

TABLE I
THE EFFECTS OF CYTIDINE-AZACYTIDINE COMBINATIONS ON TFT RESISTANCE IN LS178Y CELLS
Treatment time = 3 h, expression time = 48 h.

Cytidine conc. (molarity)	2.81×10^{-7} M (69 ng/ml) Aza Cyd		2.11×10^{-7} M (51 ng/ml) Aza Cyd	
	Relative total growth (%)	Mut. freq. $\times 10^{-6}$	Relative total growth (%)	Mut. freq. $\times 10^{-6}$
1×10^{-2}	75	26	88	37
5×10^{-3}	78	28	95	21
1×10^{-3}	80	22	83	22
5×10^{-4}	76	32	85	34
1×10^{-4}	71	28	85	25
5×10^{-5}	84	25	85	28
1×10^{-5}	78	25	85	24
5×10^{-6}	69	64	81	52
Aza Cyd only	56	84	76	109
Saline control ₁	100	14	100	26
Saline control ₂	100	22	100	31
621 μ g/ml EMS	48	767	-	-

Note: The first column indicates cytidine concentration (molarity, from top): 1×10^{-2} , 5×10^{-3} , 1×10^{-3} , 5×10^{-4} , 1×10^{-4} , 5×10^{-5} , 1×10^{-5} , and 5×10^{-6} . Also in the first column, 5-azacytidine alone, saline control 1 saline control 2, and 621 μ g/ml EMS (positive control).

Study title: 5-azacytidine induces micronuclei in and morphological transformation of Syrian hamster embryo fibroblasts in the absence of unscheduled DNA synthesis. Mutation Research, 283(1): 21-28, 1992 (Stopper *et al.*).

Key study findings:

- 5-Azacytidine induced cell transformation and micronuclei but not UDS in SHE cells

Study no.: Not applicable

Volume #, and page #: Module 4.2.3.3.1.5

Conducting laboratory and location: published article

Date of study initiation: published 1992

GLP compliance: No

QA reports: No

Drug, lot #, and % purity: Not reported

Formula/vehicle: DMSO

Methods:

Strains/species/cell line:

Syrian hamster embryo fibroblasts (SHE cells): 13 day SHE cells.

In the *in vitro* transformation assay the SHE cells were lethally irradiated at 50 Gy.

Concentration selection criteria:

Basis of concentration selection: Not defined.

Concentrations used in definitive study:

- UDS assay and morphological transformation assay: 0.2, 0.4, 1 and 2 μ M
- Induction of micronucleus formation: 5-azacytidine: 0-10 μ M

Test agent stability: No reported.

Metabolic activation system: none**Controls:**

Negative control: vehicle control DMSO (0.1% in UDS assay and *in vitro* transformation assay, 1% in *in vitro* micronucleus assay and kinetochore assay)

Positive controls:

- UDS assay and morphological transformation: 4-nitroquinoline (4-NQO) (5 and 0.01 μM , respectively)
- Presence of kinetochores in micronuclei in SHE cells: 4-NQO (0.5 μM) and aneuploidy-inducing hormone DES (50 μM)

Incubation and sampling times:

- UDS assay: incubation with DMSO/tested compound, [^3H] thymidine (10 $\mu\text{Ci/ml}$) and hydroxyurea (10 mM) for 5 h.
- *In vitro* transformation assay: 48 h incubation. After washing and removal of tested compounds the cells were incubated for a further 8 day period. After 10-12 days of incubation, fixation and staining, colonies with more than 50 cells were counted as survivors.
- *In vitro* micronucleus assay and kinetochore analysis: 5 hours for incubation. After removal of tested compounds incubation of cells was continued for various length of time until sampling. Sampling time: 0 to 36 hours after removal of 5-AZ.
- Mitotic index (cells in mitosis per 1000 cells) was determined in the same cell preparations that had been evaluated for the time course of micronucleus induction .

Study design: See above.

Analysis:

No. of replicates: triplicate plates.

Counting method:

- UDS assay: cell lysates were precipitated, filtered and washed. The filters were solubilized and radioactivity was determined in a scintillation counter.
- *In vitro* transformation assay: altered colony morphology consisting of crisscrossing and piling up of cells.
- *In vitro* micronucleus assay and kinetochore analysis: micronuclei were determined microscopic evaluation; kinetochore by immunohistochemical staining.

Criteria for positive results: not defined.

Summary of individual study findings:

Study validity: Not described

Study outcome: Note table and figures are from the article.

- No unscheduled DNA synthesis was observed
- 5-Azacytidine induced *in vitro* morphological transformation in the SHE cells. The transformation frequency peaked at 0.4 μM .
- 5-Azacytidine induced micronuclei in SHE cells in a dose-dependent manner and continued rising during the entire 36 h experimental period.

- The mitotic index decreased compared to the background value during 5-azacytidine exposure, resulting in a low mitotic index after 5-azacytidine removal. The decreased mitotic index indicates cell cycle arrest.
- Anti-kinetochore staining of micronuclei showed that 5-azacytidine-induced micronuclei reacted positively with CREST serum (CRMN⁺). The authors suggest that this may be indicative of an increase in both clastogenic events and numerical chromosomal changes.

TABLE 1
UNSCHEDULED DNA SYNTHESIS (UDS) INDUCED BY
5-AC AND 4-NQO IN SHE CELLS

Compound	Concentration	cpm/5×10 ⁵ cells
DMSO	1%	9870 ± 683
4-NQO	5.0 μM	58320 ± 1422
5-AC	0.2 μM	9853 ± 648
	0.4 μM	9829 ± 615
	1.0 μM	9141 ± 606
	2.0 μM	8323 ± 567

Each data point represents the mean of three chemically treated cultures from one experiment. The experiments were repeated three times with consistent results.

TABLE 2
MORPHOLOGICAL TRANSFORMATION OF SHE
CELLS, INDUCED BY 5-AC AND 4-NQO

Com- pound	Concen- tration	Survival (%)	Transfor- mation frequency		Signifi- cance (%)
			Ratio	(%)	
DMSO	0.1%	100	0/896	0	-
4-NQO	0.01 μM	91.2	3/817	0.37	<10
5-AC	0.2 μM	89.6	3/803	0.37	<10
	0.4 μM	88.1	8/789	1.01	<1
	1.0 μM	71.0	4/637	0.63	<2
	2.0 μM	66.5	2/596	0.34	<10

The transformation frequency is calculated from the ratio of transformed colonies to the number of surviving colonies. All experiments were repeated three times with consistent results. The present data represent the results of one experiment. All transformation frequencies were statistically significant as tested in the χ^2 test.

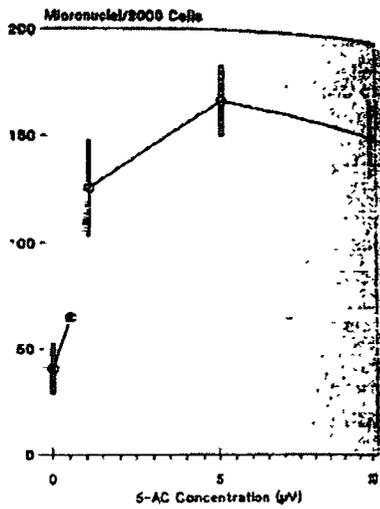
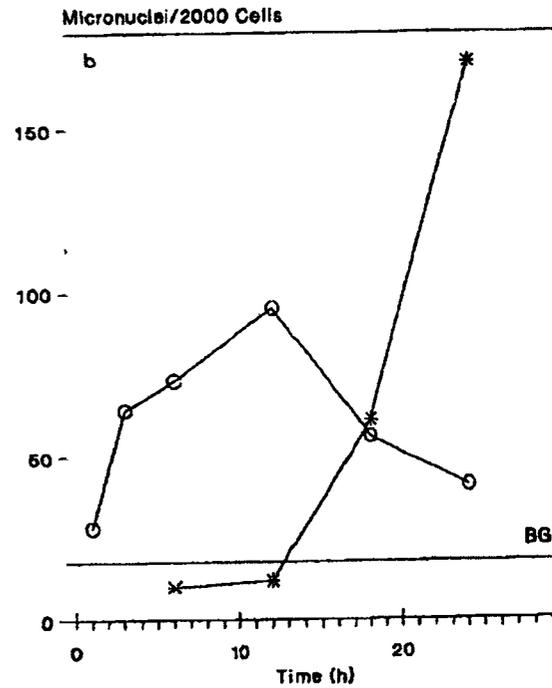
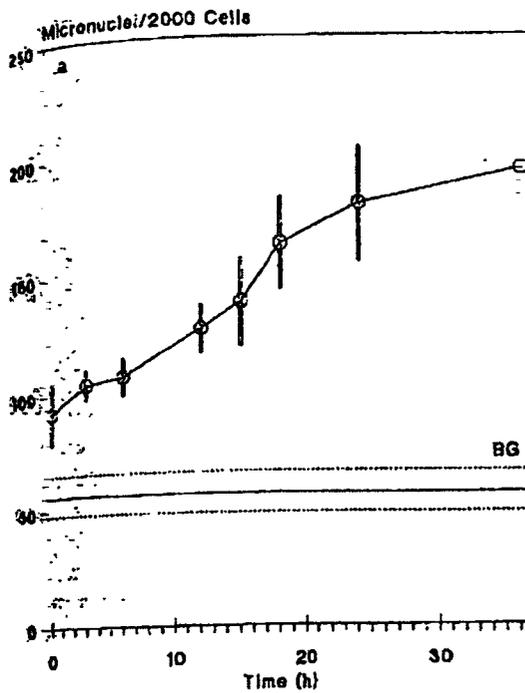


Fig. 1. Induction of micronucleus formation in SHE cells by 5-azacytidine. Data represent the mean of three treated cultures from one experiment. The experiments were repeated three times with consistent results.



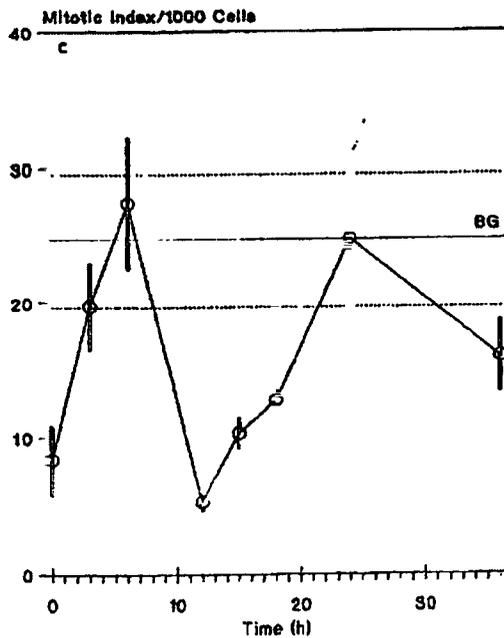


Fig. 2. (a) Time of micronucleus induction by 5-azacytidine (10 μ M) in SHE cells, starting at the time of 5-AZ removal. Each data point represents the mean of three treated cultures from one experiment. The area marked 'BG' (background) indicates the mean of the spontaneous frequency. (b) Time course of micronucleus induction in SHE cells by 4-NQO (*, 0.5 μ M) and DES (\circ , 50 μ M). The line marked 'BG' indicates the spontaneous frequency. Data taken from Schiffmann and DeBoni (Mutation Res., 246: 113-122, 1991) and Schmuck *et al.* (Mutation Res., 203: 397-404, 1988). (c) Time course of changes in mitotic index after 5-azacytidine treatment (10 μ M) in SHE cells. Each data point represents the mean of three treated cultures from one experiment. The area marked 'BG' indicates the mean of the spontaneous frequency.

TABLE 3
PRESENCE OF KINETOCHORES IN MICRONUCLEI (%) IN SHE CELLS

Compound	Dose	% CRMN ⁺	
DMSO	1% v/v	23 \pm 7	(a)
5-AC	10 μ M	29 \pm 8	(a)
DES	50 μ M	85 \pm 3	(b)
4-NQO	0.5 μ M	6 \pm 2	(b)

(a) Each number represents the mean of three counts of 100 micronuclei (with standard deviation), evaluated for kinetochore presence. (b) Data taken from Schiffmann and De Boni (Mutation Res., 246: 113-122, 1991) for comparison. These experiments were also performed with SHE cells, using the same procedure as described in this publication.

Study title: Studies of mutagenicity and clastogenicity of 5-azacytidine in human lymphoblasts and *Salmonella typhimurium*. Mutation Research, 160(1): 249-257, 1986 (Call *et al.*).

Key study findings:

- 5-Azacytidine induced mutation in the TK^{+/−} human lymphoblastoid line, TK6.
- 5-Azacytidine did not induce chromosomal aberrations in TK6 cells.
- 5-Azacytidine was mutagenic in *S. typhimurium*.

Study no.: Not applicable

Volume #, and page #: Module 4.2.3.3.1.2

Conducting laboratory and location: published article

Date of study initiation: published 1986

GLP compliance: No

QA reports: No

Drug, lot #, and % purity: Not reported

Formula/vehicle: Water

Methods:

Strains/species/cell line:

Human lymphoblasts:

HH4 cell line (TK^{+/+}, HGPRT⁺): a clone of the WIL-2 cell line.

TK6 cell line (TK^{+/-}, HGPRT⁺): derived from HH4.

Bacteria: *S. typhimurium* strain TM677.

Concentration selection criteria:

Basis of concentration selection: Not defined.

Concentrations used in definitive study:

- Mutagenicity to TK6 human lymphoblasts: 0, 0.1, 0.5, 1, 2.5, 5, 10 μ M
- Phenotypic expression time: 0, 0.5, or 5 μ M.
- Mutagenicity to HH4 human lymphoblasts: 0-1 μ M.
- Mutagenicity to TM677: 0, 0.1, 0.5, 1, 2.5, 5, and 10 μ M
- DNA incorporation studies: [¹⁴C]5-azacytidine (0-10 μ M)
- Cytogenetic damage in TK6 cells: 0-10 μ M.

Test agent stability: Not reported.

Metabolic activation system: N/A

Controls:

Negative control: None

Positive controls: None

Incubation and sampling times:

- Human lymphoblast mutation assay: 24 h treatment
- DNA incorporation studies: Human lymphoblasts were incubated with [¹⁴C]5-azacytidine for 24 hours. DNA was isolated and concentration was determined by spectrophotometry. The amount of 5-azacytidine incorporated was determined by scintillation counting.
- Cytogenetic analysis: TK6 cells were treated for 24 h.
- Bacterial mutation assay: 2 h. in suspension centrifuged and plated for selection for 2 d.

Study design: See above.

Analysis:

No. of replicates: not reported.

Counting method: see above.

Criteria for positive results: not defined.

Summary of individual study findings:

Study validity: Not described

Study outcome:

- Mutagenicity in human lymphoblasts:
 - 5-Azacytidine showed toxicity to TK6 cells at concentration greater than 0.5 μ M.

- 5-Azacytidine (0 - 10 μM for 24 h) was mutagenic at both loci, but was 5-10-fold more mutagenic at the *tk* locus than the *hgpri* locus. There was no indication about the numbers in parentheses in the figure.
- 5-Azacytidine was significantly mutagenic at 0.1 μM , the lowest concentration tested.
- 5-Azacytidine (0-10 μM for 24 h) did not increase the incidence of gaps, breaks, fragments, minute chromosomes, or exchanges. 5-Azacytidine did not increase the incidence of aberrant, polyploid, or endoreduplicated cells and did not cause chromosome decondensation. (Data not reproduced from the article.)
- The incorporation of [^{14}C] 5-azacytidine in TK6 cells as into total DNA was linear between 0 and 5 μM , but increased less steeply between 5 and 10 μM .
- 5-Azacytidine was mutagenic in *S. typhimurium* strain TM677 as measured by 8-azaguanine-resistant cells.

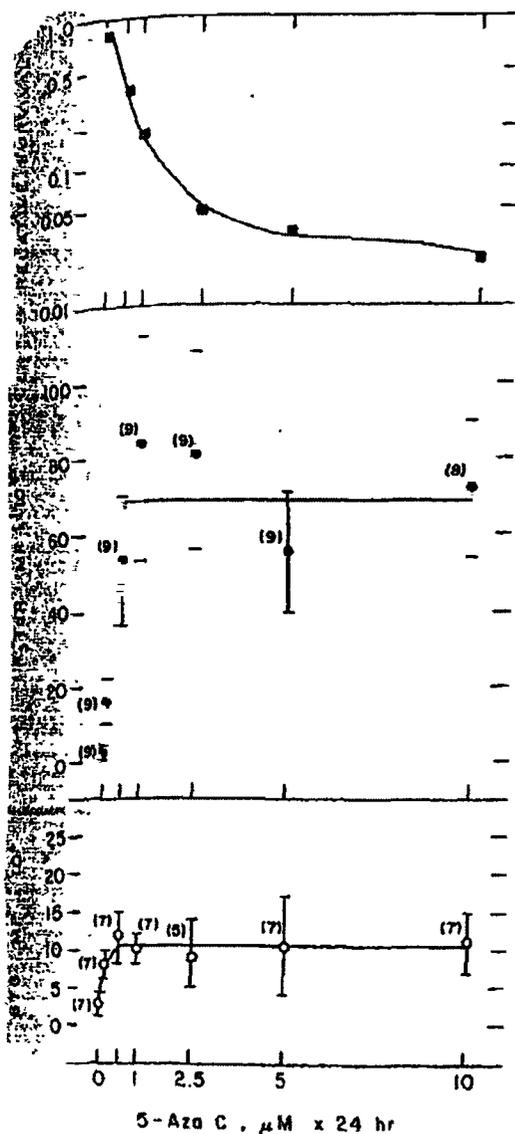


Fig. 2. Toxicity and mutagenicity of 5-AzaC in human lymphoblasts. TK6 cells were treated with 5-AzaC (0 - 10 μM for 24 h). Cultures were plated to determine survival immediately after 5-AzaC treatment. Cultures were plated in F₃TdR (1 $\mu\text{g}/\text{ml}$; 20000 cells/well) 3 days later and in 6TG (1 $\mu\text{g}/\text{ml}$, 40000 cells/well) 6 days later. Error bars are 95% confidence intervals.

Note: The Y axis indicates for the three panels (from top to bottom): Relative Survival, F₃TdR^R Mutant Fraction x 10⁶, and 6TG^R Mutant Fraction x 10⁶, respectively.

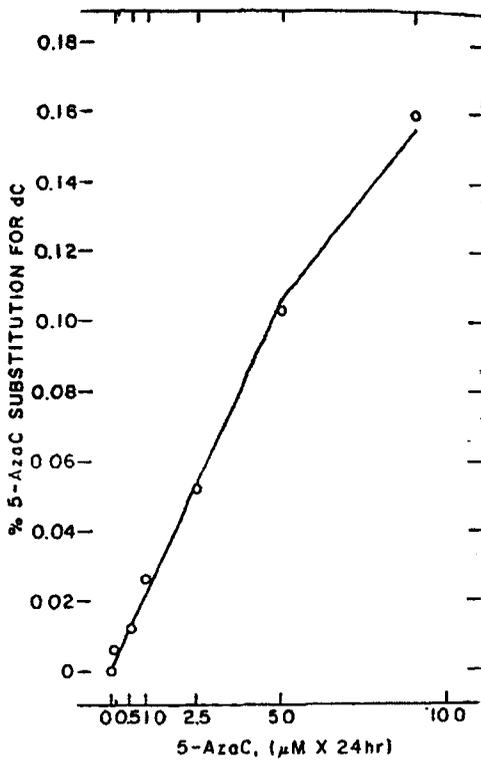


Fig. 5. Percentage substitution of 5-AzaC for dC in total DNA from TK6 cells. TK6 cells were treated with [^{14}C]5-AzaC (0-10 μM for 24 h). Incorporation of 5-AzaC into DNA was determined immediately following treatment, as described in Materials and Methods.

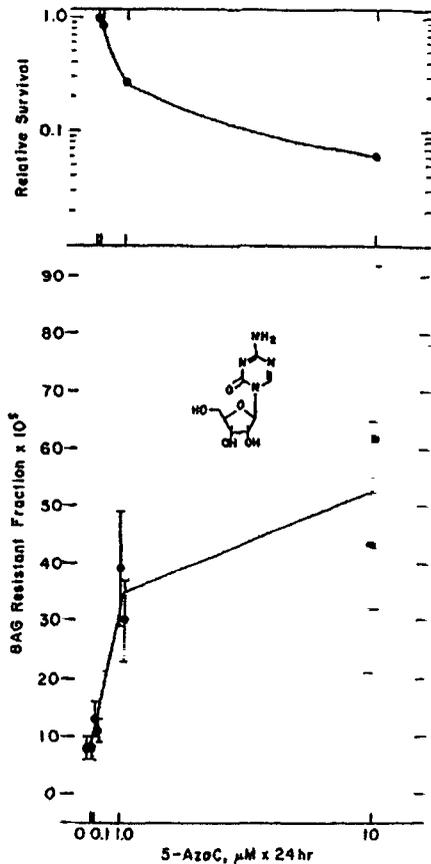


Fig. 1. Toxicity and mutagenicity of 5-AzaC in a forward mutation assay in *Salmonella typhimurium*. *Salmonella typhimurium* strain TM677 was treated with 5-AzaC (0-10 μM for 1.5 h). Cultures were then plated to determine survival and the 8-azaguanine-resistant mutant fraction. Error bars are 99% confidence intervals. The 99% upper confidence limit for the historical background mutant fraction is 25×10^{-5} .

Study title: Cell-cycle dependent micro nucleus formation and mitotic disturbances induce by 5-azacytidine in mammalian cells. Stopper et al., Mut Res 300: 165-177, 1993 Module #4.2.3.3.1.4. Summary only.

The effects of 5-azacytidine on mitosis and on micronucleus formation were investigated in mammalian cells. In mouse lymphoma L5178Y cells, 5-azacytidine induced an increase in micronuclei at doses from 0.1-5 μ M (Figure 1, below). These micronuclei contained mostly chromosomal fragments, or some may contain whole chromosomes, as detected by CREST staining and C-banding studies. By incorporating BrdU into the DNA of SHE cells, the authors also found that the induction of micronuclei in these cells occurred only when the addition of 5-AZ was in S phase.

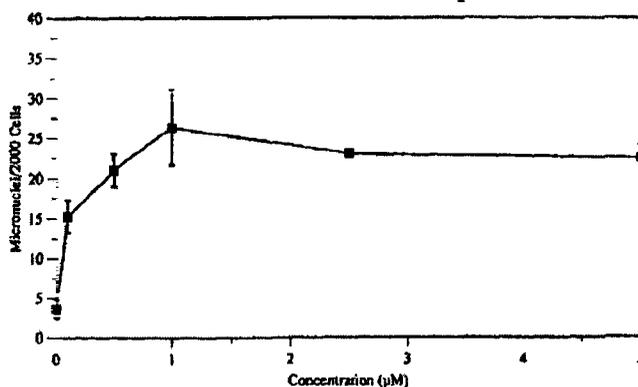


Fig. 1 Dose-response of 5-azacytidine induced micronuclei. L5178Y cells were treated with the indicated concentrations for 4 h and fixed 18 h later. Cells treated with 37 μ M DES showed 28.0 ± 6.8 micronuclei per 2000 cells.

3.4.5. Carcinogenicity

Note: No independent statistical review was conducted

Mice:

Short-term study:

Study title: 5-Azacytidine carcinogenesis in BALB/c mice, Cancer Letters, 37(1): 51-58, 1987 (Cavaliere *et al.*).

Key study findings:

- 5-Azacytidine induced a significant increase in lymphomas and skin tumors in both sexes.
- 5-Azacytidine induced lung tumors in males.
- 5-Azacytidine induced mammary carcinomas in females.

Study no.: Not applicable

Volume #, and page #: Module 4.2.3.4.2.2

Conducting laboratory and location: Not applicable

Date of study initiation: Not provided

GLP compliance: No

QA report: No

Drug, lot #, and % purity: 5-Azacytidine (Sigma Chemical Co., St. Louis, MO), purity: 99%.

CAC concurrence: No

Study Type: 50 weeks in BALB/c mice, IP injection.

Species/strain: 8-week-old intact virgin male and female BALB/c/Cb/Se mice bred at the Division of Cancer Research, Institute of Pathological Anatomy, Perugia University, Italy, were used.

Number/sex/group; age at start of study: 50/sex/group; 8 weeks old.

Animal housing: 4-5 mice/cage.

Formulation/vehicle: 5-Azacytidine in sterile saline solution.

Drug stability/homogeneity: 5-Azacytidine (powder purchased from Sigma) was stored at -20° C in the dark. No information was provided in regard of stability or homogeneity.

Methods

Doses: 2 mg/kg once a week for 50 weeks.

Basis of dose selection: Not reported

Route of administration: IP injection.

Frequency of drug administration: Once per week.

Dual controls employed: Saline control only.

Satellite groups used for toxicokinetics or special groups: None

Restriction paradigm for dietary restriction studies: n/a

Interim sacrifices: n/a

Deviations from original study protocol: not reported.

Statistical methods: The log rank test (Peto *et al.*, "Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples." Br. J. Cancer, 35: 1-39, 1977) was used to analyze survival rates and the single tumor incidence trend calculated allowing for longevity (Peto *et al.* "Guidelines for simple, sensitive significance tests for carcinogenic effects in long-term animal experiment." IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Suppl. 2, Long-term and short-term screening assays for carcinogens: A critical appraisal, pp. 811-426, Lyon, France, 1980).

Observation times

Clinical signs: observed daily for signs of toxicity and moribund.

Body weights: Not obtained

Food consumption: Not obtained

Hematology: Not obtained

Clinical chemistry: Not obtained

Organ weight: Not obtained

Gross pathology: Not obtained

Histopathology: Peer review: yes (x), no ()

Including all tumors and pathologically altered organs from animals died due to treatment, and animals killed due to moribund and at the end of study.

Toxicokinetics: n/a

Results

Mortality: the figure below is excerpted from the article depicting the survival of mice after 50-week treatment of 5-azacytidine or saline vehicle:

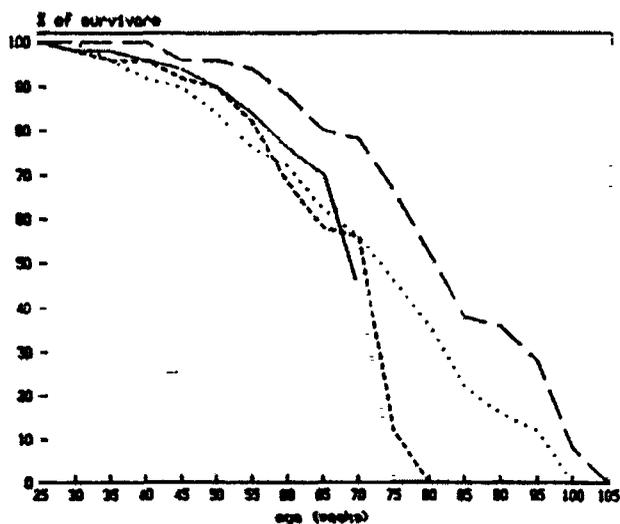


Fig. 1. Survival of 5-azacytidine-treated and control mice. —, treated males; . . . control males; - - -, treated females; — — —, control females.

Summary of experiment result:

No animals died before the 25th week. The survival was statistically reduced in treated animals of both sexes: males $P < 0.05$, females $P < 0.001$ (Fig. 1).

Clinical signs:

- 5-Azacytidine was well tolerated by all the animals and there were no gastrointestinal disturbances.

Body weights: Not obtained.

Food consumption: Not obtained.

Gross pathology: No data presented in the result.

Histopathology: The following tables indicating types, incidence of tumors and latency periods, are excerpted from the article.

TABLE 1
INCIDENCE AND SITE OF TUMOURS IN TREATED AND CONTROL MICE

Group no.	Treatment	Mice		No. of tumor-bearing mice (%)	No. of animals with tumours of:				
		No.	Sex		Lymphoreticular system (%)	Lung (%)	Mammary gland (%)	Skin (%)	Other tissues (%)
1	5-Azacytidine 2.0 mg/kg body wt.	50	M	38 (76.0)	12 (24.0)	27 (54.0)	0 (0.0)	3 (6.0)	6 ^a (12.0)
		50	F	44 (88.0)	36 (76.0)	7 (14.0)	7 (14.0)	7 (16.0)	4 ^b (8.0)
2	None	50	M	13 (26.0)	3 (6.0)	12 (24.0)	0 (0.0)	0 (0.0)	0 (0.0)
		50	F	14 (28.0)	6 (12.0)	9 (18.0)	0 (0.0)	1 (2.0)	0 (0.0)

^aOne squamous cell carcinoma of the forestomach; 1 fibrosarcoma of the seminal vesicles; 2 'large' or 'macroscopic' neoplasms type A of the cortex of the adrenal gland; 2 hepatocellular adenomas, solid type.

^bOne endometrial adenocarcinoma; 1 poorly differentiated fibrosarcoma of the dermis; 1 squamous cell carcinoma of the forestomach; 1 leiomyosarcoma of the stomach.

Note:

1. Group 1 and Group 2 refer to 5-azacytidine-treated (2 mg/kg) and saline control, respectively.
2. The percentage (in parentheses) of animals with tumor was calculated against the total number of animals of the group (i.e., 50 animals).

TABLE 2
MEAN LATENCY PERIODS (L.P.) AND RANGE IN WEEKS OF TUMOURS IN TREATED AND CONTROL MICE

Mice	Treatment	Lymphoreticular system		Lung		Mammary gland		Skin	
		Mean L.P.	Range	Mean L.P.	Range	Mean L.P.	Range	Mean L.P.	Range
Treatment	M	58.8	36-67	57.8	21-67	-	-	50.8	37-61
	F	59.1	18-69	60.1	44-69	37.1	15-58	48.0	11-68
Control	M	69.0	65-72	70.4	43-92	-	-	-	-
	F	67.1	34-88	74.6	49-88	-	-	48.0	48

Summary of histopathological findings on tumors (excerpted from the article):

- Lymphoreticular system: the rise in tumor incidence was statistically significant in both sexes (males $P < 0.01$, females $P < 0.001$). By the Pattengale and Taylor histological classification the majority of tumors were small lymphocytic lymphomas and follicular center cell lymphomas. Some immunoblastic lymphomas, and plasma cell lymphomas and granulocytic leukemias were also seen.
- Lung: statistical analysis showed that lung tumor incidence was highly significant in males ($P < 0.001$) but not in females. Histologically the lung tumors were adenomas composed of columnar or cuboid cells with scarce weakly-acidophilic cytoplasm and round or oval nuclei arranged in papillary, pseudo-glandular and cord-like structures.
- Mammary gland: seven tumors, 5 adenocarcinomas type B and 2 adenocanthomas were found only in treated female mice. The difference between treated and control animals was statistically significant ($P < 0.01$).
- Skin: the increase in skin tumor incidence was statistically significant in both sexes (males $P < 0.05$, females $P < 0.01$). The only tumor seen in a control female mouse was a squamous cell carcinoma. In treated males 2 squamous cell carcinomas and 1 keratocanthoma were found. Of the 7 tumors observed in

treated females 6 were squamous cell carcinomas and 1 a pedunculated squamous cell papilloma.

- The incidence (%) of lung tumor in female mice is 18% for the control and 14% for the treated (Table 1). In the "Result" the authors stated the incidence of lung tumors was not significant for female mice, although the authors indicated $P < 0.05$. The authors also stated in the abstract and in the discussion that the incidence of lung tumors in female mice was significant, probably based on the P value. The survival was significantly reduced in the treated males and females. However, there is no way to re-analyze the statistics of lung tumor incidence in female mice (e.g., time-adjusted analysis) without sufficient survival data. Nevertheless, the reviewer considers that the discrepancy in the report of lung tumors in female mice does not affect the overall conclusion of tumorigenicity of 5-azacytidine in the lung.

Non-neoplasm histopathological findings: not reported.

Toxicokinetics: n/a

Summary of individual study:

Administration of 5-azacytidine at 2 mg/kg/week for 50 weeks was carcinogenic in BALB/c mice. These tumors include lymphoma and skin tumors in both sexes, lung tumors in males, and mammary carcinomas in females. Other tissues with tumor findings include forestomach; seminal vesicles; adrenal gland; liver (hepatocellular adenomas, solid type), uterus (endometrial adenocarcinoma), and stomach.

Long-term Study

Study title: Bioassay of 5-azacytidine for possible carcinogenicity: Carcinogenesis testing Program, National Cancer Institute, NIH, Bethesda, MD; 1978.

Key study findings:

- Under the conditions of this bioassay, the short life span and short duration of treatment of male B6C3F1 mice precluded evaluation of the carcinogenicity of 5-azacytidine in these groups.
- The induction of tumors of the hematopoietic system in female B6C3F1 mice was associated with the administration of 5-azacytidine.
- No high dose females survived to study termination.

Study no.: NCI Report number: NCI-CG-TR-42; DHEW/PUB/NIH-78-842

Volume #, and page #: Module 4.2.3.4.1.1

Conducting laboratory and location: The bioassay was conducted by Southern Research Institute, Birmingham, AL, initially under direct contract to NCI and then under a subcontract to Tracor Jitco, Inc., prime contractor for the NCI Carcinogenesis Testing Program.

Date of study initiation: Not provided

GLP compliance: No

QA report: No

Drug, lot #, and % purity: 5-Azacytidine (Ash-Stevens, Inc., Detroit, MI), Lot No. AP-V-128, purity > 99%.

CAC concurrence: No

Study Type:

- Dose-determining study: 45 days in Swiss mice, IP, then observed for 45 days.
- 52 weeks in B6C3F1 mice, IP, then observed for 29 or 30 weeks (total 80 or 81 weeks). Female mice which survived the treatment of high dose of 5-AZ (4.4 mg/kg) were observed only for 9 weeks and killed at Week 61.

Species/strain: see above. The B6C3F1 mice were obtained from Charles River Laboratories and from A. R. Schmidt, Madison, WI.

Number/sex/group; age at start of study:

- Dose-determining study: 5/sex/group of treated animals, 10 animals in untreated control group and 10 animals used as vehicle (saline) controls. Ages of animals when 45-day study started were not reported. However, based on the age on arrival at the laboratory and the quarantine time, they should be approximate to those in the main study (see below).
- Main study: 35 mice/sex/group, 15 mice/sex in untreated control group and 15 mice/sex used as vehicle (saline) controls. All mice were 38 days of age when placed on study.

Animal housing: seven mice/cage.

Formulation/vehicle: 5-Azacytidine at the concentration of 0.2 or 0.4% was prepared in buffered saline (pH 6.9).

Drug stability/homogeneity:

The powdered 5-azacytidine was stored at 5°C in small bottles enclosed in sealed plastic bags containing Drierite®. However, no analyses of stability, homogeneity, or achieved concentrations were carried on the preparations of the test or positive control substances. Aqueous solutions of 5-azacytidine were not stored, because they are unstable at room temperature. The drug and the vehicle were mixed in a 10 ml glass Potter-Elvehjem tissue grinder with a Teflon pestle. Fresh solutions in exact amounts for administration were prepared preceding injection.

Methods

Doses:

- Dose-determining 45-day study:
 - First study: in males, 0.22, 0.6, 1.1, 2.2, and 4.4 mg/kg
 - Second study: in females, 4.4, 8.8, 17.6, and 35.2 mg/kg.
- Main study:
 - 2.2 or 4.4 mg/kg three times per week for 52 weeks (low and high dose, respectively).

Table 2. Design of Chronic Studies of 5-Azacytidine in Mice

Sex and Treatment Group	Initial No. of Animals ^a	5-Azacytidine Dose ^b (mg/kg)	Time on Study	
			Treated (weeks)	Untreated (weeks)
Male				
Untreated-Control	15	0		81-82
Vehicle-Control	15	0 ^c	52	29-30
Low-Dose	35	2.2	52	29
High-Dose	35	4.4	52	29
Female				
Untreated-Control	15	0		82
Vehicle-Control	15	0 ^c	52	29-30
Low-Dose	35	2.2	52	29
High-Dose	35	4.4	52	9 ^d

^aAll animals were 38 days of age when placed on study.

^b5-Azacytidine was administered in buffered saline by intraperitoneal injection three times per week at a volume of 1 ml/100 g body weight. Doses were based on the mean weight of the animals in each cage.

^cVehicle controls received only buffered saline solution, at the same volume as treated mice.

^dThe remaining high-dose female mice were killed at week 61.

Basis of dose selection: Based on low and high doses in 45-day studies to estimate the MTD of 5-AZ.

Route of administration: IP injection.

Frequency of drug administration: Three times per week.

Dual controls employed: untreated and saline controls

Satellite groups used for toxicokinetics or special groups: None

Restriction paradigm for dietary restriction studies: n/a

Interim sacrifices: n/a

Deviations from original study protocol: not reported.

Statistical methods: (excerpted from the report)

- Survival:
 - Probabilities of survival: product-limit procedure of Kaplan and Meier.
 - Dose-related effect on survival: method of Cox for testing two groups for equality and Tarone's extensions of Cox's methods for testing for a dose-related trend.
 - One-tailed P values have been reported for all tests except the departure from linearity test, which is only reported when its two-tailed P value is less than 0.05.
- Tumor incidence:
 - Incidence of neoplastic or nonneoplastic lesions: ratio of the number of animals bearing such lesions at a specific anatomic site (numerator) to the number of animals in which that site is examined, or the numbers of animals necropsied (denominator).
 - One-tailed Fisher exact test with P < 0.05 as significance level.
 - Linear trend in proportions: Cochran-Armitage test with continuity correction.

- A time-adjusted analysis was applied when numerous early deaths resulted from causes that were not associated with the formation of tumors.
- Life-table method: to analyze the incidence of tumors. Curves of the proportions surviving without an observed tumor were computed as in Saffiotti et al. (Cancer Res. 32 1073-1081, 1972). Cox's method of comparing these curves were used for two groups; Tarone's extension to testing for linear trend was used for three groups.
- "The approximate 95% confidence interval for the relative risk of each treated group compares to its control was calculated from the exact interval on the odds ratio. The relative risk is defined as p_t/p_c where p_t is the true binomial probability of the incidence of a specific type of tumor in a treated group of animals and p_c is the true probability of spontaneous incidence of the same type of tumor in a control group."
- "The lower and upper limits of the confidence interval of the relative risk have been included in the tables of statistical analyses. The interpretation of the limits is that in approximately 95% of a large number of identical experiments, the true ratio of the risk in a treated group of animals to that in a control group would be within the interval calculated from the experiment. When the lower limit of the confidence interval is greater than one, it can be inferred that a statistically significant result ($P < 0.025$ one-tailed test when the control incidence is not zero, $P < 0.050$ when the control incidence is zero) has occurred. When the lower limit is less than unity, but the upper limit is greater than unity, the lower limit indicates the absence of a significant result while the upper limit indicates that there is a theoretical possibility of the induction of tumors by the test chemical, which could not be detected under the conditions of this test."

Observation times

Clinical signs: observed twice daily for signs of toxicity and moribundity.

Body weights: mice weighed individually each week for 2 months and every 2 weeks for the remainder of this study. Palpation for masses was carried out at each weighing.

Food consumption: Not obtained

Hematology: Not obtained

Clinical chemistry: Not obtained

Organ weight: Not obtained

Gross pathology: including major tissues, major organs, and all gross lesions from killed animals and from animals found dead.

Histopathology: Peer review: yes (), no (x)

- Including major tissues, major organs, and all gross lesions from killed animals and from animals found dead.
- The following tissues were routinely examined microscopically; skin, muscle, lungs and bronchi, trachea, bone and bone marrow, spleen, lymph nodes, thymus, heart, salivary gland, liver, gallbladder and bile duct, pancreas, esophagus, stomach, small intestine, large intestine, kidney, urinary bladder, pituitary, adrenal, thyroid, parathyroid, mammary gland, prostate or uterus, testis or ovary, brain, and sensory organs. Peripheral blood smears were prepared from each animal. Occasionally, additional tissues were also examined microscopically.

Toxicokinetics: none

Results

Dose-determining 45-day study:

- In mice: The first set of five doses resulted in no drug-related deaths and in no weight depression exceeding the 15% guideline in the tested male mice. The result of second study in female mice was summarized in the following table:

Dose (mg/kg)	Mortality	Time of death	Comments
17.6 or 35.2	5/5	All by week 6	
8.8	3/5	By week 8	
4.4	No death		No lesions, no weight loss in surviving animals

The low and high doses for rats were set at 2.2 and 4.4 mg/kg for main study.

Mortality:

- The Kaplan and Meier curves estimating the probabilities of survival for male and female mice:

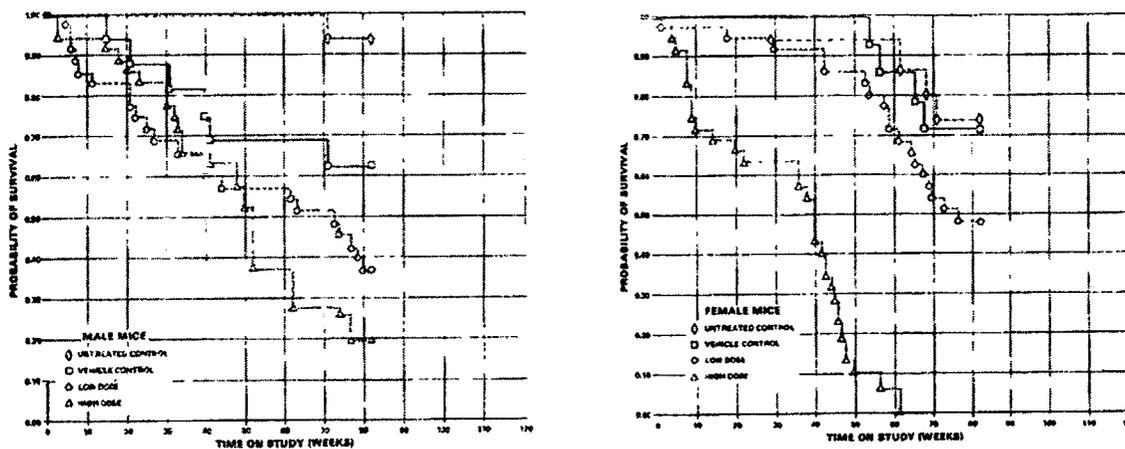


Figure 4. Survival Curves For Mice Treated With 5-Azacytidine

Summary of the results (excerpted from the report):

- In male mice, the result of the Tarone test for positive dose-related trend in mortality over the period is significant ($P < 0.001$), with 20% of the high-dose group, 37% of the low-dose group, 63% of the vehicle controls, and 93% of the untreated controls living to the end of the study. The early deaths of the treated mice may have reduced the occurrence of late-developing tumors.
- In female mice, the result of the Tarone test is also significant ($P < 0.001$), and a departure from linear trend is observed ($P < 0.001$), because of the steep increase in mortality among the high-dose mice, of which all died before the end of the study. Female mice which survived the treatment of high dose of 5-AZ (4.4 mg/kg) were observed only for 9 weeks and killed at Week 61. These early deaths of the high-dose female mice may have suppressed the occurrence of late-developing tumors. Of the low-dose female mice, 49% survived until termination of the study at week 82.
- In male mice, a time-adjusted analysis was performed on those animals living at least 52 weeks. There were 15 such animals in the untreated controls, 11 in the vehicle controls, and 18 in both the low- and high-dose groups.

Clinical signs:

No treatment related clinical signs.

Body weights:

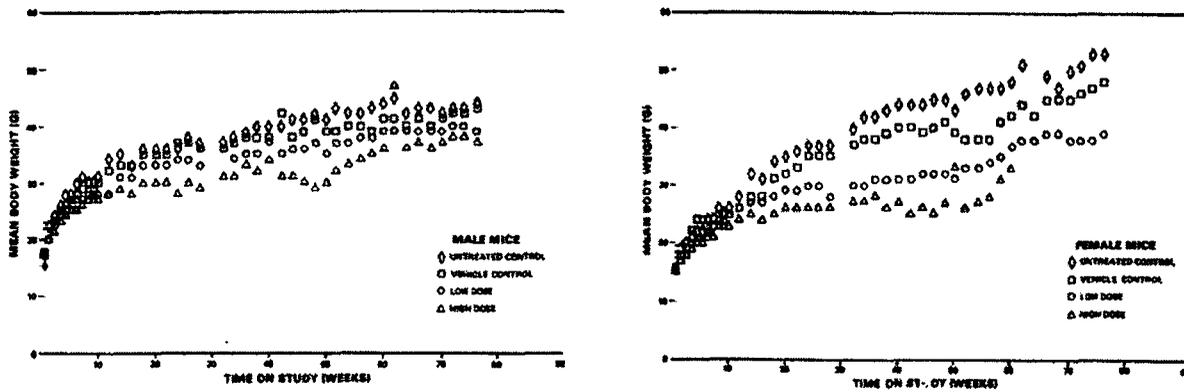


Figure 3 Growth Curves For Mice Treated With 6-Azacythine

Summary of results (excerpted from the report...)

- The mean body weights of the treated mice were dose related throughout the period of the study, and were lower than those of the vehicle controls. When treatment was stopped at week 52, some increases in the mean body weights of the remaining animals occurred. A greater difference occurred between body weights of treated and control females than between those of treated and control males.
- Fluctuations in the growth curve may be due to mortality; as the size of the group diminishes, the mean body weight may be subject to wide variations.

Food consumption: Not obtained.

Gross pathology: No data presented in the result.

Histopathology: The following tables are excerpted from the NCI contractor's report.

Neoplastic:

- Table B1 and B2 are summaries in male and female mice, respectively.
Male mice:

**APPEARS THIS WAY
ON ORIGINAL**

TABLE B1.

**SUMMARY OF THE INCIDENCE OF NEOPLASMS IN MALE MICE
GIVEN INTRAPERITONEAL INJECTIONS OF 5-AZACYTIDINE**

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ANIMALS INITIALLY IN STUDY	15	16	35	35
ANIMALS NECROPSIED	15	13	31	32
ANIMALS EXAMINED HISTOPATHOLOGICALLY	15	13	30	30
RESPIRATORY SYSTEM				
NONE				
RESPIRATORY SYSTEM				
ALVEOLI	(15)	(13)	(29)	(30)
UNDIFFERENTIATED CARCINOMA METAS	1 (7%)			
ALVEOLAR/BRONCHIOLAR ADENOMA		1 (8%)		1 (3%)
HEMATOPOIETIC SYSTEM				
MULTIPLE ORGANS				
SALIG. LYMPHOMA, LYMPHOCYTIC TYPE	(15)	(13)	(31)	(32)
SALIG. LYMPHOMA, HISTIOCYTIC TYPE			4 (13%)	2 (6%)
MEDIASTINAL L. NODE	(15)	(3)	(23)	(20)
UNDIFFERENTIATED CARCINOMA METAS	1 (7%)			
CIRCULATORY SYSTEM				
NONE				
DIGESTIVE SYSTEM				
LIVER				
UNDIFFERENTIATED CARCINOMA	(15)	(13)	(30)	(30)
HEPATOCELLULAR ADENOMA	1 (7%)	1 (8%)	1 (3%)	1 (3%)
GENITAL SYSTEM				
NONE				
NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
NUMBER OF ANIMALS NECROPSIED				

TABLE B1. MALE MICE: NEOPLASMS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ENDOCRINE SYSTEM				
# THYROID ADENOMA, NOS	(11)	(7)	(24) 1 (4%)	(20)
REPRODUCTIVE SYSTEM				
NONE				
NERVOUS SYSTEM				
NONE				
SPECIAL SENSE ORGANS				
* EAR CANAL KERATOACANTHOMA	(15)	(13)	(31)	(32) 1 (3%)
MUSCULOSKELETAL SYSTEM				
NONE				
BODY CAVITIES				
NONE				
ALL OTHER SYSTEMS				
NONE				
ANIMAL DISPOSITION SUMMARY				
ANIMALS INITIALLY IN STUDY	15	16	35	35
NATURAL DEATH		5	18	15
HORBUND SACRIFICE	1	1	4	13
SCHEDULED SACRIFICE				
ACCIDENTALLY KILLED				
TERMINAL SACRIFICE	14	10	13	7
ANIMAL MISSING				
2 INCLUDES AUTOLYZED ANIMALS				
# NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
* NUMBER OF ANIMALS NECROPSIED				

TABLE B1. MALE MICE: NEOPLASMS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
TUMOR SUMMARY				
TOTAL ANIMALS WITH PRIMARY TUMORS*	2	2	6	5
TOTAL PRIMARY TUMORS	2	2	6	6
TOTAL ANIMALS WITH BENIGN TUMORS	1	2	2	2
TOTAL BENIGN TUMORS	1	2	2	3
TOTAL ANIMALS WITH MALIGNANT TUMORS	1		4	3
TOTAL MALIGNANT TUMORS	1		4	3
TOTAL ANIMALS WITH SECONDARY TUMORS#	1			
TOTAL SECONDARY TUMORS	2			
TOTAL ANIMALS WITH TUMORS UNCERTAIN- BENIGN OR MALIGNANT				
TOTAL UNCERTAIN TUMORS				
TOTAL ANIMALS WITH TUMORS UNCERTAIN- PRIMARY OR METASTATIC				
TOTAL UNCERTAIN TUMORS				
* PRIMARY TUMORS: ALL TUMORS EXCEPT SECONDARY TUMORS				
# SECONDARY TUMORS: METASTATIC TUMORS OR TUMORS INVASIVE INTO AN ADJACENT ORGAN				

APPEARS THIS WAY
ON ORIGINAL

Female mice:

TABLE B2.

SUMMARY OF THE INCIDENCE OF NEOPLASMS IN FEMALE MICE
GIVEN INTRAPERITONEAL INJECTIONS OF 5-AZACYTIDINE

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ANIMALS INITIALLY IN STUDY	15	14	35	35
ANIMALS NECROPSIED	15	14	29	30
ANIMALS EXAMINED HISTOPATHOLOGICALLY	15	13	29	30
INTEGUMENTARY SYSTEM				
*SUBCUT TISSUE BASAL-CELL CARCINOMA	(15)	(14)	(29)	(30) 1 (3%)
RESPIRATORY SYSTEM				
#LUNG	(15)	(13)	(28)	(30)
UNDIFFERENTIATED CARCINOMA METAS	1 (7%)			
BASAL-CELL CARCINOMA, METASTATIC			1 (4%)	
ALVEOLAR/BRONCHIOLAR ADENOMA		1 (8%)		
ALVEOLAR/BRONCHIOLAR CARCINOMA		1 (8%)		
SARCOMA, NOS, METASTATIC			1 (4%)	
HEMATOPOIETIC SYSTEM				
*MULTIPLE ORGANS	(15)	(14)	(29)	(30)
MALIG.LYMPHOMA, UNDIFFER-TYPE			1 (3%)	
MALIG.LYMPHOMA, LYMPHOCYTIC TYPE	1 (7%)		4 (14%)	
GRANULOCYTIC LEUKEMIA			1 (3%)	
GRANULOCYTIC SARCOMA			9 (31%)	
#LIVER	(15)	(13)	(29)	(30)
MALIG.LYMPHOMA, LYMPHOCYTIC TYPE			1 (3%)	
*KIDNEY	(15)	(13)	(29)	(29)
MALIG.LYMPHOMA, LYMPHOCYTIC TYPE			1 (3%)	
CIRCULATORY SYSTEM				
#HEART	(14)	(12)	(28)	(30)
SARCOMA, NOS			1 (4%)	
# NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
* NUMBER OF ANIMALS NECROPSIED				

TABLE B2. FEMALE MICE: NEOPLASMS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
DIGESTIVE SYSTEM				
STOMACH				
EPITHELIOCELLULAR ADENOMA	(15) 1 (7%)	(13)	(29)	(30)
LEIOMYOSARCOMA, METASTATIC			1 (3%)	
URINARY SYSTEM				
BLADDER				
ENDOCRINE SYSTEM				
ADRENAL				
LEIOMYOSARCOMA, METASTATIC	(14)	(13)	(27) 1 (4%)	(29)
REPRODUCTIVE SYSTEM				
PRIMARY GLAND				
ADENOCARCINOMA, NOS	(15)	(14)	(29) 2 (7%)	(30)
UTERUS				
SARCOMA, NOS	(15)	(13)	(28) 1 (4%)	(27)
LEIOMYOSARCOMA			2 (7%)	
NERVOUS SYSTEM				
BRAIN				
SENSE ORGANS				
NOSE				
EPITHELIAL CELL CARCINOMA	(15)	(14)	(29) 1 (3%)	(30)
MUSCULOSKELETAL SYSTEM				
BONES				
ARTHRITIS				
LEIOMYOSARCOMA, METASTATIC	(15)	(14)	(29) 1 (3%)	(30)
NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
NUMBER OF ANIMALS NECROPSIED				

TABLE B2. FEMALE MICE: NEOPLASMS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
*PLEURA LEIOMYOSARCOMA, METASTATIC	(15)	(14)	(29) 1 (3%)	(30)
ALL OTHER SYSTEMS				
NONE				
ANIMAL DISPOSITION SUMMARY				
ANIMALS INITIALLY IN STUDY	15	14	35	35
NATURAL DEATH	1	1	14	11
HORBUND SACRIFICE	3	3	4	24
SCHEDULED SACRIFICE				
ACCIDENTALLY KILLED				
TERMINAL SACRIFICE	11	10	17	
ANIMAL MISSING				
‡ INCLUDES AUTOLYZED ANIMALS				
TUMOR SUMMARY				
TOTAL ANIMALS WITH PRIMARY TUMORS*	2	2	22	1
TOTAL PRIMARY TUMORS	2	2	24	1
TOTAL ANIMALS WITH BENIGN TUMORS	1	1		
TOTAL BENIGN TUMORS	1	1		
TOTAL ANIMALS WITH MALIGNANT TUMORS	1	1	22	1
TOTAL MALIGNANT TUMORS	1	1	24	1
TOTAL ANIMALS WITH SECONDARY TUMORS‡	1		3	
TOTAL SECONDARY TUMORS	1		6	
TOTAL ANIMALS WITH TUMORS UNCERTAIN-BENIGN OR MALIGNANT				
TOTAL UNCERTAIN TUMORS				
TOTAL ANIMALS WITH TUMORS UNCERTAIN-PRIMARY OR METASTATIC				
TOTAL UNCERTAIN TUMORS				
* PRIMARY TUMORS: ALL TUMORS EXCEPT SECONDARY TUMORS				
‡ SECONDARY TUMORS: METASTATIC TUMORS OR TUMORS INVASIVE INTO AN ADJACENT ORGAN				

Summary of histopathological findings on neoplasm:

- The administration of 5-azacytidine at the doses used was carcinogenic in the hematopoietic system in the B6C3F1 hybrid female mouse. This carcinogenic effect was manifested by the induction of lymphocytic and granulocytic neoplasms in the low-dose females (17/29). The high number of early deaths in high dose females (none surviving to study termination) and low survival rate in treated males precluded a complete evaluation of these groups.
- Several neoplasms of the hematopoietic system (both lymphocytic and granulocytic neoplasms) involving multiple organs and tissues of the spleen, lymph node, liver, and lung were present in both the male and female mice.
- In general, the hematopoietic neoplasms present in the low-dose groups were poorly differentiated, and "precise classification of cell type was extremely difficult" (the author's statement). The neoplasms observed histologically were classified into the following cell types: malignant lymphoma, lymphocytic type; malignant lymphoma, histiocytic type; malignant lymphoma, undifferentiated type; granulocytic sarcoma; and granulocytic leukemia.

- The statistical conclusion is that there is an association between the incidence of hematopoietic tumors and the administration at the low dose of 5-azacytidine to female mice. In male mice, even after time-adjusted analyses (eliminating animals that died before week 52) were performed on the incidences of lymphoma of the hematopoietic system, the incidences still remain not significant. There was no incidence of tumors at any specific site that is statistically significant in male mice. See following tables.

Male mice:

<u>Topography: Morphology</u>	<u>Vehicle Control</u>	<u>Low Dose</u>	<u>High Dose</u>
Hematopoietic System: Lymphoma ^b	0/13 (0)	4/31 (13)	3/32 (9)
P Values ^{c,d}	--	N.S.	N.S.
Relative Risk (Vehicle Control) ^e		Infinite	Infinite
Lower Limit		0.421	0.265
Upper Limit		Infinite	Infinite
<u>Weeks to First Observed Tumor</u>	--	73	74

^aTreated groups received doses of 2.2 or 4.4 mg/kg by injection three times per week for 52 weeks.

^bNumber of tumor-bearing animals/number of animals examined at site (percent).

^cBeneath the incidence of tumors in a treated group is the probability level for the Fisher exact test for the comparison of that treated group with the vehicle-control group when P < 0.05; otherwise, not significant (N.S.) is indicated.

^dSince survival in the high-dose group was short, the tests for dose-related trend are not reported.

^eThe 95% confidence interval of the relative risk between each treated group and the control group.

Female mice:

<u>Topography: Morphology</u>	<u>Vehicle Control</u>	<u>Low Dose</u>	<u>High Dose</u>
Multiple Sites: Lymphoma ^b	0/14 (0)	7/29 (24)	0/30 (0)
P Values ^{c,d}	--	P = 0.048	N.S.
Relative Risk (Vehicle Control) ^e		Infinite	--
Lower Limit		1.010	--
Upper Limit		Infinite	--
<u>Weeks to First Observed Tumor</u>	--	43	--
Hematopoietic System: Granulocytic Leukemia or Sarcoma ^b	0/14 (0)	10/29 (34)	0/30 (0)
P Values ^{c,d}	--	P = 0.010	N.S.
Relative Risk (Vehicle Control) ^e		Infinite	--
Lower Limit		1.554	--
Upper Limit		Infinite	--
<u>Weeks to First Observed Tumor</u>	--	53	--

(continued)

<u>Topography: Morphology</u>	<u>Vehicle Control</u>	<u>Low Dose</u>	<u>High Dose</u>
Hematopoietic System: Lymphoma, Granulocytic Leukemia, or Sarcoma ^b	0/14 (0)	17/29 (59)	0/30 (0)
P Values ^{c,d}	--	P < 0.001	N.S.
Relative Risk (Vehicle Control) ^e		Infinite	--
Lower Limit		2.848	--
Upper Limit		Infinite	--
<u>Weeks to First Observed Tumor</u>	--	43	--
Mammary Gland: Adenocarcinoma ^b	0/14 (0)	2/29 (7)	0/30 (0)
P Values ^{c,d}	--	N.S.	N.S.
Relative Risk (Vehicle Control) ^e		Infinite	--
Lower Limit		0.153	--
Upper Limit		Infinite	--
<u>Weeks to First Observed Tumor</u>	--	61	--

(continued)

<u>Topography: Morphology</u>	<u>Vehicle Control</u>	<u>Low Dose</u>	<u>High Dose</u>
Uterus: Sarcoma, NOS, or Leiomyosarcoma ^b	0/13 (0)	3/28 (11)	0/27 (0)
P Values ^{c,d}	--	N.S.	N.S.
Relative Risk (Vehicle Control) ^e		Infinite	--
Lower Limit		0.300	--
Upper Limit		Infinite	--
<u>Weeks to First Observed Tumor</u>	--	81	--

^aTreated groups received doses of 2.2 or 4.4 mg/kg by injection three times per week for 52 weeks.

^bNumber of tumor-bearing animals/number of animals examined at site (percent).

^cBeneath the incidence of tumors in a treated group is the probability level for the Fisher exact test for the comparison of that treated group with the vehicle-control group when P < 0.05; otherwise, not significant (N.S.) is indicated.

^dSince survival in the high-dose group was short, the tests for dose-related trend are not reported.

^eThe 95% confidence interval of the relative risk between each treated group and the control group.

Non-neoplastic:

- Table D1 and D2 are summaries in male and female rats, respectively.

Male mice:

TABLE D1.

SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS IN MALE MICE GIVEN INTRAPERITONEAL INJECTIONS OF 5-AZACYTIDINE

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ANIMALS INITIALLY IN STUDY	15	16	35	35
ANIMALS NECROPSIED	15	13	31	32
ANIMALS EXAMINED HISTOPATHOLOGICALLY	15	13	30	30
DERMATOLOGY SYSTEM				
EPIDERMAL INCLUSION CYST	(15) 1 (7%)	(13)	(31)	(32)
RESPIRATORY SYSTEM				
INFLAMMATION, INTERSTITIAL	(15)	(13) 1 (8%)	(29) 4 (14%)	(30)
BRONCHOPNEUMONIA SUPPURATIVE			1 (3%)	
PERIVASCULITIS			1 (3%)	
HYPERPLASIA, ALVEOLAR EPITHELIUM				1 (3%)
HEMATOPOIETIC SYSTEM				
BONE MARROW	(15)	(13) 1 (8%)	(29) 3 (10%)	(25)
ATROPHY, NOS				
SPLEEN	(15)	(13)	(26)	(28)
HYPERPLASIA, HEMATOPOIETIC			2 (8%)	
HYPERPLASIA, RETICULUM CELL			1 (4%)	1 (4%)
HYPERPOIESIS		1 (8%)		
THYMIC L. NODE	(15)	(3)	(23)	(20)
INFLAMMATION, SUPPURATIVE			1 (4%)	
INFLAMMATION, HEMORRHAGIC			1 (4%)	
CIRCULATORY SYSTEM				
NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
NUMBER OF ANIMALS NECROPSIED				

TABLE D1. MALE MICE: NONNEOPLASTIC LESIONS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
DIGESTIVE SYSTEM				
*LIVER	(15)	(13)	(30)	(30)
PERITYASCOLITIS			1 (3%)	
NECROSIS, NOS			1 (3%)	
NECROSIS, COAGULATIVE			1 (3%)	
NECROSIS, CENTRAL			1 (3%)	
HYPERPLASIA, NODULAR	2 (13%)			
HYPERPLASIA, HEMATOPOIETIC			1 (3%)	
URINARY SYSTEM				
*KIDNEY	(15)	(13)	(28)	(29)
PYELONEPHRITIS, ACUTE/CHRONIC			1 (4%)	
INFLAMMATION, CHRONIC		1 (8%)		
*U. BLADDER/SUBMUCOSA	(14)	(12)	(26)	(21)
HEMORRHAGE		1 (8%)		
ENDOCRINE SYSTEM				
NONE				
REPRODUCTIVE SYSTEM				
*SEMINAL VESICLE	(15)	(13)	(31)	(32)
INFLAMMATION, SUPPURATIVE		1 (8%)		
NERVOUS SYSTEM				
NONE				
SPECIAL SENSE ORGANS				
NONE				
MUSCULOSKELETAL SYSTEM				
NONE				
# NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
* NUMBER OF ANIMALS NECROPSIED				

TABLE D1. MALE MICE: NONNEOPLASTIC LESIONS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
THORACIC CAVITIES				
*PERITONEUM	(15)	(13)	(31)	(32)
INFLAMMATION, CHRONIC		1 (8%)		1 (3%)
*MESENTERY	(15)	(13)	(31)	(32)
NECROSIS, FAT				1 (3%)
OTHER SYSTEMS				
NONE				
MORPHOLOGY SUMMARY				
NO LESION REPORTED	10	9	13	22
AUTO/NECROPSY/NO HISTO			1	2
AUTOLYSIS/NO NECROPSY		3	4	3
# NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
* NUMBER OF ANIMALS NECROPSIED				

Female mice:

TABLE D2.

SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS IN FEMALE MICE GIVEN INTRAPERITONEAL INJECTIONS OF 5-AZACYTIDINE

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ANIMALS INITIALLY IN STUDY	15	14	35	35
ANIMALS NECROPSIED	15	14	29	30
ANIMALS EXAMINED HISTOPATHOLOGICALLY	15	13	29	30
INTEGUMENTARY SYSTEM				
NONE				
RESPIRATORY SYSTEM				
#LUNG	(15)	(13)	(28)	(30)
INFLAMMATION, INTERSTITIAL	2 (13%)	1 (8%)	1 (4%)	
HYPERPLASIA, LYMPHOID	3 (20%)		2 (7%)	
HEMATOPOIETIC SYSTEM				
#BONE MARROW	(13)	(13)	(28)	(29)
ATROPHY, NOS				10 (34%)
#SPLEEN	(12)	(13)	(29)	(29)
HYPERPLASIA, HEMATOPOIETIC		1 (8%)	1 (3%)	1 (3%)
HYPERPLASIA, RETICULUM CELL			1 (3%)	
HYPERPLASIA, LYMPHOID			2 (7%)	
#MESENTERIC L. NODE	(10)	(9)	(24)	(23)
HYPERPLASIA, RETICULUM CELL			1 (4%)	
#THYMUS	(7)	(8)	(10)	
HYPERPLASIA, HEMATOPOIETIC			1 (10%)	
CIRCULATORY SYSTEM				
#HEART	(14)	(12)	(28)	(30)
PERIARTERITIS	1 (7%)			
DIGESTIVE SYSTEM				
#LIVER	(15)	(13)	(29)	(30)
NECROSIS, FOCAL				1 (3%)
NECROSIS, COAGULATIVE				1 (3%)
HYPERPLASIA, FOCAL			1 (3%)	
# NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY * NUMBER OF ANIMALS NECROPSIED				

Summary of histopathological findings on non-neoplasm in mice: Among 35/sex/group treated mice, few of them (Vehicle control: 2 males and 1 female, LD: 4 males and 6 females, HD: 3 males and 5 females) were not necropsied, due to autolysis.

- Several inflammatory, degenerative, and proliferative lesions commonly seen in B6C3F1 hybrid mice occurred with approximately equal frequency in treated and control animals. The most common of these lesions were interstitial pneumonia with perivascular and peribronchiolar lymphocytic hyperplasia and extramedullary hematopoiesis in the spleen.
- Liver: necrosis, observed occasionally.
- Mice that died prior to termination of the study had no consistent lesions, except bone-marrow atrophy in high-dose females, that would account for the early deaths.

Toxicokinetics: n/a

Summary of individual study:

- The administration of 5-azacytidine, on dose schedule of 2.2 mg/kg x3/wk for 52 weeks, induced tumors of the hematopoietic system in low dose female B6C3F1 mice. No high dose (4.4 mg/kg) females survived to study termination.
- Under the conditions of this bioassay, the short life span and short duration of treatment of male B6C3F1 mice precluded evaluation of the carcinogenicity of 5-azacytidine in these groups.
- Liver necrosis and bone marrow atrophy were the main non-neoplastic lesions in rats and mice in this study.

Rats:

Study title: The tumorigenicity of 5-azacytidine in the male Fisher rat. *Carcinogenesis*, 5(12): 1583-1590, 1984 (Carr *et al.*).

Key study findings:

- 5-Azacytidine at 2.5 or 10 mg/kg, twice weekly for 9 months induced tumors in testes.

Study no.: Not applicable

Volume #, and page #: Module 4.2.3.4.2.1

Conducting laboratory and location: published article

Date of study initiation: published 1984

GLP compliance: No

QA report: No

Drug, lot #, and % purity: 5-Azacytidine (Sigma Chemical Co., St. Louis, MO)

Other reagents: Diethylnitrosamine (DEN, Eastman Chemical Co.), phenobarbital (Mallinkrodt Chemical Co.), 2-acetylaminofluorene (AAF, Aldrich Chemical Co.).

CAC concurrence: No

Study Type:

- Hepatic tumor initiation: single dose of 5-AZ in male rats, IP injection.
- Hepatic tumor promotion: 9 months in male Fisher rats, IP injection.
- Test for complete carcinogenesis: 9 months in male Fisher rats, IP injection.
- All surviving rats were sacrificed 18 months after the start of the experiment, except rats in Regimen 3A which were sacrificed 12 months after the start of the experiment.

Species/strain: Male Fisher (F344) Rats, because of the relatively low incidence of spontaneous hepatocellular carcinomas (HCC).

Number/sex/group; age at start of study:

- N=12 in all Regimen groups/subgroups excepting Regimens 6 and 7 (See Figure 1 footnote).
- Controls: DEN control (Regimen 6): n=6
Phenobarbital control (Regimen 7): n=6
- Weight at start of study: 160-180 g

Animal housing: Not reported.

Formulation/vehicle: 5-Azacytidine in sterile saline solution.

Drug stability/homogeneity: 5-Azacytidine for injection was used immediately after preparation of the solution, because it relative instability. No information was provided in regard of stability or homogeneity.

Methods

Doses:

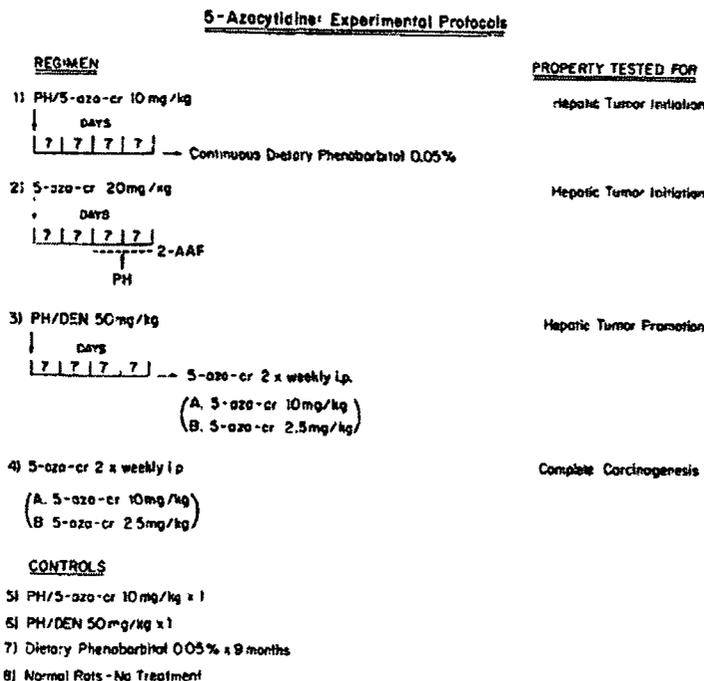


Fig. 1. Experimental protocols for 5-azacytidine treatment. 5-Aza-cr, 5-Azacytidine; PH, partial hepatectomy; 2-AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine.

Footnote: (paragraphs excerpted from the article)

- **In Regimen 1, 5-AZ (10 mg/kg) was administered IP 18 h after a partial hepatectomy (PH). Twenty-eight days after this procedure, the rats were placed on a dietary phenobarbital, 0.05% (w/w), for a subsequent 9 months. Dietary phenobarbital is a tumor promoter in experimental chemical hepatocarcinogenesis when it is administered chronically after an acute dose of a hepatic tumor initiator.**
- **In Regimen 2, a single dose of 5-AZ (20 mg/kg) was administered IP without a preceding PH. After a two week rest period, the rats were fed dietary AAF, 0.02% w/w, for two weeks only, and one week after the start of feeding AAF, a PH was performed. This protocol has been used as a procedure for inducing the selective growth of putatively initiated cells in chemical hepatocarcinogenesis studies after a variety of initiating carcinogens.**
- **In Regimen 3, rats were administered DEN, 50 mg/kg, in saline 18 h after a PH. This procedure is known to cause hepatic tumor initiation and will result in tumorigenesis if the animals are subsequently administered a chronic promoting regimen such as phenobarbital in the diet. In Regimen 3A and 3B, 10 mg/kg and 2.5 mg/kg (twice weekly for 9 months) of 5-AZ, respectively, was used as such chronic regimen to test the ability of 5-AZ as a hepatic tumor promoter.**
- **In Regimen 4, 5-AZ was administered for 9 months at either 10 mg/kg (4A) or 2.5 mg/kg (4B) IP twice weekly in saline.**
- **In Regimen 5 (5-AZ control), rats were given a single dose of 5-AZ, 10 mg/kg, 18 h after a PH followed by no further procedure.**
- **In Regimen 6 (DEN control), rats were given DEN, 50 mg/kg, 18 h after a PH followed by no further procedure.**
- **In Regimen 7 (phenobarbital control), rats were given dietary phenobarbital 0.05%, for 9 months.**
- **In Regimen 8 (age control), rats were fed standard laboratory chow and received neither DEN, nor 5-AZ, nor phenobarbital.**
- **Except for rats in Regimen 3A (sacrificed at 12 months, when gross hepatic malignancies were observed at laparotomy) all surviving rats were sacrificed at 18 months after the start of experiment.**

Basis of dose selection: based on MTD in acute dose study.

IP injection of 5-azacytidine to normal rats (160-180 gm) was used to search for the maximum tolerable acute dose. 5-Azacytidine was administered intraperitoneally at 10, 20, 30, 40 and 50 mg/kg (n=12/dose). All rats at 50 mg/kg and 40 mg/kg died within 48 hours. Eleven out of 12 rats survived 30 mg/kg and all rats survived at lower dose. Thus for chronic administration of 5-AZ, two dose levels were chosen: 10 mg/kg and 2.5 mg/kg to be administered twice weekly.

Route of administration: IP injection.

Frequency of drug administration: Single dose or twice per week (see above).

Dual controls employed: Age control (no treatment) and positive control (see above).

Satellite groups used for toxicokinetics or special groups: None

Restriction paradigm for dietary restriction studies: n/a

Interim sacrifices: n/a

Deviations from original study protocol: not reported.

Statistical methods: Not provided.

Observation times

Clinical signs:

The information about observation for signs of toxicity and moribundity is not provided. However, it was indicated that: “after the administration of various regimens, selected rats from each protocol were examined by laparotomy under light ether anesthesia, but not sacrificed, every three months to look for the appearance of internal malignancies.”

Body weights: Detailed information was not available about body weight measurement.

According to Figure 2, the observation time was: at the beginning of the study, and in 1, 2, 4, 7, 9, 12 months and at the end of observation period 18 months

Food consumption: Not obtained

Hematology: Not obtained

Clinical chemistry: Not obtained

Organ weight: Not obtained

Gross pathology: A routine post mortem examination was performed on 74 rats.

Macroscopic examination of major organ systems was performed and abnormal tissues were subjected to microscopic examination. Also see above “Clinical signs”.

Histopathology: Peer review: yes (x), no ()

See above “gross pathology”.

Toxicokinetics: n/a

Results

Mortality: the table below summarizes the mortality in each group:

Regimen	1	2	3A	3B	4A	4B	5	6	7	8
Property examined	Hepatic tumor initiation	Hepatic tumor initiation	Hepatic tumor promotion	Hepatic tumor promotion	Complete carcinogenesis	Complete carcinogenesis	5-AZ single dose	DEN control	Pheno-barbital control	Age control
Dose (mg/kg)	10	20	10	2.5	10	2.5	10	50 (DEN)	0.05% (pheno-barbital)	None
No. died	0	6	2	2	4	0	0	0	0	0

Footnote:

- Each group started with 12 rats excepting Regimens 6 and 7, in which 6 rats were used.
- The difference between 12 or 6 and the number of rats in the number examined column in Table 1 (see below), represents the number of rats died before the end of the experiment.

Clinical signs: Not reported.

Body weights:

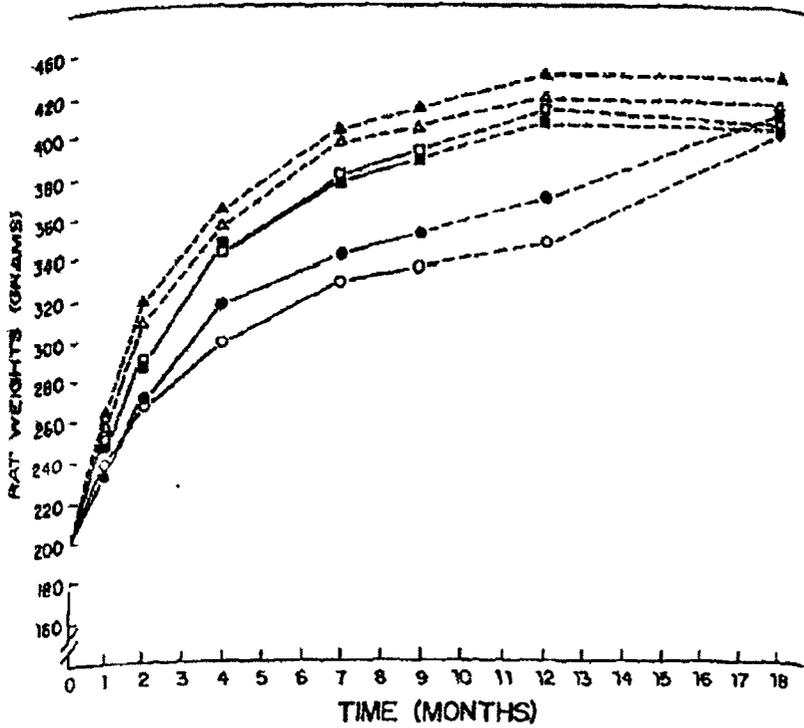


Fig. 2. Increase in rat body weight.

- (○) Azacytidine at 10 mg/kg bi-weekly, regimen 4A;
- (●) PH/DEN - azacytidine 10 mg/kg bi-weekly, regimen 3A;
- (□) Azacytidine 2.5 mg/kg bi-weekly, regimen 4B;
- (■) PH/DEN - azacytidine 2.5 mg/kg bi-weekly, regimen 3B;
- (△) PH/azacytidine once only, regimen 5;
- (▲) PH/azacytidine - phenobarbital diet, regimen 1

No information about body weight of rats in Regimens 2, 6, 7 or 8.

Weight gain was depressed in the two HD groups (i.e., Regimens 3A and 4A)

Food consumption: Not obtained.

Gross pathology: No data presented in the result.

Histopathology:

Table I. 5-Azacytidine-induced neoplasms in the male F344 rat

Regimen	Property examined	Number of rats with:			No. of rats with tumors No. of rats examined
		Hepatic tumors	Testis tumors	Other tumors	
1	Hepatic tumor initiation	0	1	1	2/12
2	Hepatic tumor initiation	0	0	0	0/6
3A	Hepatic tumor promotion	8	0	1	8/10
3B	Hepatic tumor promotion	2	2	5	7/10
4A	Complete carcinogenesis	0	1	5	5/8
4B	Complete carcinogenesis	0	9	2	7/12
5	5-Azacytidine single dose	0	3	1	2/12
6	DEN control	0	0	0	0/6
7	Phenobarbital control	0	0	0	0/6
8	Old age control	0	0	0	0/12

Total no. of 5-azacytidine rats evaluated 70
 Total no. of 5-azacytidine rats with tumors 31
 Total no. of primary tumors in 5-azacytidine rats 41
 Total no. of control rats with tumors 0/24

Table II. Spectrum of 5-azacytidine-induced tumor types

Regimen	Property examined	Tumors
1	Tumor initiation (PB)	Skin (1); testis (1)
2	Tumor initiation (Solt and Farber)	0
3A	Tumor promotion 10 mg/kg ^a	Renal (1); HCC (8)
3B	Tumor promotion 2.5 mg/kg ^a	Lung (1); testis (2); renal (2); mesothelioma (1); HCC (2); leukemia (1)
4A	Complete carcinogenesis 10 mg/kg ^a	Skin (3); lung (1); testis (1); leukemia (1)
4B	Complete carcinogenesis 2.5 mg/kg ^a	Testis (9); reticulo-endotheliosis (1); lung (1)
5	PII/5-Aza-C x 1 dose	Skin (1); testis (3)
6	Control	0
7	Control	0
8	Control	0

Number given in parenthesis is the number of rats with tumors.

^aDose of 5-azacytidine.

Note: the left most column indicates Regimen, from top to bottom: 1, 2, 3A, 3B, 4A, 4B, 5, 6, 7 and 8.

- The tumor initiator, DEN or the chronic tumor promoter, phenobarbital by itself was not tumorigenic. There were no tumors found in rats in the age control group up to 18 months after start of the experiment.
- Under the experimental scheme, 5-AZ was not a hepatic tumor initiator. Of the two rats found with tumors in Regimen 1 (initially single dose of 5-AZ followed with chronic dietary phenobarbital), one developed a squamous cell cancer of the skin at the site of injection and the other an interstitial cell tumor of the testis.
- After the initiation of treatment with DEN, 5-AZ at high dose (10 mg/kg, twice weekly, Regimen 3A) was found to be a tumor promoter. At twelve months after the start of the experiment, hepatic tumors were seen at laparotomy, and all the rats in the group of Regimen 3A were sacrificed. Table II (above) shows that 8 of 10 rats had HCC. No metastases from HCC were found in the lung or any other organ. In addition, one rat had clear cell adenocarcinoma of the kidney. The low dose of 5-AZ (2.5 mg/kg twice weekly, Regimen 3B) did not induce

tumorigenesis at 12 months, so the experiment was continued until sacrifice at 18 months. At sacrifice, 2 rats were found to have HCC, and 8 rats had a variety of other tumors including: clear cell carcinoma of the kidneys (2), interstitial cell tumor of the testes (2), abdominal mesothelioma (1), acute leukemia (1) and adenocarcinoma of the lung (1). Two rats had multiple primary tumors; one had HCC, renal cell carcinoma and an interstitial cell testicular tumor, while the other had acute leukemia, renal cell cancer and an interstitial cell testicular tumor.

- The ability of 5-AZ to be a complete carcinogen was tested at two dose levels: IP of 10 and 2.5 mg/kg, twice weekly for nine months (Regimens 4A and 4B, respectively). No mortality was observed and no rats had liver tumors at 12 months. However, by 18 months, several skin tumors were found on the abdomen around the sites of the IP injections. Tumors found in the Regimen 4A group included: skin tumors (squamous cell carcinoma (2) and skin appendage tumors (1)), acute leukemia (1), adenocarcinoma of the lung (1), interstitial cell testicular tumor (1). Tumors found in Regimen 4B group included: interstitial cell testicular tumors (9), malignant reticuloendotheliosis (1) and adenocarcinoma of the lung (1). Thus under the experimental conditions, 5-AZ was considered to be a complete carcinogenic.
- Two rats in the 5-AZ control group (single dose of 5-AZ at 10 mg/kg after PH, Regimen 5) were found with tumors: one squamous cell carcinoma of the skin and three interstitial cell testicular tumors.
- Histologic evaluation of tumors from all regimens:
 - HCC: the common histologic features of the neoplasms included:
 1. They consisted of large unencapsulated, but well-circumscribed, nodular areas in which lobular architecture was distorted.
 2. Portal triads were absent in this area and hepatic cords were more than three cells thick and the carcinoma had a “trabecular” appearance.
 3. Areas of necrosis were identified. In some areas cords and trabecula of neoplastic cells extended from the main tumor mass into the adjacent parenchyma.
 4. Foci of extramedullary hematopoiesis and areas of cystic degeneration were evident within both neoplastic and non-neoplastic parenchyma.
 - Leukemia/lymphoma: found in 2/22 rats (one in Regimen 3B and the other Regimen 4A).
 1. Spleen: There was total obliteration of splenic architecture by a monomorphous proliferation of mononuclear cells having scanty cytoplasm, irregular nuclear contours, and frequent mitotic figures. It was demonstrated that these cells were of lymphoid rather than granulocytic origin. However, it was not sure that the neoplastic cells were leukemic.
 2. Liver: the neoplasm consisted of a proliferation of small immature mononuclear cells within portal tracts, adjacent to central veins and randomly within sinusoids. The neoplastic cells were not of granulocytic differentiation. Leukemic infiltrates were found in the portal tracts in the rat of the 3B group.
 3. Lung: The rat from 3B showed pulmonary involvement by an acute mononuclear cell leukemia/lymphoma.

- **Histiocytic proliferative disease:**
It was found in spleen of one rat in Regimen 4B with the feature of cells of irregular, enlarged or reniform nuclei and moderate amounts of pale cytoplasm, and absence of small mature lymphocytes. The morphologic appearances are similar to those associated with malignant histiocytosis in humans and possibly not mast cell disease or myeloid leukemia. Also in liver of this rat, abnormality included a poorly defined reticuloendothelial proliferation within sinusoids characterized by increased numbers of atypical Kupffer cells.
- **Renal cell carcinoma:** One rat from 3A and two rats from 3B was found with RCC. In two rats the neoplasm consisted of solid sheets of uniform cells having abundant clear cytoplasm, while the carcinoma in the other animal had a more alveolar and papillary configuration with extensive areas of necrosis. Many kidneys also showed chronic pyelonephritis of varying degrees of severity.
- **Pulmonary carcinomas:** found in one rat from 3B and one from 4A. The adenocarcinoma had morphologic features consistent with the bronchiolo-alveolar type. In these neoplasms, cuboidal to low columnar neoplastic cells lined the alveolar septa giving a glandular appearance. The carcinomas were extensive and occasionally invasive to the pleural surface and the chest wall.
- **Testicular tumors:** Many treated rats were found with this neoplasm: Regimen 1 (1), 3b (2), 4A (1), 4B (9) and 5 (3). Many of the neoplasms had areas showing proliferations of both atypical Leydig and Sertoli cells and in some tumors, undifferentiated areas were present suggesting mixed gonadal stromal tumors.
- **Skin and appendage tumors:** found in three animals of Regimen group 4A. Two were with invasive squamous cell carcinoma. The third rat had a skin appendage tumor showing pilar, sebaceous and basal differentiation as well as basal cell proliferation were evident. A similar tumor of skin appendage origin was present in one rat from Regimen 5.
- **Mesothelioma:** found in one rat from Regimen 3B. the mesothelioma involved peritoneal surfaces of the pancreas, abdominal wall soft tissues as well as along the spermatic cord surface. The mesothelioma consisted of delicate fronds showing a proliferation of cuboidal cells over delicate stromal cores.
- **Possible mechanism of 5-AZ induced carcinogenesis:** It has been postulated that 5-AZ induced hypomethylation of DNA is the mechanism of 5-AZ's tumorigenicity. In this article a restriction enzyme analysis of the methylation of tumor and normal tissue DNA samples was employed to test this hypothesis. Nearly all mammalian DNA methylation occurs at cytosine in the 5' CG dimer. The restriction enzyme Hpa II is inhibited by methylation at CCGG sites whereas Msp I is not, thus an increase in Hpa II cleavage frequency (f_{Hpa}) indicated less methylation of the DNA at 5' CCGG sites. The ratio of the cleavage frequency of Hpa II to that of Msp I (f_{Hpa}/f_{Msp}) is a measure of the relative number of unmethylated 5' CCGG sites in a given tissue.

Table III. Methylation analysis at CCGG sites in tumor DNAs

DNA source	Regimen	Cleavage frequencies			Normal Tumor
		$f_{Hpa} \times 10^4 bp^{-1}$	$f_{Msp} \times 10^4 bp^{-1}$	f_{Hpa}/f_{Msp}	
Normal spleen	8	2.2	10.9	0.25	1
Spleen tumor A	4A	8.2	9.4	0.93	0.3
Spleen tumor B	4B	3.7	8.4	0.42	0.6
Normal testis	8	2.3	10.9	0.26	1
Testis tumor	4B	2.3	8.4	0.26	1
Testis tumor	4B	2.9	9.1	0.33	0.8
Normal liver	8	2.8	6.7	0.31	1
Liver tumor	3A	6.3	8.8	0.72	0.4
Liver tumor	3A	4.8	6.5	0.54	0.6
Av 8.8					

High molecular weight DNA was prepared from selected tumor types and digested to completion with *HpaII* or *MspI* and size fractionated by agarose gel electrophoresis as previously described (10). Number average molecular weights for each digest were determined from scans of gel photographs (10). Cleavage frequencies (f_{Hpa} or f_{Msp}) are the reciprocal of the number average base pair (bp) values obtained. f_{Msp} is the average

A, Acute leukemia.

B, Malignant reticuloendotheliosis.

As shown in the table above, the two spleen tumors and the two liver tumors tested are hypomethylated relative to their control tissues (f_{Hpa}/f_{Msp} ratio less than 1). The testis tumors are not significantly different in this regard from normal testis tissue. The data of HPLC analysis, which measures total 5-methylcytosine content in DNA, supports the restriction enzyme analysis result. Thus it is suggested that the mechanism underlying carcinogenesis of 5-AZ is hypomethylation of DNA.

Summary of individual study:

5-Azacytidine (10 mg/kg or 2.5 mg/kg, twice weekly for 9 months) was a complete carcinogen, inducing tumorigenesis in various organs. Of these, only testicular tumors in low dose males were associated with 5-azacytidine treatment alone (9/12 animals). Other tumors observed with 5-azacytidine treatment alone or in other regimens included skin, lung, liver, spleen, and kidney, in the forms of adenocarcinoma, leukemia/lymphoma or malignant histiocytosis. 5-Azacytidine also promoted hepatocellular carcinoma with pretreatment of partial hepatectomy and DEN (50 mg/kg). Hypomethylation of DNA is suggested as the mechanism of carcinogenicity of 5-azacytidine.

Study title: Bioassay of 5-azacytidine for possible carcinogenicity: Carcinogenesis testing Program, National Cancer Institute, NIH, Bethesda, MD; 1978.

Key study findings:

- Under the conditions of this study, the short life span and short duration of treatment of Sprague-Dawley rats of either sex precluded evaluation of the carcinogenicity of 5-azacytidine in these groups.

Study no.: NCI Report number: NCI-CG-TR-42; DHEW/PUB/NIH-78-842

Volume #, and page #: Module 4.2.3.4.1.1

Conducting laboratory and location: The study was conducted by Southern Research Institute, Birmingham, AL, initially under direct contract to NCI and then under a subcontract to Tracor Jitco, Inc., prime contractor for the NCI Carcinogenesis Testing Program.

Date of study initiation: Not provided

GLP compliance: No

QA report: No

Drug, lot #, and % purity: 5-Azacytidine (Ash-Stevens, Inc., Detroit, MI), Lot No. AP-V-128, purity > 99%.

CAC concurrence: No

Study Type:

- Dose-determining study: 45 days in female Sprague-Dawley rats, IP, then observed for 45 days.
- 34 weeks in Sprague-Dawley rats, IP, then observed for 46 or 47 weeks (total 80 or 81 weeks).

Species/strain: Sprague-Dawley rats were obtained from Charles River Laboratories, Wilmington, MA.

Number/sex/group; age at start of study:

- Dose-determining study: 5/sex/group of treated animals, 10 animals in untreated control group and 10 animals used as vehicle (saline) controls. Ages of animals when dose-determining, 45-day studies started were not reported. However, based on the age on arrival at the laboratory and the quarantine time, they should be approximate to those in the main study (see below).
- Main study: 35 rats/sex/group, 15 rats/sex in untreated control group and 15 rats/sex used as vehicle (saline) controls. Male rats and their controls were 35 days of age when placed on study; females were 42 days of age.

Animal housing: five rats/cage.

Formulation/vehicle: 5-Azacytidine at the concentration of 0.1 or 0.2% was prepared in buffered saline (pH 6.9).

Drug stability/homogeneity:

The powdered 5-AZ was stored at 5°C in small bottles enclosed in sealed plastic bags containing Drierite®. However, no analyses of stability, homogeneity, or achieved concentrations were carried on the preparations of the test or positive control substances. Aqueous solutions of 5-AZ were not stored, because they are unstable at room temperature. The drug and the vehicle were mixed in a 10 ml glass Potter-

Elvehjem tissue grinder with a Teflon pestle. Fresh solutions in exact amounts for administration were prepared preceding injection.

Methods

Doses:

- Dose-determining, 45-day study:
 - First study: 0.13, 0.33, 0.65, 1.3, and 2.6 mg/kg.
 - Second study: 2.6, 5.2, 10.4, and 20.8 mg/kg.
- Main study:
 - 2.6 and 5.2 mg/kg three times per week for 34 weeks (low and high dose, respectively).

Table 1. Design of Chronic Studies of 5-Azacytidine in Rats

Sex and Treatment Group	Initial No. of Animals ^a	5-Azacytidine Dose ^b (mg/kg)	Time on Study	
			Treated ^c (weeks)	Untreated (weeks)
<u>Male</u>				
Untreated-Control	15	0		81
Vehicle-Control	15	0 ^d	34	46-47
Low-Dose	35	2.6	34	46
High-Dose	35	5.2	34	10 ^e
<u>Female</u>				
Untreated-Control	15	0		81
Vehicle-Control	15	0 ^d	34	47
Low-Dose	35	2.6	34	46
High-Dose	35	5.2	34	11 ^e

^aMale rats and their controls were 35 days of age when placed on study; females were 42 days of age.

^b5-Azacytidine was administered in buffered saline by intraperitoneal injection three times per week at a volume of 0.25 ml/100 g body weight. Doses were based on individual weights.

^cTreatment terminated at 34 weeks rather than at 52 weeks because of high mortality in the high-dose groups.

^dVehicle controls received only buffered saline solution, at the same volume as treated rats.

^eThe remaining high-dose males were killed at week 44 and high-dose females at week 45.

Basis of dose selection: Based on low and high doses in 45-day studies to estimate the MTD of 5-AZ.

Route of administration: IP injection.

Frequency of drug administration: 3x/wk

Dual controls employed: untreated and saline controls

Satellite groups used for toxicokinetics or special groups: None

Restriction paradigm for dietary restriction studies: n/a

Interim sacrifices: n/a

Deviations from original study protocol: not reported.

Statistical methods: (from the report)

- Survival:
 - Probabilities of survival: product-limit procedure of Kaplan and Meier.
 - Dose-related effect on survival: method of Cox for testing two groups for equality and Tarone's extensions of Cox's methods for testing for a dose-related trend.
 - One-tailed P values have been reported for all tests except the departure from linearity test, which is only reported when its two-tailed P value is less than 0.05.
- Tumor incidence:
 - Incidence of neoplastic or nonneoplastic lesions: ratio of the number of animals bearing such lesions at a specific anatomic site (numerator) to the number of animals in which that site is examined, or the numbers of animals necropsied (denominator).
 - One-tailed Fisher exact test with $P < 0.05$ as significance level.
 - Linear trend in proportions: Cochran-Armitage test with continuity correction.
 - A time-adjusted analysis was applied when numerous early deaths resulted from causes that were not associated with the formation of tumors.
 - Life-table method: to analyze the incidence of tumors. Curves of the proportions surviving without an observed tumor were computed as in Saffiotti et al. (Cancer Res. 32 1073-1081, 1972). Cox's method of comparing these curves were used for two groups; Tarone's extension to testing for linear trend was used for three groups.
- "The approximate 95% confidence interval for the relative risk of each treated group compares to its control was calculated from the exact interval on the odds ratio. The relative risks is defined as p_t/p_c where p_t is the true binomial probability of the incidence of a specific type of tumor in a treated group of animals and p_c is the true probability of spontaneous incidence of the same type of tumor in a control group."
- "The lower and upper limits of the confidence interval of the relative risk have been included in the tables of statistical analyses. The interpretation of the limits is that in approximately 95% of a large number of identical experiments, the true ratio of the risk in a treated group of animals to that in a control group would be within the interval calculated from the experiment. When the lower limit of the confidence interval is greater than one, it can be inferred that a statistically significant result ($P < 0.025$ one-tailed test when the control incidence is not zero, $P < 0.050$ when the control incidence is zero) has occurred. When the lower limit is less than unity, but the upper limit is greater than unity, the lower limit indicates the absence of a significant result while the upper limit indicates that there is a theoretical possibility of the induction of tumors by the test chemical, which could not be detected under the conditions of this test."

Observation times

Clinical signs: observed twice daily for signs of toxicity and moribundity.

Body weights: rats weighed individually each week for 2 months and every 2 weeks for the remainder of this study. Palpation for masses was carried out at each weighing.

Food consumption: Not obtained

Hematology: Not obtained

Clinical chemistry: Not obtained

Organ weight: Not obtained

Gross pathology: including major tissues, major organs, and all gross lesions from killed animals and from animals found dead.

Histopathology: Peer review: yes (), no (x)

- Including major tissues, major organs, and all gross lesions from killed animals and from animals found dead.
- The following tissues were examined microscopically; skin, muscle, lungs and bronchi, trachea, bone and bone marrow, spleen, lymph nodes, thymus, heart, salivary gland, liver, gallbladder, pancreas, esophagus, stomach, small intestine, large intestine, kidney, urinary bladder, pituitary, adrenal, thyroid, parathyroid, mammary gland, prostate or uterus, testis or ovary, brain, and sensory organs. Peripheral blood smears were prepared from each animal.

Toxicokinetics: n/a

Results

Dose-determining, 45-day studies:

- The first set of five doses resulted in no deaths and in no weight depression exceeding the 15% guideline. Results of the second study are below.

Dose (mg/kg)	Mortality	Time of death	Comments
20.8	5/5	3 in week 4 and all by week 6	
10.4	2/5	1 in week 6 and 1 in week 8	
5.2 or 2.6	No death		No lesions, no weight depression exceeding the 15% guideline

The low and high doses for rats were set at 2.6 and 5.2 mg/kg for main study.

Mortality:

- The Kaplan and Meier curves estimating the probabilities of survival for male and female rats:

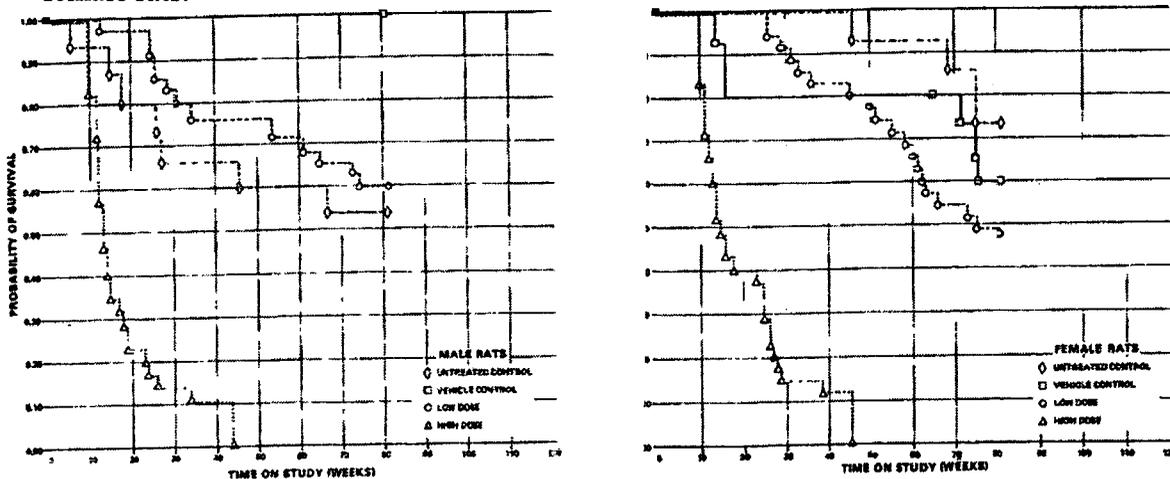


Figure 2. Survival Curves For Rats Treated With 5-Azacytidine

Summary of the result (excerpted from the report):

- In each sex, the result of the Tarone test for positive dose- related trend in mortality over the period is significant ($P < 0.001$), and a departure from linear trend is present ($P < 0.001$), due to the steep increase in mortality in the high-dose rats, of which all died before the end of the study.
- The median time on study was only 13 weeks for high dose animals. The high mortality of this group may have reduced the occurrence of late-developing tumors.
- In the low-dose rats, 21/35 (60%) of the males and 17/35 (49%) of the females lived to the end of the study at week 81.
- Time-adjusted analysis was performed on female rats that lived at least 26 weeks, which is the earliest time of an observed tumor. There were 15 such animals in the untreated controls, 14 in the vehicle controls, 31 in the low-dose group, and 10 in the high-dose group.

Clinical signs:

No treatment related clinical signs.

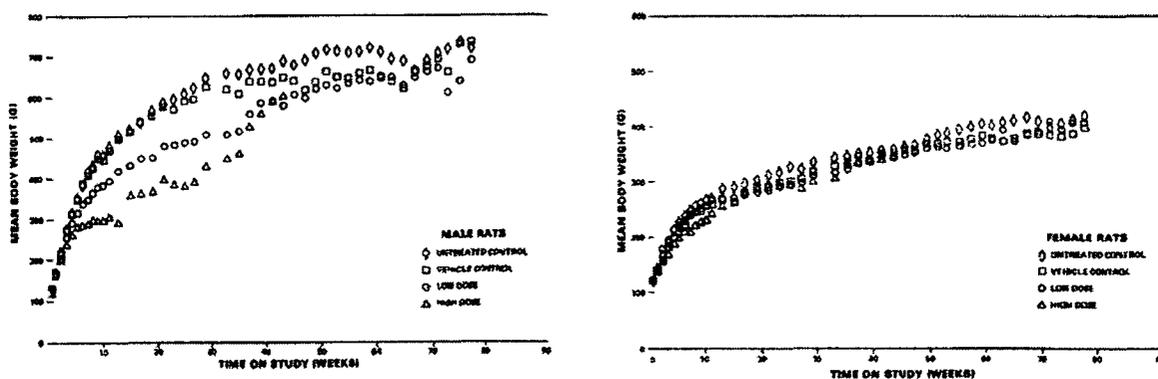
Body weights:

Figure 1. Growth Curves For Rats Treated With S-Azreyteline

Summary of the result:

- Among males, the mean body weights were lower in the treated groups than in the controls starting at about week 4 of the study, with progressively greater differences occurring as the study progressed, up to approximately week 34. When treatment was discontinued at week 34, the mean body weights of the low-dose group markedly increased, as did those of the four remaining high-dose animals.

Food consumption: Not obtained.

Gross pathology: No data presented in the result.

Histopathology: The following tables are excerpted from the NCI contractor's report.

Neoplastic:

Male rats:

TABLE A1.

**SUMMARY OF THE INCIDENCE OF NEOPLASMS IN MALE RATS
GIVEN INTRAPERITONEAL INJECTIONS OF 5-AZACYTIDINE**

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ANIMALS INITIALLY IN STUDY	15	15	35	35
ANIMALS NECROPSIED	14	15	32	33
ANIMALS EXAMINED HISTOPATHOLOGICALLY	14	15	32	33
INTEGUMENTARY SYSTEM				
SKIN				
PAPILLOMA, NOS	(14)	(15)	(32)	(33)
SQUAMOUS CELL CARCINOMA			1 (3%)	
			1 (3%)	
SUBCUT TISSUE				
UNDIFFERENTIATED CARCINOMA	(14)	(15)	(32)	(33)
BASAL-CELL CARCINOMA			1 (3%)	
SARCOMA, NOS			1 (3%)	
FIBROSARCOMA	1 (7%)			
RESPIRATORY SYSTEM				
NONE				
HEMATOPOIETIC SYSTEM				
MULTIPLE ORGANS				
MALIG. LYMPHOMA, UNDIFFER-TYPE	(14)	(15)	(32)	(33)
GRANULOCYTIC LEUKEMIA			1 (3%)	
GRANULOCYTIC SARCOMA			2 (6%)	
			1 (3%)	
CIRCULATORY SYSTEM				
NONE				
DIGESTIVE SYSTEM				
NONE				
URINARY SYSTEM				
NONE				
NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
NUMBER OF ANIMALS NECROPSIED				

TABLE A1. MALE RATS: NEOPLASMS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ENDOCRINE SYSTEM				
*PITUITARY CHROMOPHOBE CARCINOMA	(13)	(15) 1 (7%)	(29) 1 (3%)	(31)
*ADRENAL PHEOCHROMOCYTOMA	(14)	(15)	(31) 1 (3%)	(33)
REPRODUCTIVE SYSTEM				
*TESTIS ADENOCARCINOMA, NOS FIBROSARCOMA	(14)	(15)	(32) 1 (3%)	(33) 1 (3%)
*TESTIS INTERSTITIAL-CELL TUMOR	(14)	(15)	(28) 2 (7%)	(32)
NERVOUS SYSTEM				
*BRAIN ASTROCYTOMA	(14) 1 (7%)	(15)	(30)	(32)
SPECIAL SENSE ORGANS				
NONE				
MUSCULOSKELETAL SYSTEM				
NONE				
BODY CAVITIES				
NONE				
ALL OTHER SYSTEMS				
NONE				

* NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
 † NUMBER OF ANIMALS NECROPSIED

TABLE A1. MALE RATS: NEOPLASMS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ANIMAL DISPOSITION SUMMARY				
ANIMALS INITIALLY IN STUDY	15	15	35	35
NATURAL DEATH	4		11	20
ROBUSTNESS SACRIFICE	3		3	15
SCHEDULED SACRIFICE				
ACCIDENTALLY KILLED				
TERMINAL SACRIFICE	8	15	21	
ANIMAL MISSING				
* INCLUDES AUTOLIZED ANIMALS				
TUMOR SUMMARY				
TOTAL ANIMALS WITH PRIMARY TUMORS*	2	1	11	1
TOTAL PRIMARY TUMORS	2	1	14	1
TOTAL ANIMALS WITH BENIGN TUMORS			6	1
TOTAL BENIGN TUMORS			6	1
TOTAL ANIMALS WITH MALIGNANT TUMORS	2	1	9	
TOTAL MALIGNANT TUMORS	2	1	10	
TOTAL ANIMALS WITH SECONDARY TUMORS†				
TOTAL SECONDARY TUMORS				
TOTAL ANIMALS WITH TUMORS UNCERTAIN- BENIGN OR MALIGNANT				
TOTAL UNCERTAIN TUMORS				
TOTAL ANIMALS WITH TUMORS UNCERTAIN- PRIMARY OR METASTATIC				
TOTAL UNCERTAIN TUMORS				
* PRIMARY TUMORS: ALL TUMORS EXCEPT SECONDARY TUMORS				
† SECONDARY TUMORS: METASTATIC TUMORS OR TUMORS INVASIVE INTO AN ADJACENT ORGAN				

Female rats:

TABLE A2.

**SUMMARY OF THE INCIDENCE OF NEOPLASMS IN FEMALE RATS
GIVEN INTRAPERITONEAL INJECTIONS OF 5-AZACYTIDINE**

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ANIMALS INITIALLY IN STUDY	15	15	35	35
ANIMALS NECROPSIED	15	15	31	31
ANIMALS EXAMINED HISTOPATHOLOGICALLY	15	15	31	31
INTEGUMENTARY SYSTEM				
*SUBCUT TISSUE FIBROMA	(15)	(15) 1 (7%)	(31)	(31)
RESPIRATORY SYSTEM				
#LUNG ADENOCARCINOMA, NOS, METASTATIC	(15) 1 (7%)	(14)	(30) 2 (7%)	(30)
HEMATOPOIETIC SYSTEM				
*MULTIPLE ORGANS LYMPHOCTIC LEUKEMIA	(15)	(15)	(31) 1 (3%)	(31)
CIRCULATORY SYSTEM				
NONE				
DIGESTIVE SYSTEM				
NONE				
URINARY SYSTEM				
#KIDNEY TUBULAR-CELL ADENOCARCINOMA	(15) 1 (7%)	(13)	(31)	(31)
ENDOCRINE SYSTEM				
#PITUITARY CHROMOPHOBE ADENOMA CHROMOPHOBE CARCINOMA	(14) 5 (36%)	(15) 4 (27%) 1 (7%)	(30)	(27)
* NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
* NUMBER OF ANIMALS NECROPSIED				

TABLE A2. FEMALE RATS: NEOPLASMS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
REPRODUCTIVE SYSTEM				
PHARYNGEAL GLAND				
ADENOCARCINOMA, NOS	(15) 4 (27%)	(15)	(31) 9 (29%)	(31) ¹ 1 (3%)
PAPILLARY ADENOCARCINOMA			1 (3%)	1 (3%)
FIBROADENOMA	2 (13%)	3 (20%)	6 (19%)	1 (3%)
UTERUS				
LEIOMYOSARCOMA	(15)	(15)	(30) 1 (3%)	(29)
ENDOMETRIAL STROMAL POLYP			1 (3%)	
NERVOUS SYSTEM				
BRAIN				
CHROMOPHOBE CARCINOMA, METASTATIC	(14)	(14) 1 (7%)	(30)	(30)
SPECIAL SENSE ORGANS				
EYE CANAL				
KERATOACANTHOMA	(15)	(15)	(31) 1 (3%)	(31)
MUSCULOSKELETAL SYSTEM				
BONE				
BONY CAVITIES				
PRESENTERY				
LIPOMA	(15) 1 (7%)	(15)	(31)	(31)
ALL OTHER SYSTEMS				
BONE				
NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
NUMBER OF ANIMALS NECROPSIED				

APPEARS THIS WAY
ON ORIGINAL