean survival time (days) of untreated group}} \times 100$

and they correlated well with that of BEISLER[18]. He found, by means of high pressure liquid chromatographic analysis, that ribosylguanylurea was slowly formed and that less than 5% was detected when a 5-AzaCR clinical formulation was stored at room temperature for 12 hours.

With the vehicle used by LOMEN *et al.* for a 5-day continuous iv infusion, we found that the drug is quite stable in RINGER's lactate solution at room temperature for at least 4 hours. We are in general agreement with the conclusion drawn by CHAN *et al.*[20] that this drug, when stored at room temperature, was relatively stable with this formulation with a half life of 4.8 days determined by a combined procedure of high pressure liquid chromatography and carbon-13 nuclear magnetic resonance. It is of interest that this value ($t_{1/2}=4.8$ days) was in the range of those obtained by us through the two biological assays (Table 1).

Overall, our results, along with those of others[17,19], suggest that 5-AzaCR can be stored in an aqueous solution of neutral pH, *e.g.*, water or RINGER's lactate, for about 3 weeks in the freezer ($-10\sim-5$°C) and probably for a number of days at refrigerator temperature (4°C), without significant loss of its biological activity. These drug solutions can also be used for continuous iv infusion at room temperature for at least 4 hours without need of replacement with fresh solution.

Acknowledgment

We are grateful to the support in part by Contract PH 43-68-1023 and NO1-CM-43753, Division of

Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare. We also thank Mrs. JOAN W. CULP for her skillful technical assistance and Mrs. KAY ZINS and Mrs. NANCY D. LIKAM for their help in the preparation of this manuscript.

References

1) KARON, M.; L. SIEGER, S. LEIMBROCK, J. Z. FINKELSTEIN & M. E. NESBIT: 5-Azacytidine: A new active agent for the treatment of acute leukemia. Blood 42: 359~365, 1973
2) MCCREDIE, K. B.; G. P. BODEY, M. A. BURGESS, J. U. GUTTERMAN, V. RODRIGUEZ, M. P. SULLIVAN & E. J. FREIREICH: Treatment of acute leukemia with 5-azacytidine (NSC-102816). Cancer Chemother. Rep. 57: 319~323, 1973
3) VOGLER, W. R.; S. ARKUN & E. VELEZ-GARCIA: Phase I study of twice weekly 5-azacytidine (NSC-102816). Cancer Chemother. Rep. 58: 895~899, 1974
4) MOERTEL, C. G.; A. J. SCHUTT, R. J. REITEMEIER & R. G. HAHN: Phase II study of 5-azacytidine (NSC-102816) in the treatment of advanced gastrointestinal cancer. Cancer Chemother. Rep. 56: 649~652, 1972
5) VOGLER, W. R.; D. MILLER & J. W. KELLER: Remission induction in refractory myeloblastic leukemia with continuous infusion of 5-azacytidine. Proc. Am. Assoc. Cancer Res. & ASCO 16: 155, 1975
6) ISRAILI, Z. H.; W. R. VOGLER, E. S. MINGIOLI, J. L. PIRKLE, R. W. SMITHWICK & J. H. GOLDSTEIN: The disposition and pharmacokinetics in humans of 5-azacytidine administered intravenously as a bolus or by continuous infusion. Cancer Res. 36: 1453~1461, 1976
7) LOMEN, P. L.; L. H. BAKER, G. L. NEIL & N. K. SAMSON: Phase I study of 5-azacytidine (NSC-101816) using 24-hour continuous infusion for 5 days. Cancer Chemother. Rep. 59: 1123~1126, 1975
8) PITHOVA, P.; A. PISKALA, J. PETHA & F. SORM: Nucleic acid components and their analogues. LXVI. Hydrolysis of 5-azacytidine and its connection with biological activity. Coll. Czech. Chem. Commun. 30: 2801~2811, 1965
9) NOTARI, R. E. & J. L. DEYOUNG: Kinetics and mechanisms of degradation of the antileukemic agent 5-azacytidine in aqueous solutions. J. Pharm. Sci. 64: 1148~1157, 1975
10) SORM, F.; Z. SORMOVA, K. RASKA, Jr. & M. JUROVCIK: Comparison and inhibitory effects of 5-azacytidine and 5-aza-2′-deoxycytidine in mammalian tissues. Rev. Roum. Biochim. 3: 139~147, 1966
11) RASKA, K., Jr.: On the metabolism of 5-azacytidine and 5-aza-2′-deoxycytidine in mice. Coll. Czech. Chem. Commun. 30: 3001~3005, 1965
12) PITHOVA, P.; A. PISKALA, J. PITHA & J. SORM: Nucleic acid components and their analogues. LVIII. 5-Azacytidine and related compounds; study of structure, tautomerism and possibilities of pairing with purine derivatives. Coll. Czech. Chem. Commun. 30: 1626~1634, 1965
13) CIHAK, A. & F. SORM: Biochemical effects and metabolic transformations of 5-azacytidine in *Escherichia coli*. Coll. Czech. Chem. Commun. 30: 2091~2101, 1965
14) LI, L. H.; S. L. KUENTZEL, L. L. MURCH, L. M. PSCHIGODA & W. C. KRUEGER: Comparative biological and biochemical effects of nogalamycin and its analogs on L1210 leukemia. Cancer Res. 39: 4816~4822, 1979
15) GERAN, R. I.; N. H. GREENBERG, M. M. MACDONALD, A. M. SCHUMACHER & B. J. ABBOTT: Protocols for screening chemical agents and natural products against animal tumors and other biological systems (3rd Ed.). Cancer Chemother. Rep. 3: 1~103, 1972
16) HANKA, L. J.; J. S. EVANS, D. J. MASON & A. DIETZ: Microbiological production of 5-azacytidine. I. Production and biological activity. Antimicrob. Agents Chemother. -1965: 619~624, 1966
17) NEIL, G. L.; T. L. MOXLEY, S. L. KUENTZEL, R. C. MANAK & L. J. HANKA: Enhancement by tetrahydrouridine (NSC 112907) of 5-azacytidine (NSC 102816) oral activity in L1210 leukemic mice. Cancer Chemother. Rep. 59: 459~465, 1975
18) BEISLER, J. A.: Isolation, characterization, and properties of a labile hydrolysis product of antitumor nucleoside, 5-azacytidine. J. Med. Chem. 21: 205~208, 1978
19) BEISLER, J. A.; M. M. ABBASI, J. A. KELLEY & J. S. DRISCOLL: Synthesis and antitumor activity of dihydro-5-azacytidine, a hydrolytically stable analogue of 5-azacytidine. J. Med. Chem. 20: 806~812, 1977
20) CHAN, K. K.; D. D. GIANNINI, J. A. STAROSCKI & W. SADEE: 5-Azacytidine hydrolysis kinetics measured by high-pressure liquid chromatography and ^{13}C-NMR spectroscopy. J. Pharm. Sci. 68: 807~812, 1979