TABLE A2. FEMALE RATS: NEOPLASMS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ANIMAL DISPOSITION SUMMARY				
ANIMALS INITIALLY IN STUDY	15	15	35	35
NATURAL DEATHS@	1	1	10	22
MORIBUND SACRIFICE	3	5	8	13
SCHEDULED SACRIFICE				
ACCIDENTALLY KILLED				
TERMINAL SACRIFICE	11	9	17	
ANIMAL MISSING				
@ INCLUDES AUTOLYZED ANIMALS				
TUMOR SUMMARY				
TOTAL ANIMALS WITH PRIMARY TUMORS*	11	7	15	3
TOTAL PRIMARY TUMORS	13	9	20	3
TOTAL ANIMALS WITH BENIGN TUMORS	8	6	7	1
TOTAL BENIGN TUMORS	8	8	8	1
TOTAL ANIMALS WITH MALIGNANT TUMORS	5	1	11	2
TOTAL MALIGNANT TUMORS	5	1	12	2
TOTAL ANIMALS WITH SECONDARY TUMORS#	1	1	2	
TOTAL SECONDARY TUMORS	1	1	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

Summary of histopathological findings on neoplasm in rats (from the report):

- According to the statistical analyses, there was no increased incidence of tumors at any specific site in either sex that is statistically significant in the positive direction, i.e., the treated animals did not have higher frequency of neoplasm than their controls. The absence of such tumors, however, may be due to the abnormally short treatment periods and life spans in the treated animals, rather than to absence of carcinogenicity of the test chemical in the rats.
- Neoplasms were seen more frequently in the female rats than in the males. The most frequently observed neoplasms in the females involved the pituitary gland and the mammary gland. Chromophobe adenomas of the pituitary were observed frequently in the control females only. Benign and malignant mammary gland neoplasm were seen frequently in both control and low-dose female rats.
- Statistical analyses of the incidences of primary tumors that were observed in at least two animals with an incidence of at least 5% in either the vehicle-control or low dose groups:

Male rats:

Topography: Morphology	Vehicle Control	Low Dose
Hematopoietic System: Lymphoma, Granulocytic Leukemia, or Sarcoma[b]	0/15 (0)	4/32 (13)
P Values[c,d]	—	N.S.
Relative Risk (Vehicle Control)[e]		Infinite
Lower Limit		0.463
Upper Limit		Infinite
Weeks to First Observed Tumor	—	73
Testis: Interstitial-cell Tumor[b]	0/15 (0)	2/28 (7)
P Values[c,d]	—	N.S.
Relative Risk (Vehicle Control)[e]		Infinite
Lower Limit		0.168
Upper Limit		Infinite
Weeks to First observed Tumor	—	80

[a]Treated groups received doses of 2.6 or 5.2 mg/kg by injection three times per week for 34 weeks.

[b]Number of tumor-bearing animals/number of animals examined at site (percent).

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

[d]A negative trend (N) indicates a lower incidence in the treated group than in the control group.

[e]The 95% confidence interval of the relative risk between the treated group and the control group.

Female rats:

Topography: Morphology	Vehicle Control	Low Dose
Pituitary: Chromophobe Adenoma[b]	4/15 (27)	0/30 (0)
P Values[c,d]	—	P = 0.009(N)
Relative Risk (Vehicle Control)[e]		0.000
Lower Limit		0.000
Upper Limit		0.521
Weeks to First Observed Tumor	65	—
Mammary Gland: Papillary Adenocarcinoma, Fibroadenoma, or Adenocarcinoma, NOS (not otherwise specified)[b]	3/15 (20)	13/31 (42)
P Values[c,d]	—	N.S.
Relative Risk (Vehicle Control)[e]		2.097
Lower Limit		0.716
Upper Limit		10.013
Weeks to First Observed Tumor	76	26

[a]Treated groups received doses of 2.6 or 5.2 mg/kg by injection three times per week for 34 weeks.

[b]Number of tumor-bearing animals/number of animals examined at site (percent).

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

[d]A negative trend (N) indicates a lower incidence in the treated group than in the control group.

[e]The 95% confidence interval of the relative risk between each treated group and the control group.

Due to the shortened survival in the high-dose groups, results for these groups were not analyzed in these tables. In each of the 95% confidence intervals of relative risk, shown in the tables, except for the comparison involving the pituitary tumors in female rats, the value of one is included; this indicates the absence of positive significant results. It should also be noted that these intervals have upper limits greater than one, indicating the theoretical possibility of the induction of tumors by 5-azacytidine, which could not be detected under the conditions of this test.(quote from NCI contractor's report)

Non-neoplastic:

- Table C1 and C2 are summaries in male and female rats, respectively.

Male rats:

TABLE C1.

SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS 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				
NONE				
RESPIRATORY SYSTEM				
*TRACHEA	(12)	(15)	(28)	(33)
INFLAMMATION, ACUTE/CHRONIC		1 (7%)	4 (14%)	
*LUNG/BRONCHIOLE	(14)	(13)	(32)	(32)
HYPERPLASIA, LYMPHOID			1 (3%)	
*LUNG	(14)	(13)	(32)	(32)
INFLAMMATION, INTERSTITIAL		1 (8%)	4 (13%)	1 (3%)
BRONCHOPNEUMONIA SUPPURATIVE	3 (21%)		5 (16%)	
BRONCHOPNEUMONIA, CHRONIC			2 (6%)	
BRONCHOPNEUMONIA CHRONIC SUPPURA			4 (13%)	1 (3%)
HYPERPLASIA, LYMPHOID		1 (8%)	2 (6%)	
HEMATOPOIETIC SYSTEM				
*BONE MARROW	(13)	(15)	(30)	(33)
ATROPHY, NOS		2 (13%)	5 (17%)	26 (79%)
*SPLEEN	(14)	(15)	(31)	(32)
INFLAMMATION, NECROTIZING				1 (3%)
NECROSIS, NOS				3 (9%)
*LYMPH NODE	(7)	(13)	(22)	(28)
INFLAMMATION, NECROTIZING				1 (4%)
CIRCULATORY SYSTEM				
*HEART/ATRIUM	(14)	(13)	(32)	(32)
THROMBOSIS, NOS				1 (3%)

(#) NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
* NUMBER OF ANIMALS NECROPSIED

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
#MYOCARDIUM	(14)	(13)	(32)	(32)
INFLAMMATION, NECROTIZING				1 (3%)
DEGENERATION, NOS				1 (3%)
DIGESTIVE SYSTEM				
#LIVER	(14)	(14)	(31)	(33)
CONGESTION, PASSIVE				1 (3%)
CONGESTION, CHRONIC PASSIVE				1 (3%)
INFLAMMATION, NECROTIZING				1 (3%)
NECROSIS, COAGULATIVE				2 (6%)
NECROSIS, CENTRAL			5 (16%)	3 (9%)
#LIVER/CENTRILOBULAR	(14)	(14)	(31)	(33)
NECROSIS, NOS				1 (3%)
NECROSIS, COAGULATIVE				8 (24%)
#BILE DUCT	(14)	(15)	(32)	(33)
INFLAMMATION, CHRONIC			1 (3%)	
#GASTRIC SUBMUCOSA	(14)	(15)	(31)	(33)
ABSCESS, CHRONIC				1 (3%)
URINARY SYSTEM				
#KIDNEY	(14)	(15)	(31)	(32)
HYDRONEPHROSIS	1 (7%)			
INFLAMMATION, INTERSTITIAL			2 (6%)	
INFLAMMATION, SUPPURATIVE				1 (3%)
GLOMERULONEPHRITIS, MEMBRANOUS	3 (21%)	1 (7%)	1 (3%)	
PYELONEPHRITIS, ACUTE/CHRONIC				1 (3%)
INFLAMMATION, CHRONIC			1 (3%)	
ENDOCRINE SYSTEM				
NONE				
REPRODUCTIVE SYSTEM				
#PROSTATE	(14)	(15)	(28)	(30)
INFLAMMATION, SUPPURATIVE				1 (3%)
NERVOUS SYSTEM				
NONE				

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

TABLE C1. MALE RATS: NONNEOPLASTIC LESIONS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
SPECIAL SENSE ORGANS				
NONE				
MUSCULOSKELETAL SYSTEM				
NONE				
BODY CAVITIES				
NONE				
ALL OTHER SYSTEMS				
NONE				
SPECIAL MORPHOLOGY SUMMARY				
NO LESION REPORTED	7	12	4	4
AUTOLYSIS/NO NECROPSY	1		3	2

a NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY
b NUMBER OF ANIMALS NECROPSIED

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Female rats:

TABLE C2.

SUMMARY OF THE INCIDENCE OF NONNEOPLASTIC LESIONS 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				
NONE				
RESPIRATORY SYSTEM				
#TRACHEA	(14)	(15)	(29)	(28)
INFLAMMATION, ACUTE/CHRONIC			1 (3%)	
#LUNG	(15)	(14)	(30)	(30)
INFLAMMATION, INTERSTITIAL		1 (7%)	1 (3%)	2 (7%)
BRONCHOPNEUMONIA SUPPURATIVE	1 (7%)		4 (13%)	
BRONCHOPNEUMONIA NECROTIZING				1 (3%)
BRONCHOPNEUMONIA CHRONIC SUPPURA	1 (7%)			
HYPERPLASIA, LYMPHOID		1 (7%)		1 (3%)
HEMATOPOIETIC SYSTEM				
#BONE MARROW	(15)	(15)	(30)	(29)
ATROPHY, NOS			2 (7%)	15 (52%)
CIRCULATORY SYSTEM				
NONE				
DIGESTIVE SYSTEM				
#LIVER/CENTRILOBULAR	(14)	(14)	(31)	(31)
NECROSIS, NOS			1 (3%)	
NECROSIS, COAGULATIVE				4 (13%)
CYTOLOGIC DEGENERATION				1 (3%)
URINARY SYSTEM				
NONE				

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

TABLE C2. FEMALE RATS: NONNEOPLASTIC LESIONS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
ENDOCRINE SYSTEM				
NONE				
REPRODUCTIVE SYSTEM				
MAMMARY GLAND	(15)	(15)	(31)	(31)
HYPERPLASIA, CYSTIC	1 (7%)			
UTERUS/ENDOMETRIUM	(15)	(15)	(30)	(29)
INFLAMMATION, SUPPURATIVE	1 (7%)	2 (13%)	3 (10%)	
INFLAMMATION, CHRONIC SUPPURATIV			3 (10%)	
OVARY	(10)	(13)	(23)	(27)
INFLAMMATION, SUPPURATIVE			1 (4%)	
INFLAMMATION, CHRONIC SUPPURATIV			3 (13%)	1 (4%)
NERVOUS SYSTEM				
NONE				
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 C2. FEMALE RATS: NONNEOPLASTIC LESIONS (CONTINUED)

	UNTREATED CONTROL	VEHICLE CONTROL	LOW DOSE	HIGH DOSE
SPECIAL MORPHOLOGY SUMMARY				
NO LESION REPORTED	4	7	9	10
AUTO/NECROPSY/HISTO PERF				2
AUTOLYSIS/NO NECROPSY			4	4
# NUMBER OF ANIMALS WITH TISSUE EXAMINED MICROSCOPICALLY				
* NUMBER OF ANIMALS NECROPSIED				

Summary of histopathological findings on non-neoplasm in rats: Among 35/sex/group treated rats, a few of them were not necropsied, due to autolysis.

- Liver: Hepatocellular degeneration and necrosis, usually centrilobular, were present in several of the treated animals and were the only nonneoplastic lesions that appeared to be directly related to exposure to the chemical. None of the control rats had similar hepatic lesions. These lesions were present in 1/31 low-dose and 5/31 high-dose females. Necrotizing liver lesions were also present in

5/31 low-dose and 15/33 high-dose male rats. All of the animals with liver necrosis died prior to the end of this study.

- Bone marrow: Bone-marrow atrophy was present in 5/30 low-dose and 26/33 high-dose males and in 2/30 low-dose and 15/29 high-dose females, but its significance in relation to the early deaths could not be evaluated, due to the lack of clinical pathology data.
- Several inflammatory and degenerative lesions occurred with approximately equal frequency in treated and control animals.

Toxicokinetics: n/a

Summary of Individual study:

- Under the conditions of this bioassay, the short life span and short duration of treatment of Sprague-Dawley rats of either sex precluded complete evaluation of the carcinogenicity of 5-azacytidine in these groups.
- Liver necrosis and bone marrow atrophy were the main non-neoplastic lesions in rats in this study.

Histopathological inventory list:

Study	4.2.3.4.2.2.	4.2.3.4.1.1.	4.2.3.4.2.1.	4.2.3.4.1.1.
Species	Mouse	Mouse	Rat	Rat
Adrenals		X		X
Aorta				
Bone Marrow smear		X		X
Bone (femur)		X		X
Brain		X		X
Cecum				
Cervix				
Colon		X		X
Duodenum		X		X
Epididymis				
Esophagus		X		X
Eye				
Fallopian tube				
Gall bladder		X (and bile duct)		X
Gross lesions				
Harderian gland				
Heart		X		X
Ileum		X		X
Injection site				
Jejunum		X		X
Kidneys		X	X	X
Lachrymal gland				

Larynx				
Liver		X	X	X
Lungs	X	X	X	X
Lymphoreticular system	X		X	
Lymph nodes, cervical		X		X
Lymph nodes mandibular		X		X
Lymph nodes, mesenteric		X		X
Mammary Gland	X	X		X
Nasal cavity				
Optic nerves				
Ovaries		X		X
Pancreas		X	X	X
Parathyroid		X		X
Peripheral nerve				
Pharynx				
Pituitary		X		X
Prostate		X		X
Rectum		X		X
Salivary gland		X		X
Sciatic nerve				
Seminal vesicles				
Sensory organs		X		X
Skeletal muscle		X		X
Skin	X	X	X	X
Spinal cord				
Spleen		X	X	X
Sternum				
Stomach		X		X
Testes		X	X	X
Thymus		X		X
Thyroid		X		X
Tongue				
Trachea		X		X
Urinary bladder		X		X
Uterus		X		X
Vagina				
Zymbal gland				

3.4.6. Reproductive and developmental toxicology

Fertility and early embryonic development

Study title: Developmental exposure of male germ cells to 5-azacytidine results in abnormal preimplantation development in rats. Biology of Reproduction 55: 1155-1162, 1996 (Doerksen and Trasler).

Key study findings:

- Paternal administration of 5-azacytidine interfered with normal male germ cell development and resulted in alterations in fertilization and early embryo development.

Study no.: Not applicable
Volume #, and page #: Module 4.2.3.5.1.2
Conducting laboratory and location: published article
Date of study initiation: published 1996
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: 5- or 6-azacytidine (Sigma). 6-Azacytidine, although bearing similar structure as 5-azacytidine but without ability of blocking DNA methylation, served as a control. % purity: not specified.

Note: Figures and Tables are from the article.

Methods

Doses: Experiment 1: 0 (saline), 2.5 and 5 mg/kg/day, 3x weekly, 4 or 11 weeks.
Experiment 2: 0 (saline), 2.5 and 4 mg/kg/day, 3x weekly, 16 weeks.
Species/strain: Sprague-Dawley rats. Adult males (250-350 g) and virgin females (225-250 gm)
Number/sex/group:
Experiment 1: Male rats: saline (n=8), 5-AZ (n=8) or 6-AZ (n=4).
Two females to mate with each 5-AZ or 6-AZ-treated male rat.
Experiment 2: Male rats: saline (n=6), 5-AZ (n=8).
Two females to mate with 6 males/5-AZ, or 6-AZ-treated group.
Route, formulation, volume, and infusion rate: IP, 1 ml/kg.
Satellite groups used for toxicokinetics: Not performed.
Study design:
Experiment 1: At the end of treatment, 2 female rats were mated with each treated males (n=6 for saline and 5-AZ groups, n=4 for 6-AZ). Following mating, these male rats together with the rest of male rats were killed. Females were killed on Day 20 of gestation. The number of corpora lutea, implantations, resorptions, and live fetuses were determined. Fetuses were weighed, sexed, and examined for gross malformations.
Experiment 2: Six males per group were mated twice, each time with 2 female rats. Following mating, blood from the male rats was collected for

hemoglobin and leukocyte counts. The sperm count (n=3 for control, n=5 for 5-AZ) and the pregnancy outcome was assessed on 2 mated females on Day 20 of gestation. In addition, 2 females were killed on day 2 of gestation (0900-1300 h). Two-day-old embryos were assessed for abnormalities.

Parameters and endpoints evaluated:

Male body weight (twice weekly)

Male reproductive organ weight: testes, epididymides, ventral prostates, seminal vesicles, pituitaries, spleens, liver (a segment).

Sperm counts: testicular and epididymal condensed spermatid and spermatozoal numbers (hemocytometric counts).

Pregnant outcome:

Ability of male to mate (pregnancy rate)

Litter size

Preimplantation loss (mean/litter)

Postimplantation loss (mean/litter)

Embryo/fetal abnormalities:

Observations of gross morphological abnormalities, or low and high fetal birth weights (< 75% or > 125%) of average weight for a litter.

Results

Mortality: none

Clinical signs: unremarkable

Body weight and male reproductive organ weight:

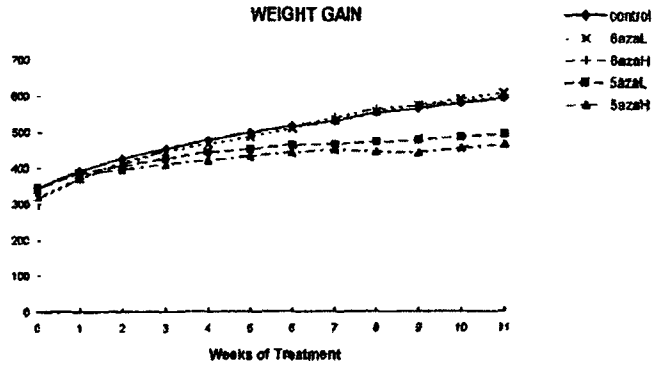

FIG. 1. Increase in rat body weight starting at Week 0, just prior to the first dosage of either saline (control), 2.5 mg/kg 6-azacytidine (6azaL), 5.0 mg/kg 6-azacytidine (6azaH), 2.5 mg/kg 5-azacytidine (5azaL), or 5.0 mg/kg 5-azacytidine (5azaH).

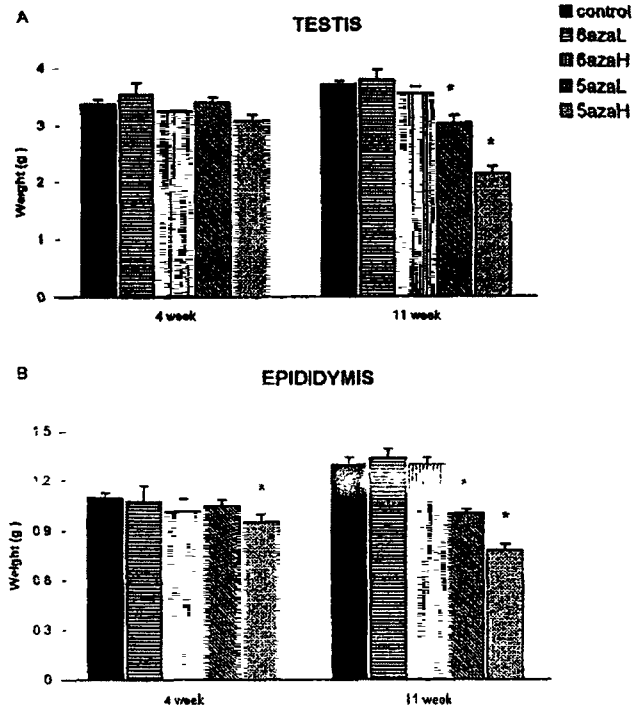

FIG 7 Effect of treatment on the weights of (A) testes and (B) epididymides after 4 wk and 11 wk of treatment with saline (control), 2 5 mg/kg 6-azacytidine (6azaL), 5.0 mg/kg 6-azacytidine (6azaH), 2.5 mg/kg 5-azacytidine (5azaL), and 5.0 mg/kg 5-azacytidine (5azaH). Bars represent means ± SEM * $p \leq 0.05$.

EFFECTS OF 5-AZACYTIDINE ON SPERMATOZOAL FUNCTION

TABLE 1. Effect of treatment on body and organ weights after 11 wk and 16 wk of treatment*

Weight	Control	5-AZA 2.5 mg/kg	5-AZA 5.0 mg/kg	6-AZA 2.5 mg/kg	6-AZA 5.0 mg/kg
11 wk					
Initial	344.8 ± 17.2	346 3 ± 15 2	343 5 ± 16.5	320.5 ± 20.8	314.5 ± 16.2
Final	593.8 ± 14.3	494 6 ± 12.2*	465 0 ± 13 3*	607.5 ± 19.0	599.3 ± 14.4
Ventral prostate	801 ± 67	658 ± 40	569 ± 49*	814 ± 110	831 ± 66
Seminal vesicles	578 ± 24	482 ± 27	451 ± 45*	669 ± 39	624 ± 50
Pituitary	14 ± 1	12 ± 1	12 ± 1	14 ± 1	14 ± 1
Spleen	916 ± 61	1011 ± 41	1131 ± 156	1190 ± 78	917 ± 67
16 wk					
Initial	331.50 ± 7.13	339.63 ± 9.75	341.75 ± 10.42		
Final	684.83 ± 33 67	607.50 ± 19 10*	549.50 ± 12.21*		
Testes	3900 ± 110	2800 ± 200*	1710 ± 250*		
Epididymides	1526 ± 48	1051 ± 78*	794 ± 55*		
Ventral prostate	947 ± 35	817 ± 41	670 ± 62		
Seminal vesicles	797 ± 171	686 ± 105	566 ± 57		
Spleen	937 ± 57	1133 ± 92	845 ± 12		

* Values represent mean in mg ± SEM.
* Denotes significance $p \leq 0.05$ vs. control values.

Summary of the experimental results:

- 5-Azacitidine reduced weight gain compared to saline and 6-AZ controls.
- Four-week treatment with 5 mg/kg of 5-AZ resulted in significant weight reduction of epididymides, but not significant in testes weights. There was a dose-related weight reduction of testes and epididymides when treatment continued for 11weeks (approximately 20% and 40% in the weights of testes and epididymides) or 16 weeks (testes: 28% and 56%, epididymides: 31% and 48%, at 2.5 mg/kg and 4 mg/kg, respectively). Eleven-week treatment with 5 mg/kg of 5-AZ resulted in significant decrease in the weights of ventral prostate and seminal vesicle.

Food consumption: Not performed

Toxicokinetics: Not performed

Necropsy: Not performed

Fertility parameters (mating/fertility index, corpora lutea, preimplantation loss, etc.):

- Effect of 5-azacytidine to testicular and epididymal sperm counts:

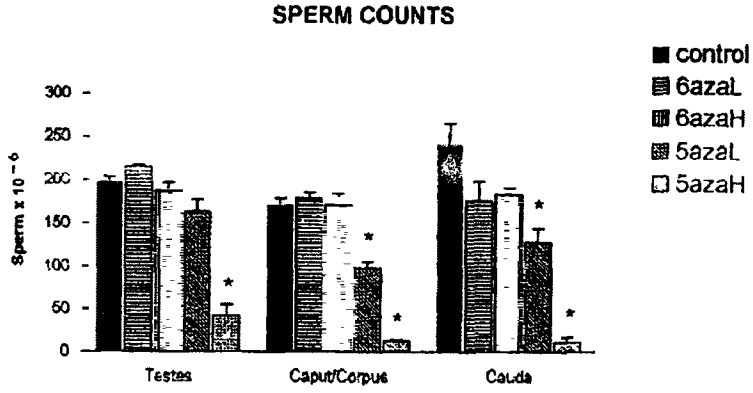

FIG. 3. Effect of 11 wk of treatment with saline (control), 2.5 mg/kg 6-azacytidine (6azaL), 5.0 mg/kg 6-azacytidine (6azaH), 2.5 mg/kg 5-azacytidine (5azaL), and 5.0 mg/kg 5-azacytidine (5azaH) on testicular and epididymal condensed spermatid and spermatozoal numbers. Bars represent mean number of sperm $\times 10^{-6} \pm$ SEM. * $p \leq 0.05$.

Sperm counts, as measured by hemocytometric counts of condensed sperm heads, in testes, caput/corpus and cauda parts of epididymides were decreased in a dose-related fashion after 4 week (non-significant) or 11 week treatment of 5-AZ. Although 2.5 mg/kg of 5-AZ did not cause significant reduction in testicular sperm counts after 11 week treatment, the counts of the rest of groups were significantly reduced (caput/corpus epididymides: 43% and 93%, cauda: 47% and 95%, at 2.5 and 5 mg/kg, respectively).

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- Effect of 5-azacytidine on pregnancy outcome:

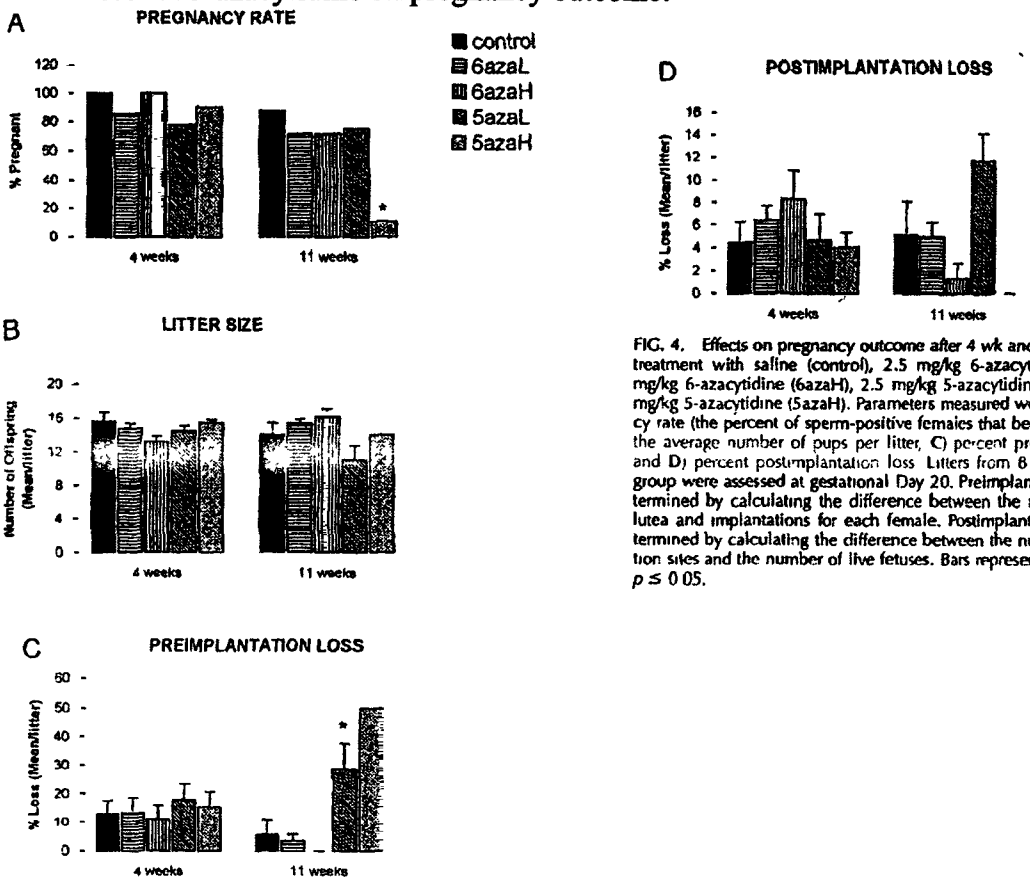


FIG. 4. Effects on pregnancy outcome after 4 wk and 11 wk of paternal treatment with saline (control), 2.5 mg/kg 6-azacytidine (6azaL), 5.0 mg/kg 6-azacytidine (6azaH), 2.5 mg/kg 5-azacytidine (5azaL), and 5.0 mg/kg 5-azacytidine (5azaH). Parameters measured were A) the pregnancy rate (the percent of sperm-positive females that became pregnant), B) the average number of pups per litter, C) percent preimplantation loss, and D) percent postimplantation loss. Litters from 8 to 12 females per group were assessed at gestational Day 20. Preimplantation loss was determined by calculating the difference between the number of corpora lutea and implantations for each female. Postimplantation loss was determined by calculating the difference between the number of implantation sites and the number of live fetuses. Bars represent means ± SEM. * $p \leq 0.05$.

Summary of experimental results:

- Pregnancy rate:
  - 4-week treatment: unremarkable
  - 11-week treatment 2.5 mg/kg/d: unremarkable
  - 11-week treatment 5mg/kg/d: 11% (1/9)
  - 16 week treatment 2.5 mg/kg/d: unremarkable
  - 16 week treatment 4 mg/kg/d: 9.1% (1/11)
- Litter size: unremarkable for either Exp 1 or 2
- Preimplantation loss:
  - Unremarkable at 4 weeks both dose groups.
  - Increased after 11-weeks dosing in both low and high dose groups (reported 5.2-fold ↑ at low dose; high dose shown in figure but not described in text).
  - ↑ non-significant in low dose 16-wk group (7.49% v 4.01% in C); ↑ in morphological abnormal 2-day old embryos at 4 mg/kg (see below)
- Postimplantation loss:
  - unremarkable at 4 weeks
  - unremarkable at 11 weeks; loss in LD animals was not statistically significant. (No historic control data provided)
  - unremarkable in 16-week LD group
- Fetal abnormalities on Day 20 gestation: unremarkable

- Effect of 5-azacytidine on early embryo development (Day 2 gestation analysis):

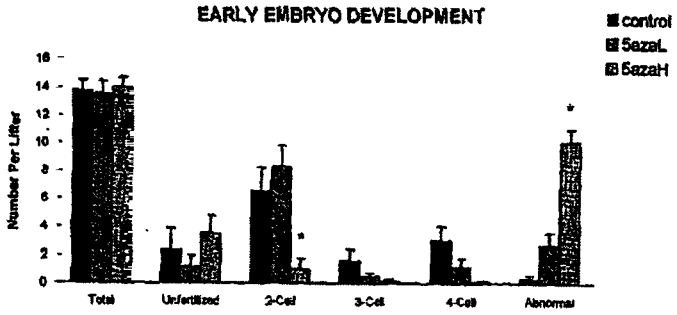


FIG. 5. Effect of paternal 5-azacytidine administration on early embryos sired by rats treated for 16 wk with saline (control), 2.5 mg/kg 5-azacytidine (5azaL), and 4.0 mg/kg 5-azacytidine (5azaH). Bars represent the average number per litter ± SEM of total number of embryos plus unfertilized oocytes recovered (represents the average number of oocytes ovulated), unfertilized oocytes, 2-cell, 3-cell, 4-cell, and abnormal embryos at Day 2 of gestation. * $p \leq 0.05$.

Embryofetal Development

Study title: Cerebral cortex of the mouse after prenatal chemical insult. Am J Anat 132(3): 335-374, 1971 (Langman and Shimada).

Key study findings:

- 5-Azacytidine treatment as single (4 mg/kg) or repeated (2 mg/kg on day 13, 14 and 15 of gestation) administration during the later stages of fetal life, result in a shortage of neurons and the presence of morphologically abnormal neurons.

Study no.: Not applicable
Volume #, and page #: Module 4.2.3.5.2.2
Conducting laboratory and location: published article
Date of study initiation: publish 1971
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: Not reported

Methods

Dose:

TABLE 1

Experimental schedule

Group	No. pregnant mice treated	No. inj. × dosage (mg/kg) / Age of gestation in days	No. pregnant mice sacrificed in hours after injection								
			1	4	8	12	24	36	48	60	72
I	6	$\frac{1\times4}{15}$		1	1	1	1		1		1
II	15	$\frac{1\times4}{15}$ + H^3 thymidine	2			1	2	3	3	2	2
III	5	control		1	1	1	1				1
			No. of offspring sacrificed in days after birth								
			1	3	5	10	15	20	30	40	
IV	9	$\frac{2\times3}{13, 14}$			1			1	3		
V	18	$\frac{3\times2}{13, 14, 15}$	4	4	7	9	9	15	10	9	
VI	5	$\frac{3\times2}{14, 15, 16}$	7	3	2	3		17	21		
VII	3	$\frac{3\times4}{15, 16, 17}$		5	5	6		5	9		
VIII	13	control	7	12	11	11	16	15	17	12	

Species/strain: Pregnant mice (DUB/ICR strain)
Number/sex/group: See table above.
Route of administration: IP
Study design:

- The dose, treatment and sacrifice schedules were tabulated above.
- In Group 2 tritiated thymidine (6 μCi/gm body weight, Sp. Act. 6.7 Ci/mM) was given 30 to 60 minutes after administration of 5-azacytidine.
- In Experiment 1 (Groups 1-3) fetal brains were examined histologically for abnormal mitotic figures or prepared for radioautography to trace ^3H-labled cells.
- In Experiment 2 (Groups 4-8) animals were treated with 5-azacytidine or saline (control) at 2 or 3 successive days of gestation and allowed to come to term. Pups were sacrificed at various times after birth. The brains of the pups were weighed, and the length and width of cerebral hemispheres and the length of the longitudinal fissure were determined. Some brains were also examined histologically.

Results

Mortality (dams): None reported

Clinical signs (dams): Not performed

Body weight (dams): Not performed

Food consumption (dams): Not performed

Toxicokinetics: Not performed

Findings in the fetal brains: (excerpted from the article)

- The abnormal mitotic figures in the neuronal cells appeared as early as 3 to 4 hours after treatment. They were found bordering the lumen of the ventricle, a few in the subependymal layer, but none in the migratory zone or cortical plate. Usually the chromosomes were clumped together, forming comma-and circle-shaped structures. Sometimes chromosome breaks were observed.
- Eight to twelve hours after treatment the effect of 5-azacytidine was diminishing. The affected cells moved away from the lumen toward the cortical plate, and a few normal looking mitotic cells began to appear.
- Compared to the control, treated brains showed fewer labeled cells in the cortex. A neuronal deficit has resulted from the treatment, although the authors state that it was difficult to pinpoint the type of affected neurons.

Findings in the offspring brains (malformations, variations, etc.):

- The neocortex and the hippocampal region of the new born by treated mothers contained fewer cells than the same structures in the controls. The thickness of the cortical layers was less than in the control.
- Many neurons in these regions were morphologically abnormal. These cells were spindle-shaped and had a dark, round to oval nucleus.
- "The length and width of the cerebral hemisphere and the length of the central longitudinal fissure of the treated animals were considerably smaller than in the controls. These differences were not significant at birth, but became evident in the course in postnatal development."

Summary of individual study findings:
An effect of 5-azacytidine on mouse brain development was demonstrated in the fetus as well as offspring of the treated animals. A single and repeated short-term administration of 5-AZ (2 mg/kg on days 13, 14 and 15 of gestation) during prenatal life caused severe microcephaly after birth. No functional tests were performed in this study.

Study title: Embryotoxicity of 5-azacytidine in mice. Phase and dose-specificity studies. Arch. Toxicol. 55: 143-147, 1984 (Schmahl et al.)

Key study findings:

- Increased embryolethality GD 10-14; maximum at GD 10.
- Increased malformations GD 10-12
- Dose-related embryotoxicity when administered on either GD 12 or 14.

Study no.: Not applicable
Volume #, and page #: Module 4.2.3.5.2.4
Conducting laboratory and location: published article
Date of study initiation: published 1984
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: Not reported

Methods

Dose:

Phase specificity experiments: 2 mg/kg

Dose dependency experiments: 0 (saline control), 0.5, 1, 2 and 4 mg/kg.

Species/strain: NMRI mice

Number/sex/group: not reported

Route of administration: IP; volume 0.2 ml/40 g bw

Study design:

- Evaluation of phase specificities:
  The most sensitive day of pregnancy to 5-azacytidine was determined by treating the dams either on days 10, 11, 12, 13, 14, 15, or 16 of pregnancy.
- Evaluation of dose dependencies.
  These experiments were performed on day GD 12 and GD 14, low and high sensitivity, respectively.
- The animals were sacrificed on GD 18 and the number of implantation sites, resorptions, and living fetuses was counted in each litter. Litters with less than four individuals have not been included.

Parameters and endpoints evaluated:

- The number of implantation sites, resorptions, and live fetuses was counted in each litter, and the weights of the fetuses were determined.
- Histological evaluations were performed to evaluate fetal malformations (see table below for details).

Note; tables from the article.

Results

Mortality (dams): None reported

Clinical signs (dams): Not performed

Body weight (dams): Not performed

Food consumption (dams): Not performed

Toxicokinetics: Not performed

Findings in phase specificity experiments:

Table 1. Phase specificity experiments with 5-azacytidine (2 mg/kg b.wt) Compilation of the embryotoxicity data

Day of treatment	Implantation sites/litter Av. no. per litter	Living fetuses Av. no. per litter	Resorptions/ implantation sites (%)	Litters with resorptions a) partially (%) b) completely (%)	Resorptions Av. % per litter	Resorptions a) per litters b) per litters with res.	Mean litter weights (g ± SEM)
– (Controls)	382/26 14.7 ± 2.4	356 13.7 ± 2.2	26/382 (6.8)	13/26 (50.0) 0 (0)	5.9 ± 9.2	1.00 ± 1.2 2.00 ± 1.1	17.7 ± 2.8
10	335/26 12.9 ± 1.8	189 7.3 ± 3.1	146/335 (43.6)	25/26 (96.1) 1.26 (3.9)	42.8 ± 6.5	5.61 ± 2.6 5.84 ± 2.8	6.8 ± 2.9
11	425/31 13.7 ± 2.2	370 11.9 ± 2.4	55/425 (12.9)	29/31 (93.5) 0 (0)	12.6 ± 8.2	1.77 ± 1.4 1.89 ± 1.4	13.1 ± 2.6
12	312/21 14.9 ± 1.7	277 13.2 ± 1.8	35/312 (11.2)	14/21 (66.6) 0 (0)	10.6 ± 9.4	1.66 ± 1 3 2.50 ± 1.4	15.0 ± 2.1
13	447/33 13.5 ± 1.9	386 11 7 ± 1 8	61/447 (13.6)	21/33 (63.6) 0 (0)	13.2 ± 8.5	1.85 ± 1.6 2.95 ± 1 2	13.1 ± 2.0
14	372/26 14 3 ± 2.4	328 12.6 ± 2.3	44/372 (11.8)	21/26 (80 7) 0 (0)	10.8 ± 4 2	1.69 ± 1.6 2.09 ± 1.3	13.6 ± 2.5
15	472/32 14.7 ± 2.4	433 13.5 ± 2.4	39/472 (8.3)	13/32 (40.6) 0 (0)	7.7 ± 8.1	1.22 ± 1.4 3.00 ± 1 6	17.5 ± 2.2
16	386/28 13.8 ± 1.8	356 12.7 ± 1 9	30/386 (7.8)	14/28 (50 0) 0 (0)	7.6 ± 8 3	1.07 ± 1.2 2.14 ± 1.5	17.1 ± 2.2

Summary of experiment findings:

- The most sensitive response occurred when the pregnant mice were treated on day 10 of pregnancy (the first day dosed). Treatment on or after day 15 did not affect embryolethality. Fetal weight (average/litter) was most affected on GD 10.

Table 2. Phase specificity experiments with 5-azacytidine (2 mg/kg body weight). Frequency of malformations (percent)

	Treatment on day		
	10	11	12
Number of fetuses	189	370	277
Malformed fetuses (%)	124 (65.6)	320 (86.5)	5 (1.8)
Malformed fetuses per litter	4.7 ± 4.1	10.3 ± 3.8	0.2 ± 1.2
Fetuses with			
Exencephaly	3 (1.6)	–	–
Cleft palate	91 (48.1)	142 (38.4)	3 (1.1)
Defects of skull bones	67 (35.4)	30 (8.1)	–
Rib anomalies	4 (2.1)	5 (1.3)	–
Fusion of sternebrae	22 (11.6)	36 (9.7)	1 (0.4)
Malformed ulna + radius	81 (42.8)	1 (0.2)	–
Malformed tibia, fibula + femur	69 (36.5)	12 (3.2)	–
Frontpaw polydactyly		1 (0.2)	–
Frontpaw syndactyly	1 (0.5)	1 (0.2)	–
Frontpaw a-/oligodactyly	2 (1.0)	107 (28.9)	–
Hindpaw polydactyly	1 (0.5)	1 (0.2)	–
Hindpaw syndactyly	1 (0.5)	1 (0.2)	–
Hindpaw a-/oligodactyly	2 (1.0)	194 (52.4)	1 (0.4)
Tail anomalies	4 (2.1)	28 (7.6)	–
Hematomas	–	17 (4.6)	–

Table 3. Phase specificity experiments with 5-azacytidine (2 mg/kg body weight). Frequencies of histopathological findings in the pre-term fetuses (day 18 p.c.) (percent)

Diagnosis	Treatment on day p.c.			
	10	11	12	13
Number of fetuses observed	26	31	21	33
Hypoplasia of the telencephalon	25/26 (96.1%)	13/31 (41.9%)	7/21 (33.3%)	9/33 (27.3%)
Hypoplasia of the basal ganglia	22/26 (84.6%)	4/31 (12.9%)	3/21 (14.3%)	3/33 (9.1%)
Formation of syncytial cell islands within the ventricular zone	1/26 (3.8%)	28/31 (90.3%)	19/21 (90.4%)	4/33 (12.1%)
Subpial heterotopias	0/26 (0%)	2/31 (6.5%)	17/21 (80.9%)	28/33 (84.8%)
Capillary ectasias and hemorrhagias	1/26 (3.8%)	14/31 (45.2%)	4/21 (19.0%)	0/33 (0%)
Necrotic cardiomyopathy	2/26 (7.7%)	17/31 (54.8%)	6/21 (28.6%)	1/33 (3.0%)

(%) represents # fetuses with findings/#total # fetuses, not % control

Summary of findings:

- Malformations observed only on GD10-12. The malformation findings in the day 10 animals consisted of cleft palate, defects of skull bones, malformed ulna, radius, tibia, fibula, and femur. Treatment on day 11 showed oligo-and adactyly of the front and hind paws and large hematomas at the distal end of the hindpaws, in addition to the occurrence of cleft palate.
- The histological findings, including severe hypoplasias of dorsal forebrain and the basal ganglia were most often seen when dams were treated with 2 mg/kg azacytidine on day 10 post conception. Findings occurred to a significantly lesser degree when treatment on day 11 or 12. Other histological findings in the day 11 /12 treatment groups included small capillary ectasias, hind paw hematomas, and extensive necrotic cardiomyopathy. No concurrent or historic control values were presented in the article. Litter incidences were not reported.

Findings in dose-dependency experiment:

Table 4. Dose dependency experiments with 5-azacytidine. Compilation of the embryotoxicity data

Day of treatment (dose, mg/kg)	Implantation sites/litter Av. no. per litter	Living fetuses Av no per litter	Resorptions/ implantation sites (%)	Litters with resorptions a) partially (%) b) completely (%)	Resorptions Av % per litter	Resorptions a) per litters b) per litters with res.
–	382/26	356	26/382	13/26 (50.0)	5.9 ± 9.2	1.00 ± 1.2
(Controls)	14.7 ± 2.1	13.7 ± 2.2	(6.8)	0 (0)		2.00 ± 1.1
12 (0.5)	304/22	278	26/304	12/26 (46.1)	8.2 ± 9.7	1.18 ± 1.2
	13.8 ± 1.8	13.1 ± 1.9	(8.5)	0 (0)		2.17 ± 1.7
12 (1.0)	324/24	291	33/324	14/24 (58.3)	9.7 ± 9.2	1.37 ± 1.9
	13.5 ± 1.5	13.0 ± 1.5	(10.2)	0 (0)		2.36 ± 1.4
12 (2.0)	312/21	277	35/312	14/21 (66.6)	10.6 ± 9.4	1.67 ± 1.3
	14.9 ± 1.7	13.2 ± 1.8	(11.2)	0 (0)		2.50 ± 1.5
12 (4.0)	268/20	155	113/268	17/20 (85.0)	40.8 ± 26.5	5.65 ± 1.6
	13.4 ± 1.9	12.8 ± 1.7	(42.2)	3/20 (15.0)		6.65 ± 2.3
14 (0.5)	296/21	272	24/296	8/21 (38.1)	7.9 ± 6.8	1.14 ± 1.1
	14.1 ± 1.6	13.4 ± 1.9	(8.1)	0 (0)		3.00 ± 1.6
14 (1.0)	313/23	285	28/313	10/23 (43.5)	8.5 ± 6.5	1.22 ± 1.2
	13.6 ± 1.6	12.9 ± 2.0	(8.9)	0 (0)		2.80 ± 1.8
14 (2.0)	372/26	328	44/372	21/26 (80.7)	13.8 ± 4.2	1.69 ± 1.4
	14.3 ± 2.4	12.6 ± 2.3	(11.8)	0 (0)		2.10 ± 1.6
14 (4.0)	310/24	218	92/310	20/24 (83.3)	27.6 ± 12.2	3.83 ± 1.9
	12.9 ± 1.7	12.4 ± 1.9	(29.7)	4/24 (16.7)		4.60 ± 1.7

Comment:

- Treatment on day 12 or day 14 with 0.5, 1, 2 or 4 mg/kg of 5-azacytidine exerted a dose-related embryotoxicity, as well as fetal weight reduction. In the day 12 group, mean fetal weight reduction was significant at greater than 0.5 mg/kg 5-AZ. The reduction was significant in all four treated groups in day 14 group.

Summary of individual study:

- The main effects of 5-azacytidine treatment on embryo-fetal development were growth retardation (embryotoxicity) and the occurrence of malformations. Both variables were dose related, "especially with regard to the resorption rate, weight increase, bone malformations, and brain malformations."
- These variables were also dependent on the time of treatment. For instance, intrauterine lethality (% of resorptions/implantation sites) was greatest when the treatment was on day 10 while other phase specific effects (skeletal abnormalities, CNS abnormalities, and the appearance of capillary ectasias, hind paw hematomas and necrotic cardiomyopathy), were observed when dosing was between day 10 to day 13 of gestation.
- Other important histological anomalies were vascular dilatations and myocardial necrosis (according to the author, resembling partly the clinically observed rhabdomyolysis after therapeutic applications of this drug to man).

Study title: Effect of 5-azacytidine administration during very early pregnancy. Fundamental and Applied Toxicology, 23: 429-433, 1994 (Cummings).

Key study findings:

- Dose-dependent embryotoxicity occurred when 5-AZ was administered to rats during Days 1-8 of pregnancy. These toxicities, including decreases in offspring survival, fetal weight and increase of incidences of malformations (microphthalmia and exencephaly), were evident on Day 20, but not on Day 9 of pregnancy.
- Postimplantation exposure to 5-AZ produced an increase in resorptions and a decrease in fetal survival and fetal weight, with no gross external malformations evident.

Study no.: Not applicable
Volume #, and page #: Module 4.2.3.5.2.1
Conducting laboratory and location: published article
Date of study initiation: published 1994
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: 5-azacytidine (5-AZ) (Sigma); lot 19F-0118.

Methods

Dose:

Early pregnancy protocol (Day 9) and developmental toxicity study (Day 20): 0 (saline control), 0.15, 0.3, 0.6, and 1.2 mg/kg/day

Pre- versus postimplantation study: 0.5 or 1 mg/kg on Days 1-3 or on Days 4-8. Saline was given to the other 3 days on which no 5-AZ was administered.

Species/strain: Female Holtzman rats at 60 days of age

Number/sex/group: n=8/group. The number of fetuses in each endpoint evaluated was shown in respective tables.

Route of administration: IP at injection volume of 0.4 ml/kg body weight.

Study design:

- Early pregnancy protocol:
  5-AZ was administered on Days 1-8 of pregnancy and the pregnant rats were killed on Day 9.
- Developmental toxicity study:
  5-AZ was administered on Days 1-8 of pregnancy and the pregnant rats were killed on Day 20.
- Pre- versus postimplantation study:
  5-AZ was administered on Days 1-3 or days 4-8 of pregnancy and dams were killed on Day 20.

Parameters and endpoints evaluated:

- Early pregnancy study:
  Measurement of potential maternal toxicity: body weight, trimmed uterine weight, number of implantation sites, number of resorptions (sites exhibiting extravasation of blood), implantation site weight (sites trimmed of intersite tissue and weighed), trimmed ovarian weight, and number of corpora lutea (CL).
  Serum for the assay of progesterone, estradiol, and luteinizing hormone (LH, sensitivity 15 pg/tube) by radioimmunoassay.
- Developmental toxicity study:
  Maternal weight, trimmed uterine weight, number of live fetuses and resorptions, fetal weight, gross external abnormalities of fetuses, trimmed ovarian weight, number of CL in the uterus.
- Pre- versus postimplantation study:
  Fetuses and maternal tissues were evaluated as in the developmental toxicity study.

Note: figures and tables are from the article.

Results

Mortality (dams): None reported

Clinical signs (dams): Not performed

Body weight (dams): see in the individual tables below

Food consumption (dams): Not performed

Toxicokinetics: Not performed

Findings in early pregnancy protocol:

TABLE 1
Early Pregnancy Protocol and 5-Azacytidine Effects in Rats[a]

Parameter[b]	Dose (mg/kg/day) 0	0.15	0.3	0.6	1.20
N	8	8	8	8	8
Maternal body weight gain (g)	16.5 ± 2.0	20.0 ± 1.7	17.9 ± 3.3	10.4 ± 1.8	12.9 ± 2.1
Implantation sites (No.)	13.0 ± 0.7	13.1 ± 0.7	12.5 ± 0.5	11.6 ± 0.5	13.3 ± 0.4
Resorptions (No.)	0.4 ± 0.3	0.0 ± 0.0	0.3 ± 0.3	1.3 ± 0.6	0.4 ± 0.3
Implantation site weight (mg)	111 ± 9	117 ± 5	113 ± 5	109 ± 4	103 ± 4
Serum progesterone (ng/ml)[c]	80 ± 6	86 ± 10	69 ± 5	66 ± 3	69 ± 6
Serum estradiol (pg/ml)[c]	10 ± 2	5 ± 2	8 ± 2	6 ± 2	8 ± 1
Serum LH (pg/ml)[c]	309 ± 46	283 ± 42	219 ± 11	265 ± 33	284 ± 40

[a] Doses of 5-azacytidine, as indicated, were administered on Days 1–8 of pregnancy.
[b] All parameters were assessed following euthanasia on Day 9 of pregnancy. Data are expressed as the mean ± SE. No significant differences between treated and control groups were found for any parameter listed.
[c] Serum was collected on Day 9, frozen, and assayed for hormones by RIA.

Comment: treatment of 5-AZ on days 1-8 of pregnancy did not cause significant effects on dams or fetuses when assessed on Day 9.

Findings in developmental toxicity study:

TABLE 2
Evaluations on Day 20 of Pregnancy after Early Pregnancy Exposure of Rats to 5-Azacytidine[a]

Parameter[b]	Dose (mg/kg/day) 0	0.15	0.30	0.60	1.20
N	8	8	8	8	8
Maternal body weight gain (g)[c]	42 ± 4	41 ± 3	35 ± 2	34 ± 2*	24 ± 3**
Ovarian weight (mg/pair)	107 ± 5	105 ± 4	112 ± 4	99 ± 5	103 ± 8
Pregnant uterine weight (g)	71 ± 4	63 ± 5	44 ± 10	21 ± 10**	2 ± 0.5**
No. corpora lutea	12 ± 0.4	12 ± 0.5	12 ± 1.0	13 ± 0.8	14 ± 0.7

[a] Doses of 5-azacytidine, as indicated, were administered to rats on Days 1–8 of pregnancy.
[b] All parameters were assessed following euthanasia of the dams on Day 20 of pregnancy. Data are expressed as the mean ± SE.
[c] Extrauterine body weight gain, Days 1–20; difference between the weight of the dam on Day 1 and the weight of the dam on Day 20 without the uterus.
* Significantly different from vehicle-treated controls ($p < 0.05$).
** Significantly different from vehicle-treated controls ($p < 0.005$).

Treatment of 5-AZ on Days 1-8 caused dose-related reduction of trimmed uterine weight when dams were killed on Day 20 of pregnancy. The other maternal toxicity was significant weight gain reduction at higher dose levels. Other parameters, e.g., ovarian weight and number of CL, were not different from the control.

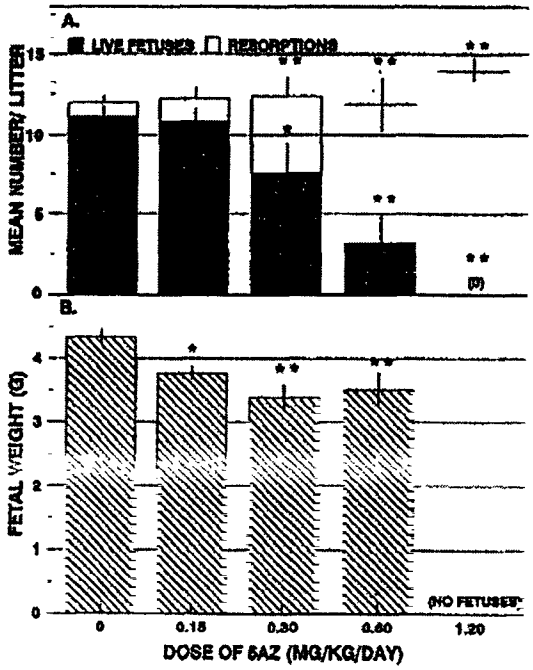


FIG. 1. Effect of 5-azacytidine on pregnancy outcome in rats. Dams received 5-azacytidine at the doses indicated during Days 1–8 of pregnancy; evaluations were performed on Day 20. (A) Fetal survival and incidence of resorptions. (B) Fetal weight. Data are shown as means ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.005$, significantly different from vehicle (0)-treated controls.

TABLE 3
Malformations Found Following 5-Azacytidine Exposure of Rats during Early Pregnancy[a]

	Dose (mg/kg/day)				
Malformation[b]	0	0.15	0.30	0.60	1.20
Umbilical hernia	0/89	0/87	1/61	1/26	No fetuses
Microphthalmia	0/89	0/87	3/61*	2/26*	No fetuses
Hydrops	0/89	0/87	1/61	0/26	No fetuses
Exencephaly	0/89	0/87	4/61*	0/26	No fetuses

[a] Doses of 5-azacytidine, as indicated, were administered on Days 1–8 of pregnancy.
[b] Fetuses were examined for gross external anomalies after removal from the dams on Day 20 of pregnancy. Data are expressed as the total number of fetuses showing each malformation/the total number of fetuses per treatment group.
* Significantly different from vehicle-treated controls ($p < 0.05$).

5-Azacitidine caused a dose-related severe reduction in the number of surviving fetuses and in weight of live fetuses. There were no surviving fetuses at dose level of 1.2 mg/kg/day. Two malformations were significantly more evident (from the 0.3 mg/kg/d group) than the control: microphthalmia and exencephaly.

Findings in pre- versus postimplantation study:

TABLE 4
Pre- vs Postimplantation Exposure of Rats to 5-Azacytidine: Evaluation on Day 20 of Pregnancy

	Dose[a] (days)				
Parameter[b]	0	0.5 (1–3)	0.5 (4–8)	1.0 (1–3)	1.0 (4–8)
N	8	8	8	8	8
Maternal body weight gain (g)[c]	36 ± 3	40 ± 3	44 ± 4	41 ± 6	33 ± 6
Ovarian weight (mg/pair)	99 ± 5	107 ± 4	100 ± 4	104 ± 3	80 ± 7
Pregnant uterine weight (g)	71 ± 4	75 ± 5	50 ± 11	72 ± 6	22 ± 13**
No. corpora lutea	14 ± 0.5	13 ± 0.6	13 ± 0.4	13 ± 0.4	13 ± 0.7

[a] Doses of 5-azacytidine, in mg/kg/day, were administered to rats on Days 1–3 (preimplantation interval) or Days 4–8 (postimplantation interval) of pregnancy.
[b] All parameters were assessed on Day 20 of pregnancy. Data are expressed as the mean ± SE.
[c] Extrauterine body weight gain, Days 1–20, difference between the weight of the dam on Day 1 and the weight of the dam on Day 20 without the uterus.
** Significantly different from vehicle-treated controls ($p < 0.005$).

Treatment of 5-AZ during the preimplantation interval (Days 1-3) had no effect on the number of surviving fetuses, the number of resorptions, or fetal weight (figure below). The pregnant uterine weight was not affected. Treatment during postimplantation interval (Days 4-8) at the dose of 0.5 mg/kg/day reduced fetal weight. Higher dose treatment (1 mg/kg/day) during this interval resulted in a significant reduction in fetal survival and an increase in the number of resorptions, but no effect on fetal weight.

Pregnant uterine weight was significantly reduced by this treatment, but neither ovarian weight, the number of CL, nor gross external malformations were affected.

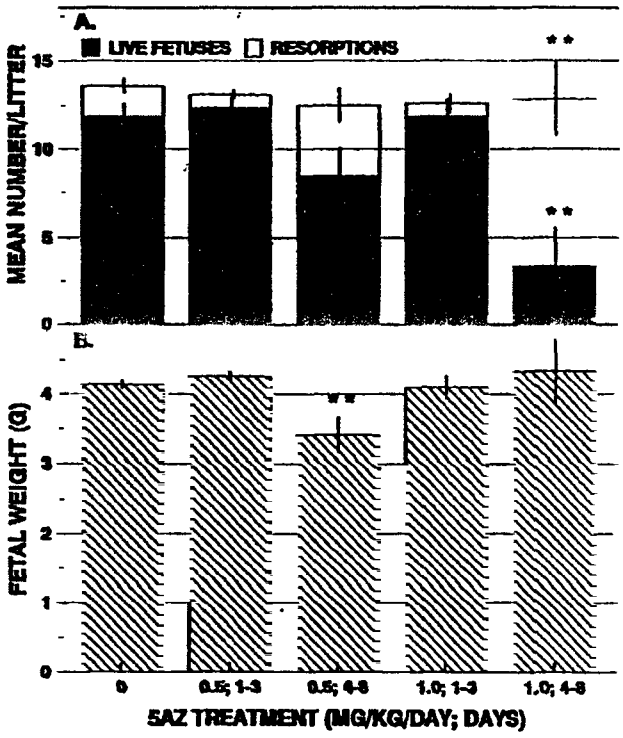


FIG. 2. Pre- versus postimplantation exposure to 5-azacytidine in rats. Dams received 5-azacytidine on Days 1–3 (preimplantation period) or Days 4–8 (postimplantation period) at either 0.5 or 1.0 mg/kg/day and were evaluated on Day 20. (A) Fetal survival and incidence of resorption. (B) Fetal weight. Data are shown as means ± SEM. $*p \leq 0.05$ and $**p \leq 0.005$, significantly different from vehicle (0)-treated controls.

Summary of individual study:
Treatment of 5- azacytidine during early pregnancy (Days 1-8) resulted in dose-related embryo- as well as maternal toxicity when observed on Day 20. The former was shown as reduction of surviving fetuses, fetal weight loss and fetal malformations (mainly microphthalmia and exencephaly), and the latter, decrease in maternal body weight gain and reduced pregnant uterine weight. Reduction in living fetuses and high incidence of resorption may also contribute to the decrease in uterine weight.

The embryotoxic effect of 5-azacytidine was related to gestation day exposure. Exposure of rats to 5-AZ, 0.5 mg/kg or 1 mg/kg, during postimplantation interval was embryotoxic. Treatment of 5-AZ at 0.5 mg/kg/day caused significant reduction in fetal weight and 1 mg/kg/day resulted in reduction of pregnant uterine weight, fewer surviving fetuses, and increased number of resorptions. In this study (pre vs post implantation dosing), an increase in resorptions was observed without a significant change in maternal body weight gain, indicating a developmental toxicity without maternal toxicity.

Study title: Teratogenicity of 5-azacytidine in the Sprague-Dawley rat. J Toxicol Environment Health, 29: 201-210, 1990 (Rosen et al.).

Key study findings:

- 5-Azacytidine was embryolethal, caused reductions in fetal weight, and affected fetal development.

Study no.: Not applicable
Volume #, and page #: Module 4.2.3.5.2.3
Conducting laboratory and location: published article
Date of study initiation: published 1990
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: Not reported

Methods

Dose:
0 (saline control), 0.5, 1, and 2 mg/kg/day
Species/strain: Time-pregnant Sprague-Dawley rats (60-90 d of age).
Number/sex/group: n=6/group in 5-AZ treated group, and n=5/group in control groups. The number of fetuses in each endpoint evaluated was shown in respective tables.
Route of administration: IP; volume 0.1 ml.
Study design:
Treatments of 5-azacytidine were administered on one of 4 days of gestation (d 9, 10, 11, or 12). Dams were killed on gestation d 20 and litters were removed and weighed.
Parameters and endpoints evaluated:
Maternal effects: number of pregnant, average live fetus per litter, average dead fetus per litter, average fetal weight per litter, and fetal abnormalities.

Note: Tables are from the article.

Results

Mortality (dams): None reported

Clinical signs (dams): Not performed

Body weight (dams): Not performed

Food consumption (dams): Not performed

Toxicokinetics: Not performed

Effect of 5-AZ on fetal survival and fetal weight when administered on various day of gestation:

TABLE 1. Effect of 5-Azacytidine on Fetal Survival and Average Fetal Weight Following Exposure on Days 9 and 10 of Gestation[a]

	Day 9 exposure (mg/kg)				Day 10 exposure (mg/kg)			
Observation	0	0.5	1	2	0	0.5	1	2
Number bred	5	6	6	6	5	6	6	6
Number pregnant	5	5	5	6	5	6	2	4
Average live per litter	12.8 ± 0.5	14.0 ± .04	2.0 ± 0.8[b]	1.2 ± 0.7[b]	13.2 ± 1.1	10.5 ± 1.6	3.5 ± 1.5[b]	8.0 ± 3.8
Average dead per litter	0.2 ± 0.2	0	11.2 ± 1.3[b]	11.3 ± 1.4[b]	0.6 ± 0.6	3.0 ± 1.5	9.5 ± 0.5[b]	5.5 ± 3.2
Average fetal weight per litter	4.37 ± 0.10	4.08 ± 0.11[b]	3.72 ± 0.13[b]	2.97 ± 0.20[b]	4.48 ± 0.09	3.31 ± 0.25[b]	2.68 ± 0.28[b]	3.11 ± 0.31[b]

[a]Averages are mean ± SE.
[b]Different from controls ($p \leq .05$).

TABLE 2. Effect of 5-Azacytidine on Fetal Survival and Average Fetal Weight Following Exposure on Days 11 and 12 of Gestation[a]

	Day 11 exposure (mg/kg)				Day 12 exposure (mg/kg)			
Observation	0	0.5	1	2	0	0.5	1	2
Number bred	5	6	6	6	5	6	6	6
Number pregnant	5	4	6	5	4	6	5	3
Average live per litter	9.0 ± 2.6	11.8 ± 1.4	11.0 ± 1.9	11.4 ± 0.7	11.3 ± 1.1	13.5 ± 0.4	12.6 ± 1.0	11.3 ± 0.3
Average dead per litter	1.2 ± 1.0	1.3 ± 0.5	1.2 ± 1.0	1.6 ± 0.6	0.8 ± 0.3	0	0	0.3 ± 0.3
Average fetal weight per litter	4.53 ± 0.29	3.92 ± 0.23	3.92 ± 0.22	3.32 ± 0.25[b]	4.53 ± 0.07	4.02 ± 0.17[b]	3.67 ± 0.07[b]	3.67 ± 0.07

[a]Averages are mean ± SE.
[b]Different from controls ($p \leq .05$).

Summary of effect of 5-AZ on fetal survival and weight:

- 5-Azacytidine was embryotoxic, causing embryonic death at 1 and 2 mg/kg on gestation d 9 and 10. This effect was most pronounced on d 9 where 2 litters in the 1 mg/kg dose group and 4 litters in the 2 mg/kg group were totally resorbed.
- Average fetal weight decreased when treatment was on d 9 or d 10 and the effect was dose-related. When treatment was on d 11 or d12, the effect of 5-AZ was not even in all the treated groups, i.e., some treated groups did not have significant reduction in fetal weight compared to the control.

TABLE 3. Incidence of Fetal Anomalies Following Exposure to 5-Azacytidine on Days 9 and 10 of Gestation

	Day 9 exposure (mg/kg)				Day 10 exposure (mg/kg)			
Necropsy observations (fetuses/litters)	0	0.5	1	2	0	0.5	1	2
Number examined	64/5	70/5	10/3	7/2	66/5	62/6	7/2	32/4
Exencephaly-encephalocele	0	7/1	3/2[a]	6/2[a]	0	1/1	3/2[a]	2/2
Micromelia	0	0	0	0	0	0	0	4/2
Club foot	0	0	0	0	0	2/1	3/1	0
Syndactyly	0	1/1	1/1	0	1/1	1/1	0	0
Oligodacytly	0	1/1	0	0	0	2/2	0	1/1
Micrognathia	0	0	0	0	0	1/1	0	0
Gastroschisis	0	1/1	0	0	0	7/3[a]	3/1	3/1
Edema	0	0	0	0	0	0	0	1/1
Fused ribs	0	0	0	0	0	42/5[a]	7/2[a]	15/3[a]
Missing ribs	0	2/1	0	0	0	1/1	1/1	5/2
Wavy ribs	6/1	0	0	0	0	12/2	4/2[a]	11/4[a]
Extra ribs or ossifications	6/2	34/5[a]	5/2	1/1	1/1	18/5[a]	0	13/2
Average number ossified sternebrae per litter (mean ± SE)	5.7 ± 0.2	5.8 ± 0.1	4.9 ± 0.3[a]	4.3 ± 0.8[a]	5.9 ± 0.1	3.9 ± 0.6[a]	2.4 ± 0.6[a]	2.3 ± 1.3[a]

[a]Different from controls ($p \leq .05$).

TABLE 4. Incidence of Fetal Anomalies Following Exposure to 5-Azacytidine on Days 11 and 12 of Gestation

Necropsy observations (fetuses/litters)	Day 11 exposure (mg/kg)				Day 12 exposure (mg/kg)			
	0	0.5	1	2	0	0.5	1	2
Number examined	45/5	47/4	66/6	57/5	45/4	81/6	63/5	34/3
Exencephaly-encephalocele	0	0	0	2/2	0	0	0	0
Micromelia	0	0	0	24/3[a]	0	5/1	0	0
Club foot	0	0	1/1	0	0	11/2	45/4[a]	16/2
Syndactyly	2/2	0	2/1	6/3	0	3/1	18/4[a]	11/2
Oligodactyly	0	0	0	7/2	0	4/1	25/4[a]	19/2
Micrognathia	0	27/3[a]	28/3[a]	16/4[a]	0	0	1/1	0
Gastroschisis	0	0	0	2/1	0	0	0	0
Edema	0	0	0	8/3[a]	0	0	0	0
Fused ribs	0	1/1	2/2	7/4[a]	0	0	0	0
Missing ribs	1/1	16/3	32/4[a]	42/4[a]	0	0	1/1	0
Wavy ribs	1/1	0	2/2	1/1	1/1	0	0	0
Extra ribs or ossifications	0	0	0	0	2/2	2/2	1/1	1/1
Average number ossified sternebrae per litter (mean ± SE)	5.8 ± 0.1	5.7 ± 0.2	5.7 ± 0.3	3.9 ± 0.8[a]	5.8 ± 0.1	5.8 ± 0.1	5.2 ± 0.3[a]	5.4 ± 0.1

[a]Different from controls ($p \leq .05$).

Summary of teratogenic effect of 5-AZ:

- Exencephaly-encephalocele: most prominent in 1 and 2 mg/kg groups when treatment was on d 9, despite the high mortality rate in these groups that made the estimation difficult. The findings were also seen on d 10 and 11.
- Extra ribs or ossifications: "supernumerary ribs ranging in size from approximately one-half of the thirteenth rib to minute ossification sites were increased on d 9 and 10."
- Limb anomalies:
  - Micromelia: 2 mg/kg group exposed on d 11 and associated with the most severely affected fetuses.
  - Club foot, syndactyly and oligodactyly: in d 12 treatment groups and the difference was significant in 1 mg/kg group compared to the control.
- Other anomalies:
  - Micrognathia and missing ribs: in d 11 treatment groups. "No gaps in the alignment of the ribs were noted, indicating that the missing rib was either the first or thirteenth."
  - Gastroschisis: noted from d 9 to d 11 but highest on d 10.
  - Fused and wavy ribs: at all doses on d 10 and 11.
  - Severe general edema: in 2 mg/kg group on d 11.
  - Delay in sternebral ossification: treated groups on d 9, 10 and 11. .
  - Average number of ossified sternebrae per litter: significantly reduced in 1 and 2 mg/kg groups on d 9, all treated groups on d 10 and 2 mg/kg group on d 11.

Summary of individual study:

5-Azacitidine was teratogenic with findings varying depending upon day of dosing. The findings are probably unrelated to maternal toxicity. While maternal body weight was not measured in this study, in some studies similar dose levels administered to non-pregnant or pregnant rats resulted in no body weight changes (e.g., see p 92 and 121 of this review). In another study body weight changes were observed in pregnant rats (table, p 120). 5-Azacitidine was administered repeated in these studies, in contrast to the single dose administered in this study.

Prenatal and postnatal development

Study title: Enhanced mortality in offsprings of male mice treated with 5-azacytidine prior to mating. Morphological changes in testes. Neoplasma 23: 53-60, 1976 (Seifertova et al.)

Key study findings:

- 5-Azacytidine administered to male mice prior to mating resulted in their decreased fertility and in the loss of offsprings at different periods of embryonic and postnatal development.

Study no.: Not applicable
Volume #, and page #: Module 4.2.3.5.3.1
Conducting laboratory and location: published article
Date of study initiation: published 1976
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: Not reported

Note: the figure and tables are from the article.

Methods

Doses:
Histology and cytology of testes: 3 mg/kg/day x3d
Induction of embryonic lethality: ♂ 3.3 mg/kg/day x3d; ♀ 3.3 mg/kg/ on d 11-13 of pregnancy
Evaluation of perinatal and postnatal mortality: 3.3 mg/kg/day x3d
Effect on fetal resorption: 1, 3 and 5 mg/kg
Species/strain: mice, random bred strain II (32-38 g)
Number/sex/group: See below "study design"
Route, formulation, volume, and infusion rate: IP injection
Satellite groups used for toxicokinetics: Not performed
Study design:

- Histology and cytology of testes.
  Males were administered 5-azacytidine and then received colchicine (40 μg/mouse) 24 h after the last injection. The animals were killed 5 hours after colchicine and testes removed and processed for the histology and chromosomal analysis.
- Induction of embryonic lethality:
  Females (n=9/group) were mated with treated males (dx3, mated 24 h after last dose; n=3/group) on 7, 14 and 21 days post-treatment with 5-azacytidine (3.3 mg/kg). Females with vaginal plugs were sacrificed for uterine analysis on d 14-16. Females dosed daily on d 11-13 of pregnancy and sacrificed on d 14 for uterine analysis were mated with untreated males.

- Evaluation of perinatal and postnatal mortality:
  Males were given either 5-azacytidine (n=9) or saline (n=6) for three consecutive days, and 24 hours after the last injection one male was caged with three females. The day of probable fertilization was determined according to the date of birth.
- Resorption of fetuses following 5-azacytidine:
  Exp 1.a. 5-Azacytidine was given daily to dams on days 11-13. Resorptions were assessed on d 14 of pregnancy. In this segment females were bred with untreated males.
  Exp 1.b. Treated males were caged with females (1M + 3F) 24 h after the last injection. Pregnancy day 1 was determined according to the presence of vaginal plugs. Female mice were sacrificed after 14 days.
  Note: There was no information in the article about the control, e.g., untreated females mated with untreated males, which gender received saline, number of the animals.
  Exp 2. Effect of the time interval on resorption of fetuses.
  5-Azacytidine (3 mg/kg/day) was given IP to groups of 9 males on 3 consecutive days. The males were mated 1, 2, 3 and 4 weeks later and after 18 days the pregnant females were sacrificed.
  Note: There was no information about the number of untreated males (control).

Results

Treated males

- Histological findings in testes of 5-AZ-treated mice:
  5-Azacytidine interfered with mature spermatozoa and spermatids at later stages of their development. A disorganization of the cellular pattern of seminiferous epithelium was noted. The authors report that "in the lumina of affected tubules the association of cells of different maturation stages was distorted, and empty spaces were formed between the cells localized on the basement membrane and the succeeding generations of cells."
- Chromosomal analysis of spermatogonia: unremarkable

F_1 litter data:

- Induction of embryonic lethality

Table 1. Embryonic lethality in progeny of males and females treated by 5-azacytidine. The drug (3.3 mg/kg) was administered to groups of males on 3 consecutive days. First group (1 male with 3 females) was caged for 7 days 24 h following 5-azacytidine, and further groups for a similar period 7, 14 and 21 days post-treatment. Mated females were killed for uterine analysis 14—15 days after vaginal plug was found. Females mated with untreated males received on days 11, 12 and 13 of pregnancy 5-azacytidine (3.3 mg/kg) and were killed after 14 days for uterine analysis

Mating post-treatment days	Number of females		Total implants among fertile females	Living embryos among fertile females	Dead implants %	Living embryos as % of control among females	
	mated	pregnant				fertile	mated
Control	9	8	8.7 ± 1.1	8.0 ± 0.9	7.6	100	100
Females	9	8	7.8 ± 1.0	4.8 ± 0.6	38.0	60.0	55.1
1—7[b]	9	4	5.2 ± 1.3	3.7 ± 0.6[a]	28.5	46.2	20.5
7—14[b]	9	6	5.6 ± 0.4	4.6 ± 0.4	17.6	57.4	39.7
14—21[b]	9	7	7.4 ± 1.3	6.5 ± 1.6	11.5	85.0	65.1
21—28[b]	9	6	8.8 ± 1.2	8.1 ± 1.1	7.5	101.0	69.2

[a] $P < 0.002$. — [b] Males treated with 5-azacytidine.

- Females mated with males treated with 5-azacytidine showed a decrease in the average number of live embryos when mating was within 1-7 days of dosing.

- Evaluation of perinatal and postnatal mortality

Table 2. Litter sizes at birth and weaning in offsprings from 5-azacytidine treated males. Males received 5-azacytidine (3.3 mg/kg) on 3 consecutive days and were caged with females (1 male with 3 females) 24 h after the last injection. The day of fertilization was determined according to the time of birth

Mating posttreatment days	Number of females	Females with implants	Mean litter-size		Lethality factor[a] %	
			1 day	21 days	1 day	21 days
Control	18	11	6.9 ± 1.1	6.5 ± 1.3	0	0
1—7	18	10	5.9 ± 0.9	2.6 ± 1.0[b]	15	60
8—13	9	4	7.5 ± 2.2	4.5 ± 2.5	—7	31

[a] Lethality factor % $= 1 - \frac{\text{live offsprings per female in experimental group}}{\text{live offsprings per female in control group}} \times 100$.

[b] $P < 0.002$.

- Mean litter size was decreased in the day 1-7 treatment group at 21 d.

Resorption of fetuses following 5-AZ

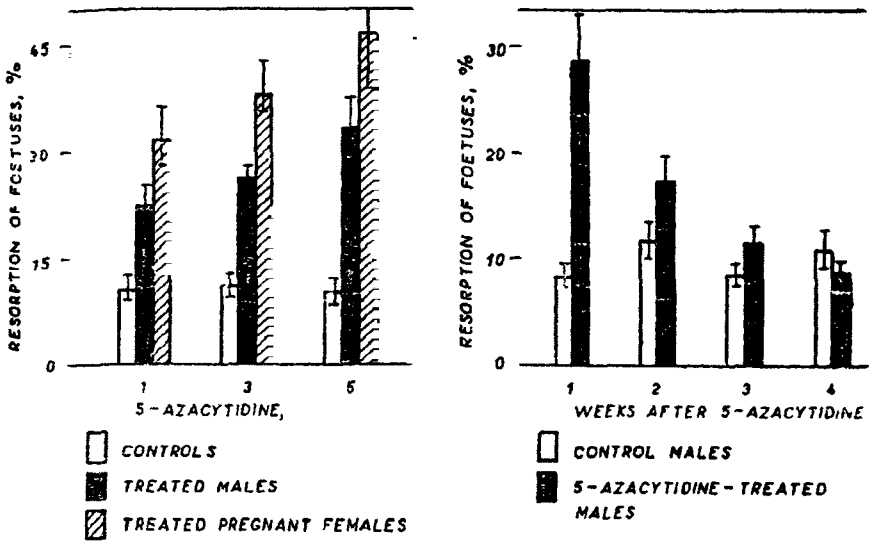


Fig. 1. Enhanced resorption of mouse foetuses in relation to the dose level of administered 5-azacytidine to males or pregnant females. 5-Azacytidine (mg/kg) was given i. p. to groups of 6 males or 18 females on 11, 12 and 13 days of pregnancy. Males were mated 24 hr after the last treatment with the drug. The number of resorptions was counted on day 14 of pregnancy and is expressed as per cent of total foetuses in the control.
Fig 2 Effect of the time interval between the administration of 5-azacytidine to males and their mating on the number of resorbed foetuses. 5-Azacytidine was given i. p. to groups of 9 males on 3 consecutive days at a dose level of 3 mg/kg. The males were mated 1, 2, 3 and 4 weeks later and after 18 days the pregnant females were sacrificed. The number of resorbed foetuses was evaluated, and is expressed as per cent of total foetuses in the control.

5-Azacytidine increased resorption of fetuses in a dose-related (males and females) and time-interval (between the treatment of males and their mating) related manner.

Study Title: 5-Azcytidine-induced exencephaly in mice. Takeuchi and Takeuchi. J Anat 140 (3): 403-412, 1985. Article summary.

For the evaluation of the teratogenicity of 5-AZ, pregnant Slc:ICR mice received 1 mg/kg 5-AZ by a single IP on Day 6.5, 7.5, or 8.5 gestation. On Day 18 the females were killed and live and dead fetuses were counted. Live fetuses were examined for malformations.

One mg/kg 5-AZ treatment on Day 8.5 caused a significant increase in the mean fetal death rate, but no significant increases if the treatment was on Day 6.5 or 7.5. Significant decreases in fetal weight occurred in all groups treated with the drug. The mean malformation rates were significantly greater in the group treated on Days 7.5 and 8.5. Types and incidences of 5-AZ induced malformations are shown in the table below.

Table 1. *Effects of 5-azacytidine on the development of mouse fetuses*

Gestational day of treatment	Number of litters	Number of implantations	Mean fetal death rate (%)	Number of live fetuses	Mean fetal body weight: mean ± S.D. (g)	Mean malformation rate (%)
			5-azacytidine (1 mg/kg)			
6·5	10	138	15·2 ± 8·8	117	1·19 ± 0·10*	4·2 ± 5·4
7·5	10	126	27·8 ± 17·0	91	1·12 ± 0·14*	93·3 ± 9·0*
8·5	10	138	73·8 ± 17·6*	35	1·17 ± 0·08*	41·1 ± 38·9†
			Distilled water (control)			
7·5	10	143	14·1 ± 5·3	122	1·38 ± 0·08	2·6 ± 5·4

* $P < 0·01$ compared with the control.
† $P < 0·05$ compared with the control.

Table 2. *Types and incidence of external malformations in mouse fetuses after exposure to 1 mg/kg 5-azacytidine*

	Gestational day			Controls treated with water
	6·5	7·5	8·5	
Total number of live fetuses	117	91	35	122
Total number of malformed fetuses	5	84	13	3
Exencephaly	1	83	4	—
Encephalocoele	1	1	—	—
Eye anomalies	1	48	3	—
Microtia	—	1	—	—
Cleft palate	3	—	5	2
Umbilical hernia	—	—	1	—
Digital anomalies	—	—	3	—
Abnormal tail	—	—	2	1

3.4.7 Local tolerance

Study title: Cutaneous irritation in the topical application of 5-azacytidine (NSC #102816) to New Zealand white rabbits, Murphy et al., NCI contractor's report #RIPS-CIPA-102816-13-76. Study done: 8/9/76-8/12/76. (Module #4.2.3.6.1)

The skin irritation and organ toxicity potential of topically applied 5-AZ was evaluated in New Zealand white rabbits (n=3/sex/group). Three concentrations of 5-AZ (1, 3, and 9%) in 1% methyl cellulose (diluent) were applied to the skin on the dorsal surface. After 224 hours the sites were cleaned, read, and scored according to the Draize scoring system. The rabbits were necropsied and skin and internal organs (kidney, liver, spleen, femur, bone marrow, large or small intestines and ovaries or testes) were examined histopathologically. Mild skin irritation was noted at a concentration of 9% of 5-AZ, but the irritation was reversible. The primary irritation indexes, PII's, was 1.5, as compared to the PII's of the control and the other two concentrations was <1. "These scores are not sufficiently large by Draize criteria to assign primary irritant properties." (quote from the report). The histopathological evaluation also supported that 5-AZ caused mild skin irritation (subacute inflammation: plus 1-2, chronic inflammation: plus 1, no different from the diluent control). The following table summarizes the PII's result:

TABLE 2

Primary Skin Irritation Potential of 5-Azocytidine

Compound	Conc. (%)	PII*
5-Azocytidine	9	1.5
	3	0.8
	1	0.9
Methyl cellulose (diluent)	1	0.1

* PII--Primary irritation index, calculated according to the method of Draize (1944).

The original histopathological report (Table 4) of internal organs was missing from report copy from NCI. According to the authors: "No lesions, either macro- or microscopic, were noted.....", thus topically applied 5-AZ did not cause observable acute organ toxicity or primary skin irritation.

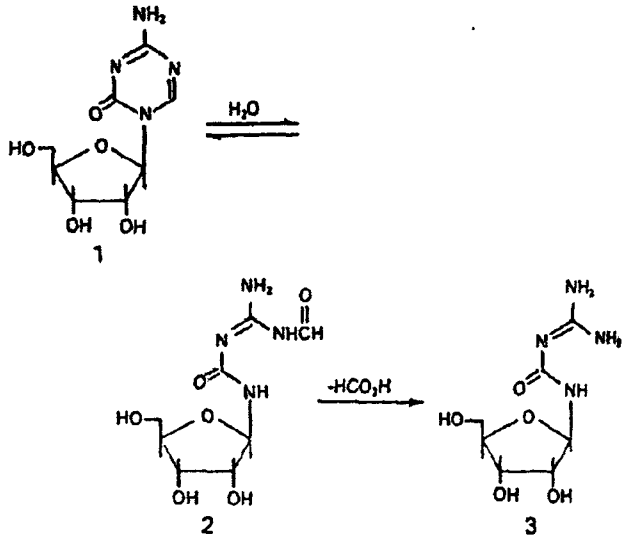
- The effect of 5-azacytidine on microcirculation in hamster cheek pouch has been reviewed by Dr. Almon Coulter (Appendix A) (Palm and Kensler, NCI Contractor's Report # PH-43-65-61, 1970, Module # 4.2.3.2.2)

3.4.8 Special toxicology studies

Study title: Isolation, characterization, and properties of a labile hydrolysis product of antitumor nucleoside, 5-azacytidine. Beisler, J Med Chem 21 (2): 204-208, 1978. Modules #4.2.2.4.1/4.2.3.7.6.1

In an aqueous environment, both *in vivo* and *in vitro*, 5-azacitidine underwent a spontaneous hydrolysis and resulted in an equilibration with a labile product, n-formylguanyl-ribosylurea (RGU-CHO), and finally the irreversible formation of guanylribosylurea (RGU). These components were isolated and identified by HPLC following analysis of 24 h-old water solution of 5-AZ. "RGU exhibited no pronounced toxicity when tested either *in vitro* or *in vivo*. Although RGU-CHO showed considerable antitumor activity against murine L1210 leukemia, hydrolysis studies indicated that all of the observed activity could be attributed to 5-AZ formed by *in vivo* equilibration from RGU-CHO." The following figure proposes the scheme of 5-AZ hydrolysis:

Scheme I



3.6 OVERALL CONCLUSIONS AND RECOMMENDATIONS

Conclusions:

The pharmacological findings reported in the literature to investigating the mechanism of action of 5-AZ as an antimetabolite and cytotoxic agent, as well as studies investigating pharmacokinetics, general toxicity, genotoxicity, carcinogenicity, and reproductive and development toxicity of 5-AZ were submitted to NDA 50-794. Although most studies were published journal articles that predated Good Laboratory Practices (21 CFR Part 58), and the study design and data obtained do not conform to current ICH guidelines, the submission is adequate to support the use of Vidaza for the proposed indication.

The administration routes in the pharmacokinetics studies were IP and IV, and in toxicology studies were oral, IP and IV. The proposed clinical administration route, subcutaneous injection, was not used in any of these submitted studies. However, clinical pharmacokinetic data have indicated comparable plasma PK profiles for IV and subcutaneous (SC) routes in humans. The plasma T_{max} was approximately 0.5 h for SC and 2 h for IV; plasma half-life was 4.2 h for SC and 3.5 h for IV. (quote from Dr. Coulter's review, Appendix A) Nevertheless, it is worth-mentioning that metabolism of 5-AZ displayed a species discrepancy, i.e., 5-AZ is more stable in the mouse liver S9 fraction compared to human.

The distribution of 5-AZ to the mouse blood was rapid after IP administration. The concentration peaked shortly after administration and depletion was rapid. The estimated T_{max} was 15 min and C_{max} at this time was 0.3-2 μg/ml, with $t_{1/2}$ estimated at 3.8 hours. Although the analysis of tissue concentration in the distributed organs was compromised by the sensitivity of the methodologies employed at that time, the distribution pattern can be correlated to some non-clinical findings:

1. There was minimal distribution to the CNS, but the amount of 5-AZ could be influential, since clinical observations in mice showed hypoactivity, ataxia, impaired righting reflex and prostration after a single or repeated IV administration.
2. The higher concentration and longer retention in lymphatic organs (e.g., spleen and thymus) may reflect blood distribution to these tissues. Alternatively, this distribution may also explain the observation that lymphoid tissue is a target of 5-AZ toxicity (e.g., increased extramedullary hematopoiesis in spleen in mice, degenerative changes in bone marrow and lymphatic tissues in dogs, and lymphoid hypoplasia of the lymph nodes and spleen in monkeys).
3. In female weaning — pigs, kidney and liver had the highest specific activities of radioactivity following IV administration (Appendix A). Liver and kidney were also target organs of 5-AZ toxicity. The histopathological findings in these two organs after single dose of 5-AZ included: cloudy swelling and focal necrosis of kidney tubule epithelium, reduced liver glycogen, and other minor degenerative liver lesions (IP to mice), increased hepatic neutral lipid at doses of ≥ 310 mg/m^2 (IV to rats), and early degenerative changes in the kidneys and liver (IV to dogs).
4. The carcinogenicity studies in rodents reported that 5-AZ increased incidences of tumors in the hematopoietic system (BALB/c mice), lung, lymphoid tissues, skin and mammary gland (B6C3F1 mice), and testicular tumors (Fisher rats). The findings could be attributed to organs containing rapidly dividing cells, thus sensitive to 5-azacitidine toxicity. The distribution and retention of 5-AZ to some of these organs may also be a factor.

5-Azacitidine and its metabolites were mainly excreted in the urine. In mice, within 6-8 h 50% of the compound (5-azacitidine and its decomposition products) was excreted, increasing to ~60% by 24 h. In a separate mouse study, approximately 45% of the radiolabeled compound was recovered in the urine and another 20% in the expired air (as $^{14}CO_2$). The HPLC chromatogram showed 6 major peaks in the urinary sample. These peaks included parent 5-azacytidine, deaminated metabolites: 5-azauracil, ribofuranosylbiuret and possibly 5-azauridine; hydrolytic products: RGU-CHO (with n-formylguanylribosylurea), RGU (guanylribosylurea), and azacytosine. The HPLC chromatogram pattern was similar in mice and dogs. Kidney excretion as the major elimination route should call the attention to the safety of administration of 5-AZ to renally-impaired patients.

5-Azacitidine did not inhibit cytochromes P450 1A2, 2C9, 2C19, 2D6, 2E1, or 3A4, when assayed in an in vitro system. However, some inhibition of enzyme activity was seen in vivo, possibly due to inhibition of gene activity. This inhibition was manifested by depression of enzymatic activity of ethoxycoumarin O-deethylase and ethylmorphine N-deethylase and an increased sleeping time in hexobarbital-treated mice.

The underlying mechanism of 5-AZ as an antimetabolite and cytotoxic agent is its ability to incorporate to DNA and demethylate (or prevent methylation) of replicating DNA. This hypomethylation action was also suggested to be the mechanism of 5-AZ's mutagenic effect in bacteria and the clastogenic effect in the induction of micronuclei

formation (chromosomal fragments) in mouse L5178Y cells and SHE cells. The submitted genotoxicity studies are all *in vitro* studies. The positive mutagenicity and clastogenicity studies of 5-AZ also support its tumorigenicity, as shown in the rodent (mice and rats) studies. Interpretation of the carcinogenicity studies is confounded by inadequate data (some doses exceeding the MTD, and hence high mortality and short exposure time, inadequate statistical analysis in some studies, as well as small number of surviving animals). However, the Executive Carcinogenicity Assessment Committee concurred with the findings reported in this review (Appendix B).

The reproductive and development toxicity of 5-AZ was tested in mice and rats. The positive toxicities of 5-AZ on male F0 rats, on embryonic growth and survival (embryotoxicity), and on fetal and newborn development (teratogenicity) have summarized (Executive Summary, p 4-5).

Unresolved toxicology issues: None

Recommendations:
The non-clinical studies submitted in NDA 50-794 adequately support the use of Vidaza for the treatment of MDS patients via subcutaneous injection.

Suggested labeling:

Proposed labeling:

Mechanism of Action

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The cytotoxicity of azacitidine is proportional to dose and exposure time. Although the mechanisms of cytotoxicity are complex and multifaceted, there is general agreement that incorporation of azacitidine into DNA and RNA, and inhibition of protein synthesis, are critically important. Cytotoxicity is greatest in cells that are proliferating (S-phase) and metabolically active. Cytotoxic effects may also be mediated through induction of the DNA damage response pathways. Non-proliferating cells are relatively insensitive to Vidaza.

FDA Recommendations:

The concentration of azacitidine required for maximum inhibition of DNA methylation in vitro does not cause major suppression of DNA synthesis. Hypomethylation may restore normal function to genes that are critical for differentiation and proliferation. The cytotoxic effects of azacitidine cause the death of rapidly dividing cells including cancer cells that are no longer responsive to normal growth control mechanisms. Non-proliferating cells are relatively insensitive to Vidaza.

Warnings

Pregnancy - Teratogenic Effects: Pregnancy Category D

Proposed labeling:

There are no adequate and well-controlled studies in pregnant women using Vidaza. If this drug is used during pregnancy, or if the patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus.

Women of childbearing potential should be advised to avoid becoming pregnant while receiving treatment with Vidaza.

Use in Males

[redacted]

FDA recommendation:

Vidaza may cause fetal harm when administered to a pregnant woman. Early embryotoxicity studies in mice revealed a 44% frequency of intrauterine embryonal death (increased resorption) after a single IP (intraperitoneal) injection of 6 mg/m^2 (approximately 8% of the recommended human daily dose on a mg/m^2 basis) azacitidine on gestation day 10. Developmental abnormalities in the brain have been detected in mice given azacitidine on or before gestation day 15 at doses of ~3-12 mg/m^2 (approximately 4%-16% the recommended human daily dose on a mg/m^2 basis).

In rats, azacitidine was clearly embryotoxic when given IP on gestation days 4-8 (postimplantation) at a dose of 6 mg/m^2 (approximately 8% the recommended human daily dose on a mg/m^2 basis), although treatment in the preimplantation period (on gestation days 1-3) had no adverse effect on the embryos. Azacitidine caused multiple fetal abnormalities in rats after a single IP dose of 3 to 12 mg/m^2 (approximately 8% the recommended human daily dose on a mg/m^2 basis) given on gestation day 9, 10, 11 or 12. In this study azacitidine caused fetal death when administered at 3-12 mg/m^2 on gestation days 9 and 10; average live animals per litter was reduced to 9% of control at the highest dose on gestation day 9. Fetal anomalies included: CNS anomalies (exencephaly/encephalocele), limb anomalies (micromelia, club foot, syndactyly, oligodactyly), and others (micrognathia, gastroschisis, edema, and rib abnormalities).

There are no adequate and well-controlled studies in pregnant women using Vidaza. If this drug is used during pregnancy, or if the patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus.

Women of childbearing potential should be advised to avoid becoming pregnant while receiving treatment with Vidaza.

Use in Males

[redacted]

Carcinogenesis, Mutagenesis, Impairment of Fertility

Proposed labeling:

[redacted]

FDA recommendation:

The potential carcinogenicity of azacitidine was evaluated in mice and rats. Azacitidine induced tumors of the hematopoietic system in female mice at 2.2 mg/kg (6.6 mg/m^2, approximately 8.8% the recommended human daily dose on a mg/m^2 basis) administered IP three times per week for 52 weeks. An increased incidence of tumors in the lymphoreticular system, lung, mammary gland, and skin was seen in mice treated with azacitidine IP at 2.0 mg/kg (6.0 mg/m^2, approximately 8% the recommended human daily dose on a mg/m^2 basis) once a week for 50 weeks. A tumorigenicity study in rats dosed twice weekly at 15 or 60 mg/m^2 (approximately 20-80% the recommended human daily dose on a mg/m^2 basis) revealed an increased incidence of testicular tumors compared with controls.

The mutagenic and clastogenic potential of azacitidine was tested in *in vitro* bacterial systems *Salmonella typhimurium* strains TA100 and several strains of *trp*E8, *Escherichia coli* stains WP14 Pro$^-$, WP3103P, WP3104P, and CC103; in *in vitro* forward gene mutation assay in mouse lymphoma cells and human lymphoblast cells; and in an *in vitro* micronucleus assay in mouse L5178Y lymphoma cells and Syrian hamster embryo cells. Azacitidine was mutagenic in bacterial and mammalian cell systems. The clastogenic

effect of azacitidine was shown by the induction of micronuclei in L5178Y mouse cells and Syrian hamster embryo cells.

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Pregnancy

Teratogenic Effects: Pregnancy Category D. See **Warnings** section.

Nursing Mothers

Proposed labeling:

It is not known whether azacitidine or its metabolites are excreted in human milk. Because of the potential for tumorigenicity shown for azacitidine in animal studies and the potential for serious adverse reactions, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

FDA recommendation:
FDA concurs with the proposed labeling.

Signatures (optional):

Reviewer Signature _Shwu-Luan Lee
Supervisor Signature_____

Concurrence Yes ___ No ___

3.7. APPENDIX/ATTACHMENTS

Appendix A:
NDA 50-569, Review 1, Almon Coulter, Ph.D., 1982

Appendix B:
Executive CAC committee Meeting Minute, March 30, 2004.

APPEARS THIS WAY
ON ORIGINAL

Executive CAC
Date of Meeting: March 30, 2004

Committee: David Jacobson-Kram, Ph.D., HFD-024, Chair
Joseph Contrera, Ph.D., HFD-901, Member
Abby Jacobs, Ph.D., HFD-024, Member
Josie Yang, Ph.D., HFD-550, Alternate Member
John Leighton, Ph.D., Team Leader
Shwu-Luan Lee, Ph.D., Presenting Reviewer

Author of Draft: Shwu-Luan Lee, Ph.D.

The following information reflects a brief summary of the Committee discussion and its recommendations. Detailed study information can be found in the individual review.

NDA # 50,794
Drug Name: Vidaza (Azacitidine, 5-Azacytidine)
Sponsor: Pharmion

Background:
5-Azcytidine is a synthesized pyrimidine analogue of cytidine. The sponsor proposes the use of 5-azacytidine for the indication of MDS (myelodysplastic syndromes) with a dose schedule of 75 mg subcutaneous daily injection for 7 days, every 4 weeks. 5-Azacytidine may exert its antineoplastic effect through hypomethylation (by inhibiting DNA methyltransferase) and cytotoxicity on abnormal hematopoietic cells in the bone marrow.

The two rat and two mouse carcinogenicity studies presented to the Exec CAC are based on the data of two published journal articles (BALB/c mice: Cavaliere *et al.* Cancer Letters, 37(1): 51-58, 1987; Fisher rats: Carr *et al.* Carcinogenesis, 5(12): 1583-1590, 1984) and one NCI report (1978). The NCI studies predated GLP, but were well documented, and the studies in the literature did not follow GLP.

Genotoxicity
The mutagenic and clastogenic potential of 5-azacytidine has been investigated in bacterial mutation assays in strains of *Salmonella typhimurium* and *Escherischia coli*, a mouse lymphoma mutation assay, an *in vitro* human lymphoblast mutation assay, and an *in vitro* micronuclei and transformation assay in Syrian hamster embryo (SHE) fibroblasts. 5-Azacytidine was positive for mutagenicity in the bacterial mutation assay, , positive for clastogenicity in the in vitro micronucleus assay and positive for transformation in the SHE cell assay.

Mouse Carcinogenicity Study:

Study #1, a study conducted for the NCI, used 35 $B6C3F_1$ mice/sex (n=15/sex for the controls) at doses of 2.2 and 4.4 mg/kg administered by intraperitoneal injection, 3 times per week, for 52 weeks. The administration of 5-azacytidine at the low dose was

associated with an increased incidence of neoplasms of the hematopoietic system (lymphomas, and granulocytic leukemia and sarcomas) in female mice. The doses chosen, exceeding the MTD, resulted in the low survival rate and shorter life span of the animals (especially the high dose animals, and hence impacted the outcome of the study and precluded any evaluation of male mice.

Study #2 employed 50/sex/group BALB/c mice in a 50-week test. The dose used was 2 mg/kg administered i.p. once weekly and was associated with an increased incidence of neoplasms in the lymphoreticular system, lung (males only) , mammary gland (females only), and skin in treated males and females.

Rat Carcinogenicity Study:

Study #1 was from the same NCI report as mouse study #1 (see above). Sprague Dawley rats (n=35/sex/group, n=15/sex/group in the control group) were given 2.6 mg/kg or 5.2 mg/kg of 5-azacytidine by intraperitoneal injection, 3 times per week, for 34 weeks. No conclusion can be drawn from the study due to inadequate data (see mouse study #1).

Study #2 was a published article (Carr *et al.*, see above). The committee only discussed the treatment in Regimens 4A and 4B in which 5-azacytidine was administered as a sole agent. Twelve male Fischer rats were given 5-azacytidine at 10 mg/kg (4A) or 2.5 mg/kg (4B) twice per week by intraperitoneal injection for 9 months. The control was an age-control, 12 untreated male rats. 4/12 rats in the high dose group died. Drug-related tumors included testicular tumors. The small numbers of animals precluded drawing any other conclusions.

Executive CAC Recommendations and Conclusions:

General comment:

The Committee noted that some of the studies were research studies from the literature with small numbers of animals. Also the Committee noted that these studies, including the NCI studies, would be considered inadequate by current standards.

Mice:

1. The Committee agreed that in the Study #1 there were drug related hematopoietic system neoplasms in female B6F3C1 mice, but no conclusions were possible about the males.
2. The Committee agreed with the conclusion in the Study #2 that the drug was associated with increased incidence of tumors in the lymphoreticular system, lung (males only), mammary gland (females only) and skin in the BALB/c mice.

Rat:

1. The Committee could not make any conclusions on Study #1 (Sprague-Dawley rats) due to high mortality in both dose groups.

2. In Study #2, the Committee found that due to small number of animals, it was difficult to draw any definitive conclusions. However, it did appear that testicular tumors in Fischer rats were increased as compared to age-matched controls.

David Jacobson-Kram, Ph.D.
Chair, Executive CAC

cc:\
/Division File, HFD-150
/John Leighton, Ph.D. Team leader, HFD-150
/Shwu-Luan Lee, Ph.D. Pharm-Tox Reviewer, HFD-150
/Amy Baird, CSO/PM, HFD-150
/Adele Seifried, HFD-024

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/s/

David Jacobson-Kram
4/6/04 10:09:48 AM

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/s/

Shwu-Luan Lee
4/27/04 04:27:55 PM
PHARMACOLOGIST

John Leighton
4/28/04 04:36:37 PM
PHARMACOLOGIST