CENTER FOR DRUG EVALUATION AND RESEARCH

APPROVAL PACKAGE FOR:

APPLICATION NUMBER

50-794

Pharmacology Review(s)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

PHARMACOLOGY/TOXICOLOGY REVIEW AND EVALUATION

NDA NUMBER:	**50-794**
SERIAL NUMBER:	**000**
DATE RECEIVED BY CENTER:	**12/26/03**
DRUG NAME:	Vidaza **(Azacitidine, 5-Azacytidine)**
INDICATION:	**Myelodyplastic Syndromes (MDS)**
SPONSOR:	**Pharmion Corporatation**
DOCUMENTS REVIEWED:	**Electronic Module 4**
REVIEW DIVISION:	**Division of Oncology Drug Products (HFD-150)**
PHARM/TOX REVIEWER:	**Shwu-Luan Lee, Ph.D.**
PHARM/TOX SUPERVISOR:	**John Leighton, Ph.D., D.A.B.T.**
DIVISION DIRECTOR:	**Richard Pazdur, M.D.**
PROJECT MANAGER:	**Amy Baird**

Date of review submission to Division File System (DFS): April 27, 2004

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EXECUTIVE SUMMARY

1. Recommendations

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

1.2 Recommendation for nonclinical studies: None

1.3 Recommendations on labeling: Refer to Section 3.6: Overall Conclusion, pages 148-152, for labeling recommendations on the Mechanism of Action, Warning, Carcinogenesis, Mutagenesis, Impairment of Fertility, Pregnancy, and Nursing Mothers sections of the product label.

2. Summary of nonclinical findings

2.1 Brief overview of nonclinical findings

5-Azacytidine, a pyrimidine analogue of cytidine, has been studied as an antineoplastic since the 1960s. The safety of 5-azacitidine has been investigated in multiple non-clinical single and repeat dose toxicity studies in mice, rats, dogs and monkeys. Other safety studies submitted in this application include genotoxicity, carcinogenicity, reproductive and development toxicity studies. Pharmacokinetic and ADME studies have also been conducted. These studies were submitted to support the use of 5-azacytidine (subcutaneous) in the treatment of myelodysplastic syndromes (MDS). These studies were for the most part published articles.

The administration routes used in the general toxicity studies include oral, IP and IV. The highest dose used in repeated dose toxicity studies administered parenterally in mice (134 mg/m^2/day for 5 days), dogs (22 mg/m^2/day, for 5 days and continued for 2 cycles), and monkeys (26.4 mg/m^2/day for 14 days), on a mg/m^2 basis, were approximately 1.8, 1/3 and 1/3 the recommended human daily dose (75 mg/m^2/day for 7 days), respectively. Although data of the systemic drug exposure (AUC) was not assessed in nonclinical studies, the C_{max} observed in blood after IP administration in mice ranged from 0.3-2 μg/ml was comparable to the values observed in clinical testing (~0.7 μg/ml).

The blood concentration of 5-azacytidine peaked at 15 min after IP administration and was undetectable by 1 hour as measured in a microbiological assay. When administered as a radiolabeled compound, parent and metabolites/degradation products were detectable in blood even after 24 hr. The administered 5-azacytidine distributed to solid tissues (e.g., thymus, spleen, kidney, liver), but minimally to the brain and muscle. At 24 h the concentration was highest in spleen and thymus. The urine was the major excretion route for 5-azacytidine and its metabolites. Approximately 45% radiolabeled 5-azacytidine and its

metabolites were found in the urine, which peaked at 8 h and then the excretion reached plateau. Another 20% of the radioactivity recovered in the expired CO_2.

In aqueous solutions 5-azacytidine undergoes a spontaneous hydrolysis and results in equilibration with n-formylguanylribosylurea (RGU-CHO), and finally the irreversible formation of guanylribosylurea (RGU). Another important metabolic pathway of 5-azacytidine was deamination. The stability of 5-azacitidine displayed a species discrepancy, i.e., 5-azacitidine is more stable in the mouse liver S9 fraction as compared to human.

The acute and repeated dose toxicology studies have identified bone marrow (hypoplasia), liver (increased liver enzymes, ALT and AST, increased neutral lipid, cloudy swelling), kidney (cloudy swelling and focal necrosis of tubules) and lymphoid tissues (increased extramedullary hematopoiesis in spleen, and lymphoid hypoplasia of the lymph nodes and spleen) as the target organs/tissues. Clinical signs, blood chemistry and other hematological changes were slight and reversible.

The mutagenic and clastogenic potential of 5-azacytidine has been investigated in test systems including bacterial mutation assays in strains of *Salmonella typhimurium* and *Escherichia coli*, a mouse lymphoma mutation assay, an *in vitro* human lymphoblast mutation assay, and an *in vitro* micronuclei and transformation assay in mouse lymphoma and the Syrian hamster embryo (SHE) fibroblasts. 5-Azacytidine was mutagenic in the bacterial Ames assay, and increased revertant colonies in the mouse and human lymphoma cells. 5-Azacytidine was also clastogenic, increasing micronuclei formation in mouse L5178Y cells and SHE cells. An in vivo test for chromosomal damage was not conducted.

Two mouse and two rat carcinogenicity studies were submitted. Although no conclusions could be drawn from some of these studies, the tumor induction of the hematopoietic system in female B6F3C1 mice (at 6.6 mg/m^2 for 52 weeks) was considered significant. The study conducted in the BALB/c mice indicated that IP administration of 5-azacytidine at 6 mg/m^2 for 50 weeks increased the number of tumors in lung, lymphoid system, skin and mammary glands. In male Fisher rats, 5-azacytidine (15 or 60 mg/m^2) administered as a single IP injection or twice weekly for 9 months increased the incidence of tumors in testis.

5-Azacitidine treatment exerted reproductive and development toxicity in mice and rats. 5-Azacytidine has direct effect on male sex organs, sperm counts and spermatogenesis. The affected sperms may function to fertilize oocytes, but the fertilized oocytes did not develop (decreased surviving at day 2 gestation). The embryotoxicity and development toxicity (teratogenicity) of 5-azacytidine is dose related and phase-specific. Decreased fetal weight was the most sensitive indication of embryotoxicity, i.e., doses as low as 1.5 mg/m^2/day in mice and 0.9 mg/m^2/day in rats decreased fetal weights. Other embryotoxicity findings

included increased resorption and reduced litter size. These effects were significant when 5-azacytidine was administered to males 1-7 days prior to mating or to dams on Days 4-8 gestation. Teratogenicity induced by 5-azacytidine included CNS anomalies (exencephaly), limb and skeletal anomalies (missing ribs, oligodactyly, and club foot) and other fetal abnormality (cleft palate, cardiomyopathy, and hind paw hematoma). The findings were observed when female mice were treated with a single dose of 5-azacitidine as early as Day 7.5 gestation or on Days 10-14 gestation, and rat dams treated on Days 9-12 gestation. The developmental toxicity was observed in the absence of maternal toxicity.

2.2 Pharmacologic activity

The role of 5-azacytidine in gene expression, cell differentiation, tumorigenicity, cytotoxicity, and antineoplastic effects are all centered on its primary pharmacodynamic property, i.e., hypomethylation of replicating DNA. With incorporation of 5-azacytidine into the newly synthesized strand of DNA, the inhibition of DNA methylation (via the inhibition of DNA methyltransferase 1, DNMT 1) is highly efficient, and yet noncompetitive. The major and pivotal point of DNA methylation is at the CpG islands. Roughly half of 5' promoter proximal elements contain CpG islands, and (hyper)methylation of CpG islands possibly leads to genetic instability, or silencing of a number of tumor suppression genes. 5-Azacytidine may serve as a potent inducer of differentiation by causing demethylation and re-expression of genes silenced by hypermethylation.

Recent discovery of the relationship of hypermethylation of $p15^{INK4B}$ (at the CpG island) and MDS (early event), and the capacity of 5-azacitidine to inhibit the methylation of DNA in proliferating hematopoietic cells, has suggested a possible role of 5-azacytidine in the treatment of MDS.

2.3 Nonclinical safety issues relevant to clinical use

- The general toxicology studies of 5-azacytidine have identified the target organs of toxicity to be bone marrow, liver, kidney and lymphoid tissues. Toxicity in these nonclinical studies occurred at doses less than the proposed clinical dose, when compared on a body surface area basis. These studies were generally conducted with few animals per dose group and were not conducted to current standards (e.g., application of GLP) or through use of modern analytical techniques. However, the long history of clinical use of this compound and its intended patient population, mitigates the need for the conduct of additional general toxicology studies.
- The main excretion route of 5-azacytidine and its metabolites is urine (~45%). Risk of toxicity may be greater for patients with impaired renal function.
- The findings of positive genotoxicity in vitro and carcinogenicity in both sexes at multiple sites in multiple animal species, with a mechanism of action likely relevant to humans, indicates the potential risk of secondary cancers in humans.

- The teratogenic and spermatogenic effects of 5-azacitidine in the rodent caution the use of 5-azacytidine in pregnant women or women of childbearing potential, as well as men whose partners are of childbearing potential.

PHARMACOLOGY/TOXICOLOGY REVIEW

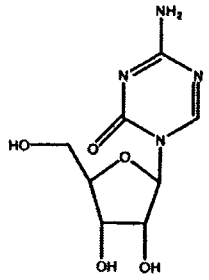
3.1 INTRODUCTION AND DRUG HISTORY

NDA number: N 50-794
Review number: Review 1
Sequence number/date/type of submission: N 000, letter date: December 26, 2003.
Information to sponsor: Yes (X) No ( ) (labeling review)
Sponsor and/or agent: Pharmion Corporation, Boulder, CO 80301
Manufacturer for drug substance: Ben Venue Laboratories, Inc., Bedford, OH 44146

Reviewer name: Shwu-Luan Lee, Ph.D.
Division name: Oncology Drug Products
HFD #: 150
Review completion date: April 26, 2004

Drug:

Trade name: Vidaza
Generic name: 5-azacitidine, azacitidine, 5-azacytidine
Code name: U-18,496, NSC-102816
Chemical name: 4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one
CAS registry number: 320-67-2
Molecular formula/molecular weight: $C_8H_{12}N_4O_5$/244.21
Structure:



Relevant INDs/NDAs/DMFs: IND 7574
IND 64,251 (reviewed by Dr. Anwar Goheer)
[redacted] (reviewed by Dr. Almon Coulter)

Drug class: Antimetabolite

Indication: "Vidaza is indicated for treatment of patients with MDS. It is effective for all five subtypes: refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS) requiring transfusions, with thrombocytopenia or significant clinical hemorrhage, or with neutropenia and infection requiring treatment with antibiotics; refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMMoL)."

Clinical formulation: Vials of Vidaza contain 100 mg of 5-azacitidine and 100 mg mannitol as a sterile lyophilized powder for reconstitution with sterile water upon injection.

Route of administration: Subcutaneous injection

Proposed use: 75 mg/m^2/day x 7, every four weeks.

Disclaimer: Tabular and graphical information are constructed by the reviewer unless cited otherwise.

Studies reviewed within this submission:

Module #	Title
Pharmacology	
4.2.1.1.5	The mechanism of inhibition of DNA (cytosine-5)-methyltransferases by 5-azacytosine is likely to involve methyl transfer to the inhibitor
4.2.1.1.6	Azacitidine. 10 years later
4.2.1.1 7	Biochemistry of azacitidine: a review
4.2.1.1.8	DNA methyltransferase inhibitors-state of the art
4 2.1.1.10	Distinct patterns of inactivation of p15^{INK4B} and p16^{INK4A} characterize the major types of hematological malignancies
4.2.1.1.12	Cancer epigenetics comes of age
4.2.1 1.24	DNA methylation: past, present and future directions
	Towards a pharmacology of DNA methylation
4.2.1.1.31	Hypermethylation of the p15^{INK4B} gene in myelodysplastic syndromes
Drug-Drug Interaction	
4.2.1.4.1	Depression of the hepatic cytochrome P-450 monooxygenase system by treatment of mice with the antineoplastic agent 5-azacytidine
4.2.2.4.3	Investigation of the potent inhibitory effect of Azacitidine on the metabolism of cytochrome P450 (CYP) model substrates
Reference #117	In vitro Evaluation of 5-Azacitidine as an Inducer of Cytochrome P450 Expression in Cultured Human Hepatocytes
Pharmacokinetics	
4.2.2.1.1	Deamination of 5-azacytidine by a human leukemia cell cytidine deaminase
4.2.2.1.3	5-azacytidine: microbiological assay in mouse blood
4.2 2.1.4	On the metabolism of 5-azacytidine and 5-aza-2'-deoxycytidine in mice
4.2.2.4.1	Isolation, characterization, and properties of a labile hydrolysis product of the antitumor nucleoside, 5-azacytidine
4.2.2.4.2	Species Comparison of the In Vitro Metabolism of 5-Azacitidine
4.2.2.5.2	Characterization of the urinary metabolites of 5-azacytidine in mice
General Toxicology	
4.2.3.1.1*	Preclinical toxicology of NSC-102816 (5-Azacytidine) in mice, hamsters, and dogs
4.2.3.1.2	Toxicity of S-Triazin-2-(1-H)-1 ,4-Amino-1-b-D-Ribofuranosyl-5-Azacytidine (NSC-102816) Clinical Formulation in Mice and Rats Administered a Single Dose by the Intravenous Route
4 2.3.1.3	Task II Preclinical Single Dose Range-Finding and Lethality Study of Azacitidine (NSC-102816) in CD2F1 Mice
4 2.3.2.1	Repeated-dose toxicity of S-triazin-2(1 H)-1 ,4-amino-1-b-D-ribofuranosyl-5-azacytidine (NSC 102816) in the rhesus monkey
4.2.3 2.2*	Preclinical toxicology of NSC-102816 (5-azacytidien) in mice, hamsters, and dogs.

Genotoxicity	
4.2.3.3.1.1	The mutagenicity of 5-azacytidine and other inhibitors of replicative DNA synthesis in the L5178Y mouse Lymphoma cell
4.2.3.3.1.2	Studies of mutagenicity and clastogenicity of 5-azacytidine in human Lymphoblast and *Salmonella typhimurium*
4.2.3.3.1.3	Mutagenicity of 5-azacytidine in *Salmonella typhimurium*
4.2.3.3.1.4	Cell-cycle dependent micronucleus formation and mitotic disturbances induced by 5-azacytidine in mammalian cells
4.2.3.3.1.5	5-azacytidine induces micronuclei in and morphological transformation of Syrian hamster embryo fibroblasts in the absence of unscheduled DNA synthesis
Reference #94	Induction of malignant transformation and mutagenesis in cell cultures by cancer chemotherapeutic agents
Reference #10	Effects of the *umuDC*, *mucAB*, and *samAB* operons on the mutational specificity of chemical mutagenesis in *Escherichia coli*: II. Base substitution mutagenesis
Reference #5	Mutagenic effects of 5-azacytidine in bacteria
Reference #11	A comparison of mutagen spectra detected by the *Escherichia coli* Lac$^-$ reversion assay and the *Salmonella typhimurium* His$^+$ reversion assay
Carcinogenicity	
4.2.3 4.1.1	Bioassay of 5-azacytidine for possible carcinogenicity: Carcinogenesis Testing Program, National Cancer Institute, NIH
4.2.3.4.2.1	The tumorigenicity of 5-azacytidine in the male Fischer rat
4.2.3.4.2.2	5-Azacytidine carcinogenesis in BALB/c mice
4.2.3.4.2.3*	Test for carcinogenicity of food additives and chemotherapeutic agents by the pulmonary tumor response in strain A mice
4.2.3.4.3.1*	Transformation of BHK 21/13 cells by chemical carcinogens and mutagens
4.2.3.4.3 2*	Induction of morphological transformation in mouse C3H/10T1/2 clone 8 cells and chromosomal damage in hamster A(T1)C1-3 cells by cancer chemotherapeutic agents
Reproductive and Developmental Toxicity	
4.2.3.5.1.2	Developmental exposure of male germ cells to 5-azacytidine results in abnormal preimplantation development in rats
4.2.3.5.2.1	Effect of 5-azacytidine administration during very early pregnancy
4.2.3.5.2.2	Cerebral cortex of the mouse after prenatal chemical insult
4.2.3.5.2.3	Teratogenicity of 5-azacytidine in the Sprague-Dawley rat
4.2.3.5.2.4	Embryotoxicity of 5-azacytidine in mice. Phase- and dose-specificity studies
4.2.3.5.3.1	Enhanced mortality in offsprings of male mice treated with 5-azacytidine prior to mating. Morphological changes in testes.
Reference #125	5-Azacytidine-induced exencephaly in mice
Local Tolerance	
4.2.3.6.1	Cutaneous irritation in the topical application of 5-azocytidine (NSC#102816) to New Zealand white rabbits
4.2.3.2.2.*	Preclinical toxicology of NSC-102816 (5-azacytidien) in mice, hamsters, and dogs.
Other Toxicity Studies	
4.2.3.7.6.1	Isolation, characterization, and properties of a labile hydrolysis product of the antitumor nucleoside, 5-azacytidine

* reviewed by Dr. Almon Coulter, see Appendix A

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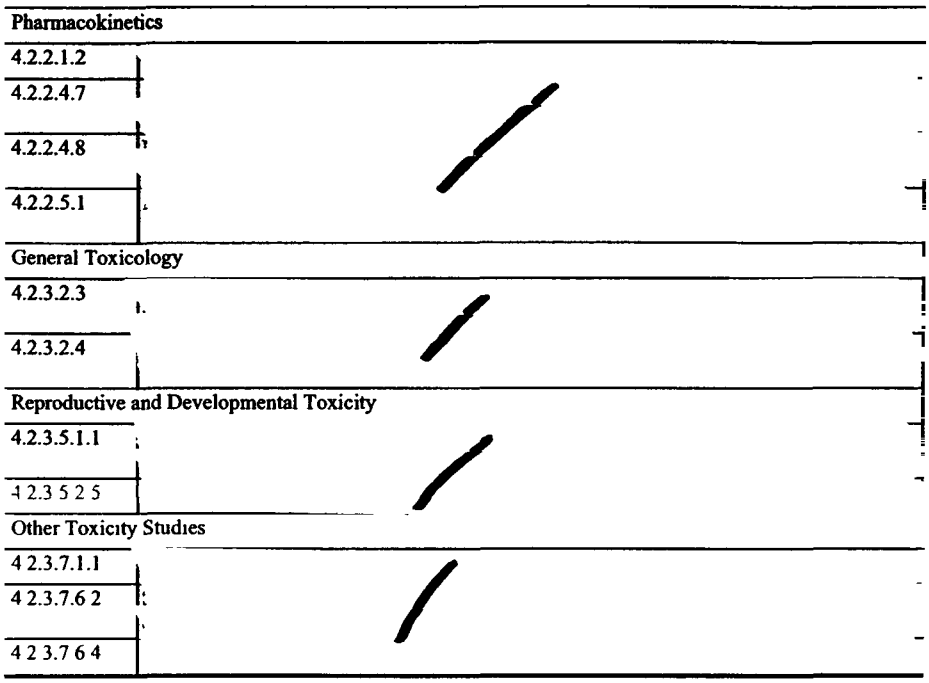


3.2 PHARMACOLOGY

3.2.1 Brief summary

5-Azacytidine has been under investigation in since the 1960s, and studies and detailed reviews have been published regarding its pharmacological properties (e.g., see Glover *et al.*, (1987) Azacitidine: 10 years later. Cancer Treat Rep 71: 737-746). Pharmacology studies have revealed that the potent antineoplastic effect of 5-azacytidine is likely due to its primary pharmacodymic properties, i.e., inhibition of the synthesis of pyrimidines, DNA, RNA, and hence proteins. Since the more recent recognition of the possible correlation between hypermethylation of DNA (silencing gene expression) and development of cancer, research on the effects of 5-azacytidine on cancer cells has received considerable interest. This research suggests that the DNA hypermethylation may be selective for certain genes, and that 5-azacitidine may block cytosine methylation in newly replicated DNA, thus affecting primarily cells undergoing cell division.

Pharmacologic drug interactions through direct action on cytochrome P450s would not be expected as 5-azacitidine did not inhibit or induce these enzymes. Several studies showed that 5-azacitidine decreased drug enzyme activity in vivo, possibly through inhibitory off target effects on general protein synthesis.

3.2.2 Primary pharmacodynamics

Note: The review of this section was done by Dr. Haleh Saber-Mahloogi.

5-Azacitidine is phosphorylated by uridine-cytidine kinase and reduced by ribonucleotide reductase prior to incorporation into DNA. After incorporation into DNA, 5-azacitidine non-competitively inhibits DNA methyltransferase, causing a block in cytosine

methylation in newly replicated DNA. Treatment with 5-azacitidine results in hypomethylation of the DNA.

Detailed mechanism of action:

1. Inhibitory effect on methyl-transferases:

DNA methylation (addition of a methyl group to the 5 position of cytosine) is an epigenetic process with several effects, including chromatin structure modulation, transcriptional repression and the suppression of transposable elements. In malignancy, methylation patterns change, resulting in global hypomethylation with regional hypermethylation. This can lead to genetic instability and the repression of tumor suppressor genes (J Goffin and E Eisenhauer. DNA methyltransferases-state of the art. Ann Oncol 2002; 13: 1699-1716).

The process of methylation is carried out by DNA methyltransferases (DNMT), DNMT1 being the most abundant one. These enzymes catalyze the covalent addition of a methyl group from a donor S-adenosyl-methionine to the 5 position of cytosine, predominantly within the CpG islands. While roughly half of 5' promoter proximal elements contain CpG islands, they are not usually methylated in normal tissues. After development, DNA methylation remains a permanent component of the genome (J Goffin and E Eisenhauer. ibid). Cancers display a particular pattern of methylation. They can be either hypomethylated or hypermethylated (KD Robertson and PA Jones. DNA methylation: past, present and future directions. Carcinogenesis 2000; 21: 461-467). Hypermethylation is evident in the repetitive or parasitic elements, possibly leading to genetic instability through failed inhibition of homologous recombination and allowing transcription of these normally repressed genes. In addition, methylation of the CpG islands can repress transcription in a manner analogous to a mutation or deletion (J Goffin and E Eisenhauer. ibid). Methylation of the CpG islands can also repress transcription by interfering with transcription initiation (PA Jones and PW Laird. Cancer epigenetics comes of age. Nature Genetics 1999; 21:163-167). Hypermethylation has been demonstrated to silence a number of tumor suppressor genes, resulting in malignancies. The abnormal DNA methylation pattern may be the product of DNMT1 levels present in tumors (KD Robertson. DNA methylation, methyltransferases and cancer. Oncogene 2001; 20: 3139-315) or the timing of DNMT1 induction (M Szyf. Towards a pharmacology of DNA methylation. Trends Pharmacol Sci 2001; 22: 350-354), which is normally elevated in S-Phase.

To demonstrate the inhibitory effect of 5-azacitidine on DNMT, an oligonucleotide duplex containing a single target site for the EcoRII methyl- transferase was synthesized, in which the target base was 5-azacytosine (S Gabbara and A Bhagwat. The mechanism of inhibition of DNA (cytosine-5)-methyltransferases by 5-azacytosine is likely to involve methyl transfer to the inhibitor. Biochem J 1995; 307:87-92). This substrate formed a stable covalent complex with EcoRII methyl-transferase in the absence and in the presence of the cofactor S-adenosylmethionine. The complex formed in the presence of the cofactor was resistant to SDS and moderate heat treatment, and a methyl group was incorporated into the complex. Enzyme titration and kinetic studies of inhibition suggest that methyl transfer to the complex occurred only during the first turnover of the reaction.

These results suggest that, when the enzyme binds to 5-azacytosine in the presence of the cofactor, a methyl group is transferred to the N-5 position of the base, resulting in the inactivation of the enzyme.

In conclusion, after incorporation into DNA, 5-azacitidine non-competitively inhibits DNA methyltransferase, causing a block in cytosine methylation in newly replicated DNA. In addition, alterations in the pattern of cytosine methylation are causally related to gene expression and cell differentiation. This hypomethylation of DNA is thought to be the mechanism underlying the differentiating activity of 5-azacitidine, resulting in one mechanism by which 5-azacitidine may exert its tumorigenic effects (AB Glover et al. Azacitidine: 10 years later. Cancer Treatment Reports 1987; 71: 737-746).

2. Effect on DNA Synthesis and Relation to Cytotoxicity:

Drugs such as 5-azacitidine prevents methylation but they also act through other mechanisms. For instance, after phosphorylation they can be incorporated into nucleic acids, preferentially into RNA. When in DNA, they covalently link with DNMT which may induce cell death by obstructing DNA synthesis. However, these may also induce DNA damage through structural instability at the site of incorporation. DNMT is depleted by being bound to these agents and is thereby unavailable for methylation, resulting in significant demethylation after repeated replication (J Goffin and E Eisenhauer. ibid).

Although 5-azacitidine is incorporated into DNA to a lesser extent than into RNA, its inhibitory effect on DNA synthesis, as measured by incorporation of tritiated thymidine or deoxyadenosine, is greater than its effect on RNA synthesis at the same concentrations as measured in L1210 leukemia cells. In rats, when administered shortly after partial hepatectomy, 5-azacytidine led to complete inhibition of DNA synthesis in regenerating liver, although if 5-azacitidine was administered before hepatectomy, increased mitotic activity and thymidine incorporation was observed. In L1210 cells, the 5-azacytidine-induced inhibition of DNA synthesis, but not of RNA synthesis, paralleled the inhibition of mitosis with lethality predominantly in the S Phase. These findings suggest that inhibition of DNA synthesis does contribute to the cytotoxicity of 5-azacitidine (A Glover and B Leyland-Jones. Biochemistry of azacitidine: a review. Cancer Treatment Reports 1987; 71: 959-964).

After incorporation into DNA, 5-azacitidine inhibits DNA methyltransferase non-competitively, causing a block in cytosine methylation in newly replicated DNA, but not in resting nondividing cells. This inhibition of methylation occurs at concentrations which do not cause major suppression of DNA synthesis, as measured by thymidine incorporation (A Glover and B Leyland-Jones. ibid).

3. Drug activity related to proposed indication:

Cyclin-dependent kinase inhibitor genes $p15^{INK4B}$ and $p16^{INK4A}$ (candidate tumor suppressor genes) are frequently inactivated by genetic alterations in various malignant tumors. Distinct patterns and types of inactivation of $p15^{INK4B}$ and $p16^{INK4A}$ genes occur in specific subtypes of hematological malignancies, e.g. selective hypermethylation of

p15^{INK4B} was seen in AML but not in CML (J Herman et al. Distinct patterns of inactivation of p15^{INK4B} and p16^{INK4A} characterize the major types of hematological malignancies. Cancer Res 1997; 57: 837-841). Hypermethylation of the 5' CpG island of the *p15^{INK4B}* gene was shown to occur frequently in patients with MDS (16/32 patients or 50%) and overt leukemia evolved from MDS (14/18 or 78%). Methylation status of the *p15^{INK4B}* gene progressed with the development of MDS in most patients examined. None of the patients showed apparent hypermethylation of the p16 gene. Thus, *p15^{INK4B}* appeared to be involved in the pathogenesis of MDS (T Uchida et al. Hypermethylation of the *p15^{INK4B}* gene in myelodysplastic syndromes. Blood 1997; 90: 1403-1409).

3.2.3 Secondary pharmacodynamics

There were no studies specific to secondary pharmacodynamics relevant to this review.

3.2.4 Safety pharmacology

No pre-clinical safety pharmacology studies have been conducted.

3.2.5 Pharmacodynamic drug interactions

Study title: Investigation of the potential inhibitory effect of azacitidine on the metabolism of cytochrome P450 (CYP) model substrates. (— Study Report DXNI1001, Module 4.2.2.4.3) Report dated 5/3/03. Conducted in compliance with GLP.

Key findings: 5-Azacitidine is unlikely to cause clinically significant inhibition of CYPs 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4.

Methods:

- Test substance: 5-Azacytidine in water was diluted in the incubation mixture at a final concentration of 0.1 to 100 μM.
- Hepatic microsomes were obtained from pools of 15 and 20 human livers from two different commercial vendors.
- Following substrate incubation in hepatic microsomes with either model inhibitor or 5-azacitidine, the levels of model substrate metabolites were quantified using — HPLC. The inhibitory effect of 5-azacitidine was expressed as the percentage inhibition of CYP model substrate activity of duplicate samples compared to control activity.

Result:

- IC_{50} values could not be determined.
- The following summarizes the results at 100 μM 5-azacitidine.

CYP	Substrate	Conc (μM)	Inhibitor	Conc (μM)	% inhibition*	
					Inhibitor	5-AZ
CYP1A2	Ethoxyresorufin	0.4	Furafyline	10	53.7%	19.4%
CYP2C9	Tolbutamide	100	Sulphaphenazole	10	80.4%	-9.1%
CYP2C19	S-mephenytoin	96.4	Tranylcypromine	50	51.8%	-7.2%
CYP2D6	Bufuralol	10	Quinidine	1	80.8%	-20.9%
CYP2E1	Chlorzoxazone	40	Diethyldithiocarbamate	50	75.0%	27.1%
CYP3A4	Testosterone	65	Ketoconazole	1	70.8%	-8.7%

***% relative to vehicle control**

Conclusion:

Positive controls worked as expected. Some inhibition of CYPs 1A2 and 2E1 was observed at the maximum 5-azacitidine concentration of 100 μM. Inhibition of other P450s examined was not observed.

Study title: *In vitro* evaluation of 5-azacytidine as an inducer of cytochrome P450 (CYP) expression in cultured human hepatocytes. (— Study Report —)23018-1). Draft report dated 4/20/03. Non-GLP; no study deviations noted that impact study validity.

Key findings:

- Treatment of cultured human hepatocytes with 5-azacytidine decreased the activities of 7-ethoxyresorufin O-dealkylase (CYP1A2), S-mephenytoin 4'-hydroxylase (CYP2C19) and testosterone 6β-hydroxylase (CYP3A4/5).

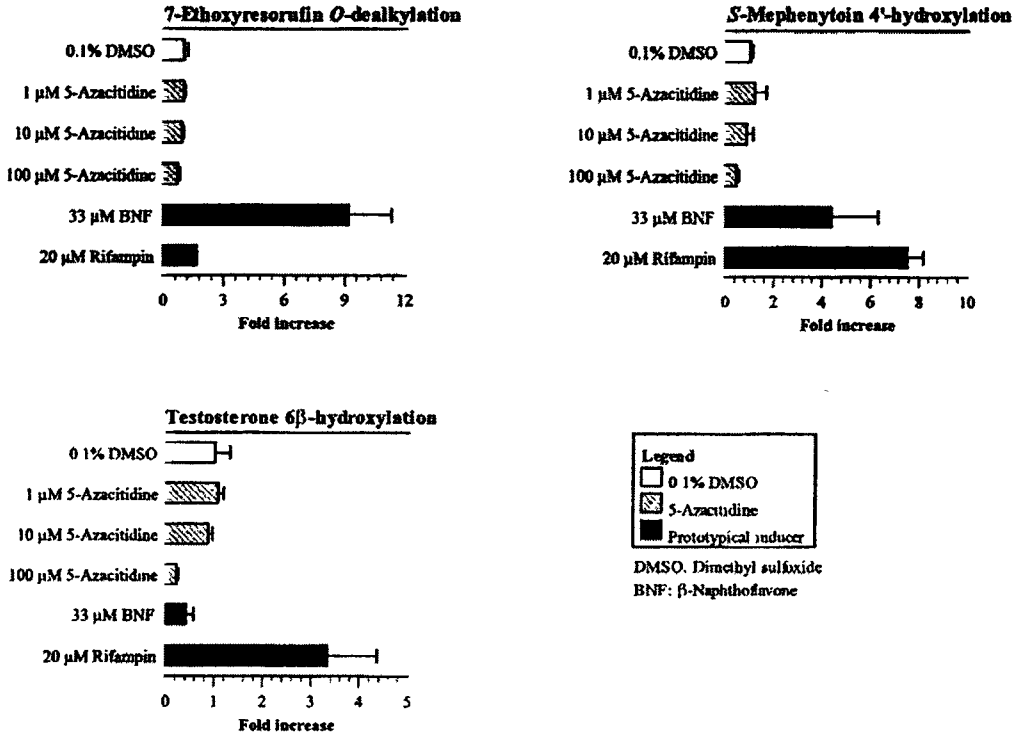
Methods:

- Three preparations of cultured human hepatocytes from three separate human livers were treated with DMSO (vehicle), one of three concentrations of 5-azacitidine (1.0, 10 or 100 μM) or one of two known human P450 inducers (33 μM β-naphthoflavone or 20 μM rifampin) once daily for three days. Twenty-four hours after the final treatment, cells were harvested to prepare microsomes for the analysis of 7-ethoxyresorufin O-dealkylation (CYP1A2), S-mephenytoin 4' -hydroxylation (CYP2C19) and testosterone 6β-hydroxylation (CYP3A4/5).
- Cells were photographed 24 h after the final treatment to document morphological integrity and signs of toxicity.

Results:

The following figure (Figure 5) from the article summarizes the experimental results.

Figure 5: The effect of treating cultured human hepatocytes with 5-azacitidine on cytochrome P450 expression



- No effect on morphology by 5-azacitidine was observed.

Conclusion:

5-Azacitidine decreased the activity of CYPs 1A2, 2C19, and 3A4/5. It is uncertain whether the effects of 5-azacitidine represents direct effects on the respective P-450s or a general effect on protein synthesis.

Study title: Depression of the hepatic cytochrome P-450 monooxygenase system by treatment of mice with the antineoplastic agent 5-azacytidine. Gooderham and Mannering, Cancer Research, 45 (4): 1569-1572, 1985 (Module 4.2.1.4.1)

Key findings:

- A single IP injection of 5-azacytidine (25 mg/kg) to Swiss mice caused 50% depression of microsomal cytochrome P450 and drug metabolic enzymes ethylmorphine N-demethylase and ethoxycoumarin O-deethylase activity. The depression was greatest at 24 hours after administration.
- 5-Azacytidine prolonged hexobarbital sleeping time in mice.

Methods:

- Animals: Swiss-Webster mice (male, 22-27 g)
  Sprague-Dawley rats (180-200 g)
- Saline or 5-azacytidine (5, 10, 25, and 50 mg/kg) was administered via IP injection to mice and body weight, liver weight and hepatic microsomal protein were determined 24 hours later.
- Animals were administered a single IP injection of 5-50 mg/kg (mice) or 25 mg/kg (rats). Cytochrome b_5, P-450, ethylmorphine N-demethylase, and 7-ethoxycoumarin O-deethylase were determined in hepatic microsomes from animals sacrificed 24 h later.
- Mice administered 25 mg/kg of 5-azacytidine (IP) were sacrificed at various intervals to assess alterations in the cytochrome b_5, P-450, and drug metabolism.
- Mice were sacrificed 24 h after receiving a single dose of 5-azacytidine (25 mg/kg) or saline (controls) and enzyme activity measured.
- Groups of 8 mice were given injections IP of saline or 25 mg/kg of 5-azacytidine. Sodium hexobarbital (100 mg/kg) was administered 12 or 24 hours later and the sleeping time of the mice was measured.

Results: Refer to the data figures and tables reproduced from the article.

- Twenty-four hours after treatment of 5-azacytidine the liver weight and hepatic microsomal protein was depressed in a dose-dependent fashion.
- 5-Azacytidine depressed liver cytochromes and ethylmorphine N-demethylase in a dose-dependent manner.
- 5-Azacytidine selectively depressed certain enzymes in the microsomal P-450-mediated monooxygenase system (e.g., cytochrome b_5, P-450 and NADPH P-450 (c) reductase activity), but not others (e.g., NADPH-cytochrome b_5 (c) reductase), as shown in Table 1.
- 5-Azacytidine at 25 mg/kg also exerted a similar depression of these components in rat livers.
- Maximum depression occurs at 24 h post dosing and then rebounds.
- 5-Azacytidine prolonged hexobarbital sleeping time of mice.

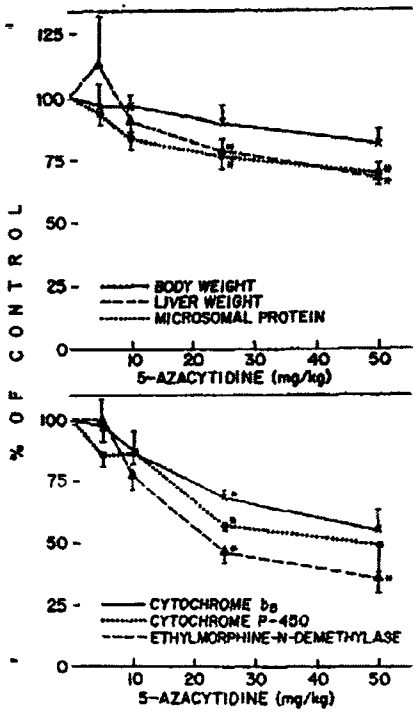


Chart 1. Effect of various doses of 5-AC on mice. Control values: body weight, 27.4 ± 1.1 g; liver weight, 1.8 ± 0.1 g, microsomal protein, 15.5 ± 0.5 mg/g liver; cytochrome b_5, 0.29 ± 0.05 nmol/mg microsomal protein; P-450, 0.89 ± 0 17 nmol/mg microsomal protein; ethylmorphine *N*-demethylase, 10.97 ± 2.08 nmol HCHO formed per mg microsomal protein per min. Animals were given single doses of 5-AC (i.p.) and killed 24 h later. *Points*, mean; *bars*, SE (n = 3). *, significantly different from control values ($P < 0.05$).

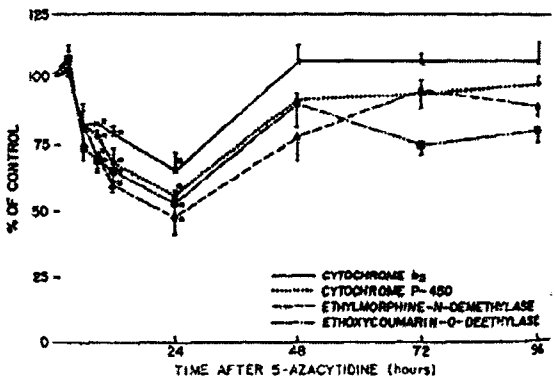


Chart 2. Loss and recovery of microsomal cytochrome b_5 and P-450 and drug metabolizing activities in mice after a single i.p. dose of 5-AC (25 mg/kg). Mice were killed at various times after the administration of 5-AC. Control values (time): cytochrome b_5, 0.29 ± 0.02 nmol/mg microsomal protein; P-450, 0.77 ± 0.05 nmol/mg microsomal protein; ethylmorphine *N*-demethylase, 10.95 ± 1.3 nmol HCHO formed per mg microsomal protein per min; 7-ethoxycoumarin *O*-deethylation, 1.40 ± 0.14 nmol 7-hydroxycoumarin formed per mg microsomal protein per min. *Points*, mean; *bars*, SE (n = 4). *, significantly different from control values ($P < 0.05$).

Table 1

Effect of 5-AC on components of the murine microsomal P-450 monooxygenase system

Mice were killed 24 h after receiving a single dose of 5-AC (25 mg/kg). Control animals received saline.

Component	Treatment	
	Control	5-AC
Cytochrome b_5 (nmol/mg protein)	0.27 ± 0.02[a]	0.22 ± 0.01[b]
P-450 (nmol/mg protein)	0.85 ± 0.04	0.65 ± 0.05[b]
NADPH-cytochrome *c* reductase (nmol cytochrome *c* reduced/min/mg protein)	201.7 ± 18.7	133.8 ± 7.7[b]
NADH-cytochrome *c* reductase (nmol cytochrome *c* reduced/min/mg protein	588.9 ± 28.4	586.8 ± 82.1

[a] Mean ± SE (n = 6).
[b] Significantly different from control ($P < 0.05$).

Table 2

Effect of 5-AC on components of hepatic microsomes of rats

Rats were given i.p. injections of 5-AC (25 mg/kg) or saline, and hepatic microsomes were harvested.

Treatment	Microsomal protein (mg/g liver)	Cytochrome b_5 (nmol/mg microsomal protein)	P-450 (nmol/mg microsomal protein)	Ethylmorphine *N*-demethylase (nmol HCHO formed/mg microsomal protein/min)	7-Ethoxycoumarin *O*-deethylase (nmol/mg microsomal protein/min)
Saline	10.6 ± 0.5[a]	0.80 ± 0.02	1.40 ± 0.04	12.14 ± 0.37	2.05 ± 0.07
5-AC	8.2 ± 0.5[b]	0.51 ± 0.03[b]	0.98 ± 0.05[b]	7.43 ± 0.74[b]	1.03 ± 0.09[b]

[a] Mean ± SE ($n = 3$).
[b] Significantly different from saline ($P < 0.05$).

- 5-Azacytidine effect on hexobarbital sleeping time of mice.

Treatment	Hexobarbital (100 mg/kg)	
	12 hours later	24 hours later
Saline	(not done)	36 ± 3 (SE) min
5-Azacytidine (25 mg/kg)	78 ± 16 min*	90 ± 14 min*

* $p < 0.05$, as compared to saline treatment.

3.3 PHARMACOKINETICS/TOXICOKINETICS

3.3.1. Brief summary:

Several studies were conducted in the 1960s and 1970s that examined the distribution, metabolism and excretion of 5-azacytidine. While these studies would not meet standards of sensitivity of modern analytical chemistry, they are nevertheless informative.

Plasma half-life of 5-azacitidine was rapid, suggesting that accumulation with repeat dosing was unlikely. Limited tissue distribution studies showed that radiolabeled 5-azacitidine distributed into all tissues examined. A major metabolic route is through deamination with subsequent elimination through the kidney.

3.3.3 Absorption: no studies reviewed

3.3.4 Distribution

Study title: 5-Azacytidine: microbiological assay in mouse blood. Pittillo and Woodley, Appl Microbiol 18 (2): 284-286, 1969 (Module 4.2.2.2.1).

Key findings:

- Blood concentration of 5-azacytidine peaked <15 minutes after drug administration and not detected by 30 minutes and 1 hour for 9.5 mg/kg and 4.75 mg/kg, respectively.
- 5-Azacitidine was not detected in the tissues examined

Methods:

- Microbiological assays. The rationale of the assay utilized the antibiotic ability of 5-azacytidine. The growth of a 6-chloropurine-resistant strain of *E. coli* ATCC9637 was assessed at 0.01-3 μg/filter-paper disc.
- 5-Azacytidine at 9.5 (the LD_{10}) and 4.75 mg/kg was administered to BDF mice (mixed sexes, 18 to 22) via IP injection. Blood samples and organs (liver, lung, brain, spleen, kidney) were collected.

Results:

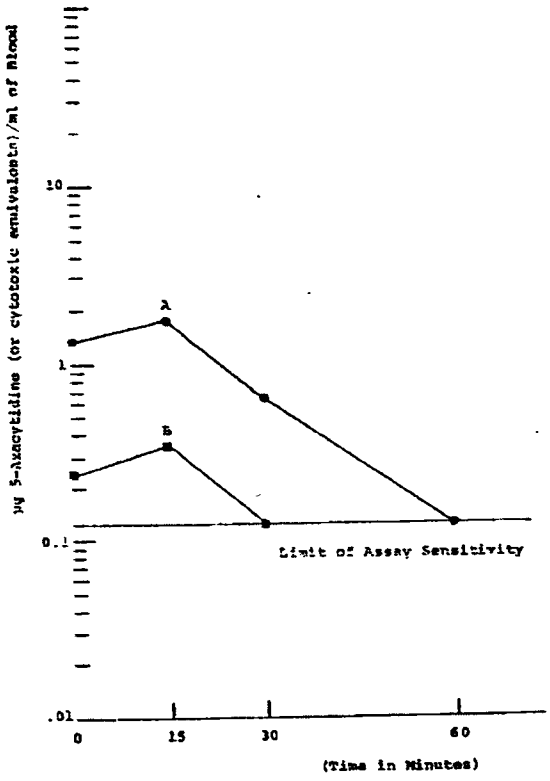


Fig. 2. Concentrations of 5-azacytidtne detected in the blood of mice by microbiological assay. Each point represents the mean drug level of five mice. Assay organism: *Escherichia coli* ATCC 9637/6-chloropurine. (A) Animals were injected with a single LD_{10} intraperitoneal dose (9.5 mg/kg) of drug. (B) Animals were injected with a single 0.5 LD_{10} intraperitoneal dose (4.75mg/kg) of drug.

- No detection of 5-azacitidine in any tissue monitored (data not shown in article).

Study title: On the metabolism of 5-azacytidine and 5-aza-2'-deoxycytidine in mice. Raska et al., Collect. Czech. Chem. Commun. 30:3001-3006, 1965 (Module 4.2.2.2.2).

Note: some of the data on 5-aza-2'deoxycytidine are shown but are not relevant to this review.

Key findings:

- Total radioactivity was rapidly cleared from blood after ip administration.
- Total radioactivity depleted slowly from solid tissue over the 24 h time period.

Methods:

- Radioisotope assays. Tissue levels of 5-azacytidine were measured in mice using ^{14}C-labeled compound. Tissue samples were oxidized by the thermal decomposition and measured by liquid scintillation counting. Radioactivity was expressed as CPM

of CO_2 released on combustion of 10 mg fresh tissue or 0.1 ml of blood. Specific activity of the labeled compound was 1 μCi/μM.

- 5-Azacytidine (260 μg, 10^6 CPM) was administered to AKR mice (females, 18 to 20 g) via IP injection. At 2, 8, 16, and 24 h, tissue samples (liver, thymus, muscle, brain, spleen, and kidneys) were collected, and tissue radioactivity determined.
- Urine samples were collected (at 2, 4, 6, 8, 16 and 24 h), chromatographed, and radioactivity determined from eluted zones.

Results:

Data are presented in the figures and table below from the article.

- The level of radioactivity in all organs decreased slowly. At 24 h the concentration was highest in spleen and thymus of all the tissues examined. 5-Azacytidine slightly penetrated the CNS.
- The maximum blood level was seen at 2 h. The authors estimate the $t_{1/2}$ of 3.8 hours.
- Within 6-8 h one-half of the compound was excreted to the urine, then the excretion practically ceased. By 24 hour, approximately 60% of 5-azacytidine (and its decomposition products) was excreted to the urine.
- Compounds identified by chromatographic analysis included 5-azacitidine and spontaneous decomposition products, guanyl-urea ribonucleoside and guanidine; 5-azauracil and its decomposition products, biuret and 1-formylbiuret.

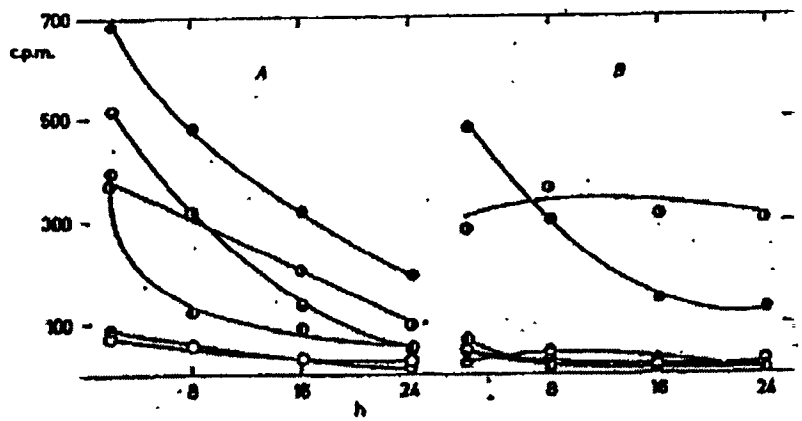


Fig. 1

Distribution of 5-Azacytidine and 5-Aza-2'-deoxycytidine in Tissues

The values refer to counts/min $^{14}CO_2$ released by combustion of 10 mg fresh tissue. ◑ Thymus, ● spleen, ◐ kidney, ◒ liver, ◓ brain and ○ muscle. *A*, distribution of 5-azacytidine; *B* distribution of 5-aza-2'-deoxycytidine; *h* time in h.

Table I

Level of Radioactive Substances in Blood
The values refer to counts/min of $^{14}CO_2$ released on combustion of 0·1 ml blood

Antimetabolite	Interval Following Application of Radioactive Antimetabolites (h)			
	2	8	16	24
5-Azacytidine	457	154	82	75
5-Aza-2'-deoxycytidine	65	6	8	3

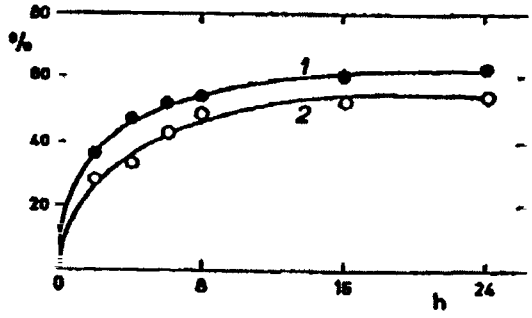


Fig. 2
Excretion of 5-Azacytidine and 5-Aza-2'-deoxycytidine in the Urine
The values refer to the percentage of radioactive compounds, in relation to time in hours (h), excreted after doses of antimetabolites; 1, 5-aza-2'-deoxycytidine; 2, 5-azacytidine.

3.3.5 Metabolism

Study title: Species comparison of the in vitro metabolism of 5-azacytidine. Module 4.2.2.4.2.

Key findings:

- 5-AZ is more stable in the mouse S9 fraction compared to human S9.
- Similar results were obtained with intact and denatured S9 for both the mouse and human incubations.

The study was conducted by — In vitro metabolism was investigated using mouse and human liver S9 fractions (n = 3). The metabolism of 64 μM 5-AZ after an incubation time of 0, 45 and 90 min was monitored by — The percent remaining after incubation with human S9 averaged 10.5% after 45 min and 5.2% after 90 min. The percent remaining after incubation with mouse S9 averaged 81.7% after 45 min and 66.4% after 90 min. Approximately 82% of the 5-AZ remained after incubation with buffer. In denatured human S9 fraction, 6.8% remained after the 90 min incubation. In denatured mouse S9 fraction, 68.7% remained after the 90 min incubation. This was a non GLP study.

Study title: Characterization of the urinary metabolites of 5-azacytidine in mice. Kelly et al., Biochem Pharmacol. 29 (4): 609-615, 1980 (Module 4.2.2.5.2).

Key findings:

- Six peaks were observed by HPLC-GC/MS analysis.

Methods:

- Test agents: [4-^{14}C]-5-azacytidine: 50 mCi/mmole (radiochemical purity, 96%) and [6-^{14}C]-5-azacytidine: 9.5 mCi/mmole (radiochemical purity, 87%).
- Female BDF_1 mice (mean weight: 20 g) were given 5-azacytidine (4-^{14}C or 6-^{14}C), 50 mg/kg (in saline, 2.6-6.2 μCi), alone or in combination with THU (tetrahydrouridine, a deaminase inhibitor), 10 mg/kg, via IP injection. Expired CO_2 was trapped (8 hour collection) and the amount of $^{14}CO_2$ present was determined by liquid scintillation. The urinary collection (for 8 hours) was analyzed by HPLC.

Results:

- HPLC analysis of urine from mice treated with [4-^{14}C]-5-azacytidine showed 6 major peaks. Peaks 1-4 were radioactive and UV absorbent, and peaks 5 and 6 were radioactive and non-UV absorbent.

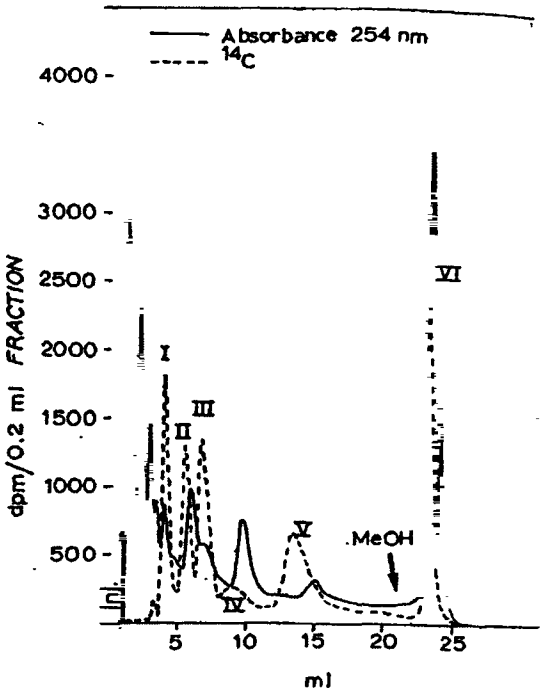


Fig. 1. High-pressure liquid chromatographic analyses of urine collected from BDF_1 mice after dosing with [4-^{14}C]-5-azaCR (3.7 μCi/mg), 50 mg/kg. The column was 15 cm × 4.2 mm i.d., packed with 5 μm silica. Eluents were chloroform–methanol–water–acetic acid (30:10:1.7: 0.42) (0–22 ml) and methanol (22–27 ml), flow rate 1 ml/min. ^{14}C peaks are labeled I–VI, where IV is 5-azaCR.

- THU administered with 5-azacytidine changed levels of radioactivity in HPLC peaks.

Table 1. Effect of THU on the urinary metabolites of [4-^{14}C]- and [6-^{14}C]-5-azacytidine in BDF$_1$ mice*

Treatment	High-pressure chromatographic fractions I	II	III	IV	V	VI	Per cent of administered dose
[6-^{14}C]-5-azaCR	28.9 ± 5.4	24.7 ± 3.2	16.0 ± 4.3	8.3 ± 2.8	0	8.8 ± 0.6	40.5 ± 11.4
[6-^{14}C]-5-azaCR + THU	7.2 ± 2.4	7.5 ± 0.9	20.4 ± 4.8	33.3 ± 3.9	4.6 ± 5.2	18.9 ± 4.8	41.4 ± 3.7
[4-^{14}C]-5-azaCR	23.2 ± 0.9	19.9 ± 6.0	4.6 ± 0.1	3.6 ± 2.0	26.5 ± 5.0	19.1 ± 1.8	45.0 ± 10.9
[4-^{14}C]-5-azaCR + THU	6.0 ± 1.9	2.7 ± 1.2	8.6 ± 1.7	21.4 ± 1.8	9.3 ± 1.2	47.9 ± 4.2	29.3 ± 11.8

* Conditions for dosing animals were the same as those shown in Table 2. The values shown in h.p.l.c. peaks I–VI represent the per cent recovery of radioactivity from the column (from three or more analyses, mean ± S.D.). The urine samples shown in Table 2 were used for analyses in these studies.

Fold changes in urine due to THU:

HPLC peaks	1	2	3	4	5	6
[6-^{14}C]-5 AZ	↓ 4X	↓ 3X	No change	↑ 4X	0→ detectable	↑ 2X
[4-^{14}C]-5 AZ	↓ 4X	↓ 7X	↑ 2X	↑ 6X	↓ 3X	↑ 2.5X

Table 2. Effect of THU on the metabolism of [4-^{14}C]- and [6-^{14}C]-5-azacytidine in BDF$_1$ mice*

Treatment	Per cent recovery of administered dose Urine	$^{14}CO_2$	Total
[6-^{14}C]-5-azaCR	47 ± 14	19 ± 4	67 ± 17
[6-^{14}C]-5-azaCR + THU	45 ± 5	21 ± 3	66 ± 10
[4-^{14}C]-5-azaCR	46 ± 11	0.5 ± 0.1	47 ± 12
[4-^{14}C]-5-azaCR + THU	31 ± 12	0.5 ± 0	31 ± 12

* Mice were dosed i.p. with THU, 10 mg/kg, immediately prior to 5-azaCR (2.6–6.2 μCi), 50 mg/kg, and urine and CO_2 were collected over an 8-hr period. Three or more animals were used for each experiment and each experiment was run in triplicate. The values shown are expressed as the per cent recovery of administered radioactivity (mean ± S.D.).

Summary of the results:

- Since THU is a deaminase inhibitor, the result suggested that peaks 1, 2, and 5 represent metabolites via deamination.
- The increasing level of radioactivity in peaks 4 and 6 indicated that peaks 4 and 6 retained the 4-amino group.
- Less than 1% of the dose of [4-^{14}C]-5-azacytidine administered was recovered as $^{14}CO_2$, whereas approximately 20% of the dose of [6-^{14}C]-5-azacytidine was recovered as $^{14}CO_2$. These authors conclude that the triazine ring of 5-azacitidine was cleaved at the number 6-carbon which the animals expired as CO_2.
- THU had no effect on the recovery of radioactivity in expired air or urine.
- The HPLC-GC/MS analysis revealed 6 peaks in the urine samples of BDF mice received [4-^{14}C] or [6-^{14}C]-5-azacytidine (50 mg/kg, IP injection). Peaks 5 and 6 were the major peaks. They contained components with the 6-carbon absent.
- Peaks 1, 2, and 5 were deaminated metabolites.
- Peaks 1, 3, and 4 were 5-azauracil, 5-azacytosine, and 5-azacytidine, respectively.

Study title: Deamination of 5-azacytidine by a human leukemia cell cytidine deaminase. Chabner *et al.*, Biochem Pharmacol 22 (21): 2763-2765, 1973.

Deamination of 5-azacytidine was assayed by measuring the rate of ammonia production accompanying the conversion of 5-azacytidine to 5-azauridine. The product was separated from substrate by ion exchange chromatography. NH_4^+ production was determined by the velocity of glutamic dehydrogenase (measured by the decrease in absorbance at 340 nm) which was directly proportional to NH_4^+ generation. Deamination of 5-azacytidine by the extract from human leukemic leukocytes was linear with time and protein concentration. A double reciprocal plot of substrate concentration versus NH_4^+ production yielded a K_m value of 4.3×10^{-4} M.

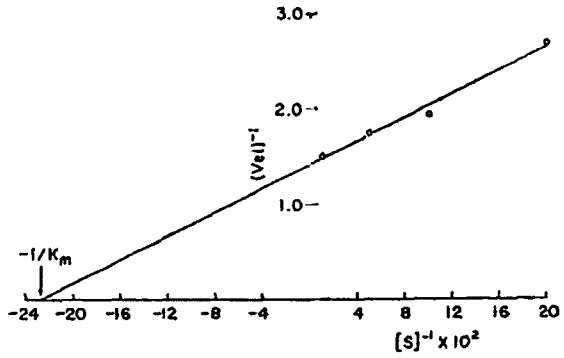


FIG. 1. Double reciprocal plot of 5-azacytidine concentration vs reaction velocity. A K_m value of $4{\cdot}3 \times 10^{-4}$ M was determined from this plot.

* Units of cytidine deaminase = nmoles of substrate consumed per hr.

The specificity of the enzyme, cytidine deaminase, was challenged in the presence of THU (tetrahydrouridine, a deaminase inhibitor). THU inhibited deamination of 5-azacytidine. The IC_{50} for THU inhibition was 5×10^{-5} M.

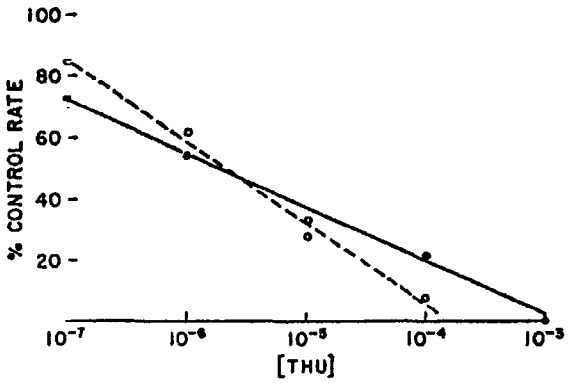


FIG. 4. Inhibition of the deamination of 5-azacytidine, 2 mM (O- - -O) and cytidine-2-^{14}C, 0·7 mM (●——●) by tetrahydrouridine. Assays were performed as described in text, except that tetrahydrouridine was added to reaction solution in concentration indicated 5 min prior to addition of substrate.

The hydrolysis decomposition of 5-azacytidine was summarized in Section 3.4.8 "Special Toxicology Studies", page 150 ("Isolation, characterization, and properties of a labile hydrolysis product of antitumor nucleoside, 5-azacytidine." Module #4.2.2.4.1).

3.3.6 Excretion

Studies were reviewed above. After IP administration of radiolabeled 5-AZ, approximately 60% (parent and metabolites) was found in the urine by 24 h. Six peaks were identified by HPLC-GC/MS. Several of the metabolites were identified as 5-azauracil, 5-azacytosine, and 5-azacytidine. Another possible metabolite is 5-azauridine.

3.3.7 Pharmacokinetic drug interactions

Data reviewed above suggest that 5-AZ may inhibit cytochrome P-450. No specific drug-drug interaction studies were conducted.

3.3.10 Tables and figures to include comparative TK summary

There are no study data concerning toxicokinetics.

Conclusions

- There was some distribution into the CNS. In toxicology studies, a single IV injection to mice caused hypoactivity and ataxia, and repeated IV administration (daily x 5) caused hypoactivity, impaired righting reflex and prostration.
- The significance of the higher concentration and longer retention in the lymphatic organs (i.e., spleen, thymus and bone marrow. This could reflect blood distribution to these tissues.
- According to sponsor's toxicology summary: Single dose IP to mice induced cloudy swelling and focal necrosis of kidney tubule epithelium, reduced liver glycogen, and other minor degenerative liver lesions. Single dose of IV to rats caused increased hepatic neutral lipid at doses of ≥ 51.7 mg/kg. Single IV administration to dogs caused early degenerative changes in the kidneys and liver. Thus the statement of no morphological changes in liver and kidneys was not in agreement with histopathological findings in other toxicology studies.
- Blood concentration of 5-azacytidine peaked shortly after administration and depletion was rapid, as measured in a microbiological assay. In a separate study, radiolabeled material of undefined composition was detected in blood even after 24 h administration.
- Tissue concentrations of 5-AZ were measured by microbiological and radiological methods. No residues were found by microbiological methods, whereas total radioactivity was observed. As there are methodological limitations to both analytical methodologies, firm conclusions as to the presence of parent 5-AZ in tissue cannot be drawn.
- A different tissue distribution data was found in female weaning [redacted] pigs. "Among the whole organs sampled following IV administration, kidney and liver had the highest specific activities of radioactivity." (quote from Dr. Coulter's review, Appendix A)
- Metabolism and urinary excretion of 5-azacytidine: ^{14}C-labeled 5-azacytidine was administered to mice and dogs. The HPLC chromatogram pattern was similar in both species.

- In mice, within 6-8 h one-half of the compound was excreted to the urine. By 24 hour, approximately 60% of 5-azacytidine (and its decomposition products) were excreted to the urine. In a separate mouse study, approximately 45% of the radiolabeled compound was recovered in the urine and another 20% in the expired air (as $^{14}CO_2$).
- In female weaning —— : pigs, 73% of the radioactive 5-AZ was excreted via the urine and 29% via the feces after 7 days (Dr. Coulter's review, Appendix A).
- Summary of pharmacokinetics in the mouse and dog (from the sponsor's submission):

Table 2.6-9: Pharmacokinetics in the Mouse and Dog

Species	Route	Dose (mg/kg)	T_{max}	C_{max}	T½	Elimination
Mouse[157]	IP	9.5 4.75	15 min 15 min	— µg/mL — µg/mL	-	-
Mouse[158]	IP	~13.7	-	-	3.8 hr	~60% in urine after 24 hr
Mouse[163]	IP	50	-	-	-	~47% in urine after 8 hr
Dog[164]	IV	0.5	-	-	-	~33% in urine after 4 hr

3.4 TOXICOLOGY

3.4.1 Overall toxicology summary

General toxicology:
Non-clinical toxicity studies were done in mice, rats, dogs and monkeys via the administration routes of PO, IP, and IV. Most studies had a single animal or only a few animals per sex per dose. No studies that investigate toxicity via the subcutaneous route (the proposed administration route of this NDA) have been conducted. However, pharmacokinetic data demonstrate rapid adsorption after intraperitoneal administration. The studies have identified bone marrow, liver, kidney and lymphoid tissues (e.g., spleen) as the target organs for 5-AZ toxicity. The time of death would be consistent with bone marrow depletion. The following table summarizes the findings:

Single dose Toxicity

Species	Route	Dose (mg/kg) schedule	STD10/HNSTD (mg/kg)	Major Findings
Mice	PO	431, 519, 624, 750	455	Wt. loss, reduced liver glygogen
Mice	IP	79.2, 99.7, 125.6, 158.1	89	Wt. loss, degeneration of kidney tubules and hepatocytes
Mice	IV	62.9, 79.2, 99.7, 125.6, 158.1	not assessed in the review	Wt. loss, increased extramedullary hematopoiesis in spleen
Rats	IV	41.0, 46.1, 51.7, 58.0, 65.1	not assessed in the review	Wt. loss, increased hepatic lipid
Dogs	IV	3.32, 6.65, 13.3	6.65	Wt. loss at high dose, decreased WBC, increase in ALT, AST (high dose), BUN (high dose), PT, degenerative changes in bone marrow, lymphatic tissues, kidney, and liver.

Repeat dose Toxicity

Species	Route	Dose (mg/kg) schedule	STD10/HNSTD (mg/kg)	Major Findings
Mice	PO	3.0, 4.16, 5.04, 6.0, daily x 5	3.1	Inactivity and alopecia, death (mean: Day 16)
Mice	IP	1.1, 1.61, 2.35, 3.42, 5.0 daily x 5	1.3	Inactivity and alopecia, death (mean: Day 13)
Dogs	IV	0.28, 0.55, 1.1, 2.2, 4.4, daily x 5	2.2	Wt. loss, anorexia, emesis and hypotension at HD. Decrease in WBC, hematoconcentration, thrombocytopenia, increase in ALT, AST, BUN, prolonged PT. Histopathological findings: liver (focal cloudy swelling, fatty vacuolization, marked fatty metamorphosis, diffuse reduction of glycogen, focal necrosis, granuloma), bone marrow and lymph nodes (hypoplasia)

Dogs	IV	0.28, 0.55, 1.1, daily x 5 with 9 days wash-off, 2 cycles	0.55	Death (HD, Day 15). Decrease in WBC and RBC (anemia), leukopenia, increase in ALT, BUN. Histopathological findings; Kidney (acellular, eosinophilic, globular precipitate), liver (congestion, decreased glycogen), lymph nodes and bone marrow (hypoplasia, hemorrhage in lymph nodes), injection site (focal edema), lung (secondary inflammation and infestation due to myelosuppression).
Monkeys	IV	0.28, 0.55, 1.1, 2.2, 14 days	1.1	Death (HD). Vaginal bleeding, salivation. Decreased hematocrit, leukocytes, increase in ALT, AST and BUN, Histopathological findings: bone marrow (hypoplasia, at greater than 0.55 mg/kg), kidney (tubular cloudy swelling), and liver (fatty metamorphosis, cloudy swelling and focal necrosis of tubules), lymph nodes and spleen (lymphoid hypoplasia at 2.2 mg/kg, focal necrosis in lymph nodes).

Genetic toxicology:

Mutagenicity Assessment

Assay	Activation	Strains	Results	Comments
E. coli	None	WP14 *Pro*$^-$, WP2 *Try*$^-$	+ in WP 14 *Pro*$^-$	Uridine and cytidine neutralized mutagenic effect
E. coli	None	CC101-106	+ in CC102, 103, 104, 105	Primarily GC-CG transversions; + in CC103 with or without *mucAB*; + in other strains only with *mucAB*
E. coli	None	WP3101P-WP3106P	+ in WP3103, WP3104	Similar to study above (see text for additional details)
S. typhimurium	None	TA7002, 7004, 7005	negative	
S typhimurium	None	TA100	Positive	
S typhimurium	None	TA98, TA100 *trpE8* and derivatives, DG1163, DG1197, *trpE8*/pKM101	+ for all 4 *trpE8* derivatives	
CHO	None	V79	Positive	
Mouse lymphoma	+S9	L5178Y	Positive	
Mouse lymphoma	None	L5178Y	Positive	
Human lymphoblasts	None	TK6	Positive at the *tk* and *hgprt* loci	

Clastogenicity assays

Assay	Results	Comments
Human lymphoblasts TK6	Negative	
SHE	Positive for micronuclei	↑ Kinetochores
Mouse lymphoma	Positive for micronuclei	↑ Kinetochores

Transformation assays

Assay	Results	Comments
SHE	positive	Negative for UDS

Carcinogenicity:

Studies in mice:

5-Azacitidine was positive for carcinogenicity in both studies submitted in this application. In BALB/c mice, 5-azacytidine at a dose of 2 mg/kg/wk for 50 weeks induced a significant increase in lymphomas and skin tumors in both sexes, lung tumors in males, and mammary carcinomas in females. Several other tumors in females were also observed. In B6C3F1 mice dosed at 2.2 mg/kg 3x/wk for 52 weeks, and then

observed for an additional 29-30 weeks, the induction of tumors of the hematopoietic system in female mice was associated with the administration of 5-azacytidine. High mortality in males and high dose females precluded a complete evaluation of this study.

Studies in rats:

In two studies reviewed, 5-azacitidine was positive carcinogenesis in one study (male Fisher rats, IP at 2.5 or 10 mg/kg x 2/week, for 9 month; females were not included in the study), inducing tumors in various organs. Most commonly these tumors were seen in skin, testis, lung, liver, spleen, and kidney, as adenocarcinoma, leukemia/lymphoma or malignant histiocytosis. The purpose of this study was to investigate the initiation and promotion of carcinogenesis; thus, a number of co-treatments in addition to 5-azacitidine exposure were studied. Restriction enzyme analysis and DNA methylcytosine content analysis indicated that hypomethylation may be the mechanism of carcinogenicity of 5-azacytidine. In a second study in which male and female Sprague-Dawley rats were given 5-azacitidine for 80-81 weeks, equivocal results were obtained because the dose of 5-azacytidine used decreased life span in the higher dose animals.

Reproductive toxicology:

5-Azacytidine caused reproductive and development toxicity in mice and rats. As indicated in the following tables, the embryotoxicity and development toxicity of 5-azacytidine is dose related and specific to the gestational age of the fetus. Of particular note is the increased number of resorptions noted in several studies when treated males were mated with untreated females and the effects on the CNS. The developmental toxicity findings occurred after single and repeat dosing and in the apparent absence of maternal toxicity.

Species	Dose (mg/kg)	Schedule	Major findings
Mouse	2-4	Single, repeat dose on GD 13-15	Neuronal deficit
mouse	2	Single dose GD 10-16	↑ embryolethality GD 10-14, peak at GD 10; ↓ mean litter wt peak on GD 10; ↑ malformations GD10-12: max % GD11; GD with maximum findings below GD10: cleft palate; defects skull bones; malformed bones in fore and hind legs; GD11: oligo and adactyly of front/hind paws; hematomas Additional CNS histopathological findings with GD peak: GD10: hypoplasia telencephalon and basal ganglia GD11: syncytial cell islands within ventricular zone; capillary ectasias and hemorrhages, necrotic cardiomypathy GD11: syncytial cell islands within ventricular zone; subpial heterotopias GD12: subpial heterotopias
	0.5, 1, 2, 4	Single dose GD 12, 14	↑ dose dependent embryolethality

Mouse	♂ 3	Dx3	Germ cell development affected when examined 24 h after last dose. Testicular cytology affected.
	♂ 3.3; ♀ 3.3	♂ dx3 24 h prior to mating; ♀ daily GD 11-13	↑ embryolethality when treated ♂ or ♀ mated with untreated animals, peak embryolethality when treated ♂ were mated with untreated ♀ within 1-7 days post dosing; recovery to control levels at 21-28 days post dosing
	♂ 3.3	Dx3 24 h prior to mating	↓ mean litter size when mated to untreated ♀ 1-7 post dosing; no effect when mated at later post dosing periods
	♂ 1, 3, 5	Dx3 24 h prior to mating	↑ resorptions dose-related; peak resorption within 1 week of dosing compared to later time post dosing
Rat	♂ 2.5, 5	3x/wk, 4 or 11 wk	↓ sperm counts dose related at 11 wk; ↓ pregnancy rate 5 mg/kg at 11 wks; ↑ preimplantation loss 11 weeks; no effect on postimplantation loss; no fetal abnormalities
	♂ 2.5, 4	3x/wk, 16 wk	↓ pregnancy rate 4 mg/kg; ↑ in abnormal 2-day embryos at 2 d a 4 mg/kg
Rat	0.15. 0.3, 0.6, 1.2	Daily DG 1-8	↑ embryolethality and resorptions from 0.3 mkd when examined on GD20 but no effect when examined on GD9; malformations found GD20 were microphthalmia and exencephaly
	0.5, 1	GD1-3 or 4-8	↑ embryolethality and resorptions when examined GD20
Rats	0.5, 1, 2	Single dose GD9-12	↑ embryolethality GD9 and 10, at 1 and 2 mg/kg; ↓ avg fetal wt/litter most dose groups GD9-12; ↑ malformations include (GD with peak occurrence noted): GD9: exencephaly-excephalocele from 0.5m/k GD10: extra ribs or ossifications from 0.5 m/k GD11: micromelia at 2 m/k GD12: club foot, syndactyly and oligodactyly from 0.5 m/k

Doses reflect treatment of females unless otherwise noted.

Special toxicology:

Topical application of 5-AZ to the rabbit skin and the hamster cheek pouch indicated that 5-AZ did not induce local irritation. 5-Azacitidine administered via IV injection to the jugular vein of hamsters did not cause thromboembolism.

Note: studies reviewed below are studies that have not been reviewed previously or capture data in greater detail (monkey study). All other studies were reviewed in the original NDA review (see Appendix A).

3.4.2 Single-dose toxicity

Study title: Toxicity of S-triazin-2-(1-H)-1,4-amino-1-β-D-ribofuranosyl-5-azacytidine (NSC 102816) clinical formulation in mice and rats administered a single dose by the intravenous route. NCI Contractor's report #ADL-NCI-73-43, Module 4.2.3.1.2.

The study was conducted by ——— under contract from the NCI (study date 6/22/73). 5-Azacitidine was administered by iv injection at 62.9-158.1 mg/kg (mice) and 41-65.1 mg/kg (rats). Ten animals/sex/group were treated. The only findings considered drug related were extramedullary hematopoiesis in the spleen in mice and an increase in the amount of hepatic neutral lipid in rats.

3.4.3 Repeat-dose toxicity

Study title: Repeated-dose toxicity of S-triazin-2-(1H)-1,4-amino-1-β-D-ribofuranosyl-5-azacytidine (NSC 102816) in the rhesus monkey.

Key study findings:

- A dose of 26.4 mg/m^2 NSC 102816 was lethal in the rhesus monkey. Clinical chemistry data from these two monkeys shows both hepatic and renal toxicity. Non-lethal doses showed mild hematological changes.

Study no.:	ADL-NCI-72-35
Study reviewed by:	Kimberly Benson, PhD
Volume #, and page #:	Module 4.2.3.2.1
Conducting laboratory and location:	/ / /
Date of study initiation:	Study report dated April 1972
GLP compliance:	No
QA report:	Yes ( ) no (X)
Drug, lot #, and % purity:	NSC 102816; Bulk lot # 920084; clinical formulation lot # 70-224; no % purity given

Methods

Doses:	6 dose groups • 0.28, 0.55, 1.1, 2.2 mg/kg 5-azacytidine • PVP solution equivalent to that in 0.55 mg/kg dose • Distilled water
Species/strain:	Rhesus monkeys
Number/sex/group or time point (main study):	1/sex/dose
Route, formulation, volume, and infusion rate:	IV; clinical formulation
Satellite groups used for toxicokinetics or recovery:	None
Age:	Not given
Weight (nonrodents only):	2.5-4.5 kg
Unique study design or methodology:	None

Observation times and results

Mortality:

Both HD monkeys died. The female monkey died one day after the Day 8 dose of 2.2 mg/kg and the male monkey died one day after the Day 14 dose.

Clinical signs:

Animals were examined daily for clinical signs of toxicity.

- Vaginal bleeding - HD (2.2 mg/kg) female that died, exhibited throughout treatment period.
- Salivation – 1 MD2 (1.1 mg/kg); 1 MD1(0.55 mg/kg) and 1 LD (0.28 mg/kg) monkey also seen in both PVP control monkeys
- Injection of sclera – 1 MD1 monkey 1 PVP control monkey

Body weights:

No significant effects of the drug treatment on body weight gains, measured daily during treatment and weekly thereafter.

Food consumption: No information was given on food consumption.

Ophthalmoscopy: Not conducted.

EKG: Not conducted.

Hematology: Blood samples were obtained every 4 days during treatment, and weekly thereafter.

Changes in Hematology Following NSC 102816 Administration – Percent Change From Sterile Water Control

Parameter	Dose Level of NSC 102816			
	LD (0.28 mg/kg)	MD1 (0.55 mg/kg)	MD2 (1.1 mg/kg)	HD (2.2 mg/kg)
Hemoglobin		↓ 33% (day 15) ♂ only		↑ 41% (day 8)
Hematocrit	↓ 20% (day 8) ♀ only ↓ 14% (day 15) ♀ only ↓12% (day 22) ♀ only	↓ 20% (day 4) ♂ only ↓ 19% (day 15) ♂ only	↓ 7% (day 4)	
Erythrocytes			↓ 9% (day 4)	
Leukocytes	↓66% (day 43) ♀ only	↑ 123% (day 4) ♂ only	↑ 107% (day 4) ♂ only	

Clinical chemistry: Blood samples were obtained every 4 days during treatment, and weekly thereafter.

Changes in Clinical Chemistry Following NSC 102816 Administration – Percent Change From Sterile Water Control

Parameter	Dose Level of NSC 102816			
	LD (0.28 mg/kg)	MD1 (0.55 mg/kg)	MD2 (1.1 mg/kg)	HD (2.2 mg/kg)
SGOT (AST)	↑ 59% (day 4) ↑ 14% (day 8) due mostly to ♀	↑ 25% (day 15) ♀ only	↑ 69% (day 4)	↑ 669% (day 8)
SGPT (ALT)	↑ 97% (day 8) ↑ 85% (day 15) ♀ only			↑ 207% (day 8)
BUN		↑ 70 % (day 8) ♀ only	↑ 83 % (day 8) ♀ only	↑ 380% (day 8)

Urinalysis: Urine was examined weekly. No effect of drug treatment was seen.

Gross pathology:

- HD female adrenals – hemorrhagic and mushy.
- HD female lungs – congested
- HD female liver – pale, enlarged
- MD2 male kidneys – multiple focal petechiae and pale cortex

Organ weights: Only significant change in organ weights was seen in the spleen weights of the two HD monkeys, which were ↓ 64% from water control.

Histopathology: Adequate Battery: yes (X), no ( )
Peer review: yes ( ), no (X)

Changes in Histopathology Following NSC 102816 Administration

Finding	Dose Level of NSC 102816			
	LD (0.28 mg/kg)	MD1 (0.55 mg/kg)	MD2 (1.1 mg/kg)	HD (2.2 mg/kg)
Kidney				
Autolysis				2/2
Liver				
Inflammation		2/2		
Abscess with infiltrates		1/2[&]		
Focal necrosis		1/2[&]		
Diffuse congestion				2/2
Early autolytic changes				1/2

Lung				
Focal emphysema		1/2*	1/2*	2/2
Foci of inflammatory cells		2/2		2/2
Congestion				1/2
Atelectasis				
Bone marrow				
Erythroid hypoplasia, slight		1/2&	1/2*	
moderate			1/2&	
Marked				2/2
Hypocellularity				
Lymph nodes				
Hyperplasia, slight	2/2			
Hypoplasia, slight-moderate				1/2
				1/2
Focal necrosis				1/2
Congestion, diffuse				
Spleen				
Autolysis, marked				1/2
Lymphoid hypoplasia				1/2
Testes				
Aspermatazoa				1/1
Trachea				
Focal squamous metaplasia			1/2&	1/2
Autolysis				

*Day 5 euthanized animal
&Day 46 euthanized animal

Toxicokinetics: Not conducted

Other: No additional parameters measured

Study Conclusions

The highest dose tested was lethal in both monkeys, 2.2 mg/kg or 26.4 mg/m^2. These monkeys both exhibited signs of liver and kidney toxicity, evident in the significant increases in BUN, SGOT (AST) and SGPT (ALT). Hematological changes were fairly minimal and in most cases, recoverable once dosing stopped. The concomitant erythroid depletion, seen in the bone marrow, was only evident in the monkeys euthanized immediately following drug administration, and not in those that were euthanized on Day 46. Increased WBCs were seen in the male monkeys in the two mid-dose groups in the early stages of dosing. These animals, as well as most others treated with NSC 102816,

showed signs of inflammation upon necropsy. Histopathology also showed autolysis of several organs in the animals that died from NSC 102816 administration.

Doses as high as 13.2 mg/m^2 were tolerated by the monkeys and signs of toxicity were slight to moderate and in many cases not observable once the recovery period had ended. Some histopathological examples of inflammation and erythroid hypoplasia persisted throughout the recovery period.

Histopathology inventory

Study Species	Rhesus monkey
Adrenals	X*
Aorta	X
Bone Marrow smear	X
Bone (femur)	
Brain	X*
Cecum	X
Cervix	
Colon	X
Diaphragm	X
Duodenum	X
Epididymis	
Esophagus	
Eye	X
Eye muscles, extrinsic	X
Fallopian tube	
Gall bladder	X
Gross lesions	
Harderian gland	
Heart	X*
Ileum	X
Injection site	X
Jejunum	X
Kidneys	X*
Lachrymal gland	
Larynx	
Liver	X*
Lungs	X*
Lymph nodes, axillary	X
Lymph nodes, bronchial	X
Lymph nodes, cervical	X
Lymph nodes, mandibular	
Lymph nodes, mesenteric	X
Mammary Gland	
Muscle, striated	X
Nasal cavity	
Optic nerves	X
Ovaries	X*

Pancreas	X
Parathyroid	X
Peripheral nerve	
Pharynx	
Pituitary	X
Prostate	X*
Rectum	
Salivary gland	X
Sciatic nerve	X
Seminal vesicles	
Skeletal muscle	
Skin	
Spinal cord	
Spleen	X*
Sternum	
Stomach	X
Testes	X*
Thymus	X*
Thyroid	X
Tongue	
Tonsils	X
Trachea	X
Urinary bladder	X
Uterus	X*
Vagina	
Zymbal gland	

X, histopathology performed
*, organ weight obtained

3.4.4. Genetic toxicology

Study title: Mutagenic effects of 5-azacytidine in bacteria. Collect. Czech. Chem. Commun., 30: 2883-2886, 1965 (Fucik *et al*).

Key study findings:
- 5-Azacytidine was mutagenic in *E. coli* strain WP 14 Pro^- , but not in WP 2 Try^-.
- Addition of uridine and cytidine to the culture medium decreased the mutagenic effect of 5-azacitidine.
- Mutagenic effect was not observed for decomposition products of 5-azacytidine

Study no.: Not applicable
Study type: AMES
Volume #, and page #: Module 4.3, References
Conducting laboratory and location: published article
Date of study initiation: published 1965
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: Not reported
Formula/vehicle: DMSO

Methods:

Strains/species/cell line:

Escherichia coli tester strains: WP 14 *Pro*$^-$ and WP 2 *Try*$^-$.

Dose selection criteria:

Basis of dose selection: Not defined.

Doses used in definitive study: 0, 0.4, and 4 μg/ml (Table 1)

Test agent stability: Not reported

Metabolic activation system: none

Controls:

Negative control: information not provided.

Positive controls: not included

Incubation and sampling times:

Incubation time: 16 hours.

Study design:

Preincubation method.

Analysis:

No. of replicates: The number of mutants was an average of 3-4 plates for each dilution. All assays were performed twice to confirm reproducibility.

Counting method: not provided.

Criteria for positive results: not defined in the article.

Summary of individual study findings:

Study validity: Not described. Tester strain titers used in the experiment were ≥ 3 x 10^8 cell/ml.

Study outcome:

The application of 5-azacytidine resulted in no increase in the number of revertants in WP 2 *Try*$^-$ (data not shown in the article). The following data tables are from the article.

Table I

The Effect of 5-Azacytidine on the Frequency of Revertants to Prototrophy in *Escherichia coli* WP 14 Pro$^-$

Experiment	Mutagen Concentration in the Medium μg/ml	Number of Viable Cells per ml	Number of Mutants per 10^8 Viable Cells
1	0	$5{\cdot}2 \cdot 10^9$	15
	0·4	$4{\cdot}7 \cdot 10^9$	135
	4·0	$3{\cdot}8 \cdot 10^9$	2 580
2	0	$1{\cdot}0 \cdot 10^{10}$	12
	0·4	$7{\cdot}8 \cdot 10^9$	38
	4·0	$7{\cdot}9 \cdot 10^9$	445

There was an increased number of mutants in the treated plates as compared to the control (experiment 1: 9-fold and 172-fold, experiment 2: 3-fold and 37-fold increase at 0.4 and 4 μg/ml of 5-azacytidine, respectively). No statistical information was provided. There was no explanation for the obvious difference in number of mutants of the two experiment results. No historical data of this laboratory was provided and a positive

control was not included. The authors report that concentrations used in the given medium possess a very low bacteriostatic activity.

Table II

Reversion of the Mutagenic Effect of 5-Azacytidine due to Natural Pyrimidine Nucleosides

Experiment	Mutagen and Nucleoside Concentration in the Medium	Number of Viable Cells per ml	Number of Mutants per 10^8 Viable Cells
1	0	$9{\cdot}6 \cdot 10^9$	50
	5-azacytidine 4 μg/ml	$7{\cdot}4 \cdot 10^9$	3080
	5-azacytidine 4 μg/ml + + uridine 80 μg/ml	$1{\cdot}6 \cdot 10^{10}$	450
	5-azacytidine 4 μg/ml + + cytidine 80 μg/ml	$1{\cdot}6 \cdot 10^{10}$	487
2	0	$1{\cdot}4 \cdot 10^{10}$	113
	5-azacytidine 5 μg/ml	$8{\cdot}8 \cdot 10^9$	3151
	5-azacytidine 4 μg/ml + + uridine 80 μg/ml	$1{\cdot}0 \cdot 10^{10}$	827
	5-azacytidine 4 μg/ml + + cytidine 80 μg/ml	$9{\cdot}2 \cdot 10^9$	491

The table above illustrates the mutagenic mechanism of 5-azacytidine may be related to its "antimetabolite character", since the addition of pyrimidine nucleosides (20 fold concentration), uridine and cytidine, neutralized its effect.

The following table shows the effects of decomposition products of 5-azacytidine, guanidyl-urea riboside and guanidine:

Table III

Examination of the Mutagenic Effect of the Decomposition Products of 5-Azacytidine

Experiment	Concentration of Compound in the Medium	Number of Viable Cells per ml	Number of Mutants per 10^8 Viable Cells
1	0	$2{\cdot}8 \cdot 10^{10}$	8
	5-azacytidine 4 μg/ml	$2{\cdot}2 \cdot 10^{10}$	103
	guanidyl-urea riboside 3 μg/ml	$2{\cdot}0 \cdot 10^{10}$	10
	guanidine 2 μg/ml	$2{\cdot}8 \cdot 10^{10}$	11
2	0	$1{\cdot}0 \cdot 10^{10}$	12
	5-azacytidine 4 μg/ml	$9{\cdot}1 \cdot 10^9$	157
	guanidyl-urea riboside 3 μg/ml	$9{\cdot}0 \cdot 10^9$	15
	guanidine 2 μg/ml	$1{\cdot}2 \cdot 10^{10}$	18

Study title: Effects of the *umuDC*, *mucAB*, and *samAB* operons on the mutational specificity of chemical mutagenesis in *Escherichia coli*: II. Base substitution mutagenesis. Mutation Research, 314(1): 39-49, 1994 (Watanabe *et al.*).

Key study findings:

- 5-Azacytidine was a primarily an inducer of GC→CG transversions.
- GC → TA transversions, AT-TA transversions, and to a lesser extent GC-AT transitions, as measured by an increase in Lac^+ revertants, were increased by 5-AZ only in the presence of pGW1700.

Note: data tables are from the article.

Study no.: Not applicable
Study type: bacterial mutagenesis
Volume #, and page #: Module 4.3, References
Conducting laboratory and location: published article
Date of study initiation: published 1994
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: Not reported

Formula/vehicle: DMSO

Methods:
Strains/species/cell line:

E. coli tester strains: CC101-CC106 (*E. coli* Lac$^-$ phenotype). Each of the 6 strains of CC101-CC106 is designed to detect one of the 6 types of base substitution; two transitions and four transversions. Each strain is constructed so that it contains a different mutation at the same codon in the *lacZ* gene (see Table 1).

The spontaneous mutation frequencies in each tester strain of *E. coli* with or without plasmid are presented in the following table. The plasmids enhance or are required for the bacterial response to uv (*umuDC, samAB*) or chemical mutagenesis (*mucAB*). The background level of each transition and transversion event was less than 2 Lac$^+$/10^8 cells. The introduction of plasmid pSEll7 (*umuDC*), pGW1700 (*mucAB*) or pYG8011, (*samAB*) did not change the frequency of spontaneous mutations.

TABLE 1

EFFECT OF PLASMIDS ON SPONTANEOUS MUTATION FREQUENCY

Strain	Reversion event	Plasmid	Viable cells /plate	Lac$^+$ revertants/ plate	Mut. fre. ($\times 10^{-9}$)
CC101	A·T→C·G	none	125	1	0.08
		pSE117	115	1	0.09
		pGW1700	92	2	0.22
		pYG8011	147	1	0.07
CC102	G·C→A·T	none	109	1	0.09
		pSE117	114	1	0.09
		pGW1700	65	1	0.15
		pYG8011	124	1	0.08
CC103	G·C→C·G	none	108	2	0.19
		pSE117	111	1	0.09
		pGW1700	96	1	0.10
		pYG8011	102	0	<0.10
CC104	G·C→T·A	none	129	1	0.08
		pSE117	117	2	0.17
		pGW1700	91	1	0.11
		pYG8011	101	1	0.10
CC105	A·T→T·A	none	124	0	<0.08
		pSE117	108	1	0.09
		pGW1700	80	1	0.13
		pYG8011	114	0	<0.09
CC106	A·T→G·C	none	131	0	<0.08
		pSE117	139	1	0.08
		pGW1700	137	0	<0.08
		pYP8011	157	1	0.08

Representative values are shown.

Dose selection criteria:

Basis of dose selection: Not defined in the article.

Doses used in definitive study: Lac$^+$ reversion test: 0, 1, 5, and 10 µg/plate (see Table 7 below)

Test agent stability: Not reported
Metabolic activation system: none
Controls:
Negative control: not indicated
Positive controls: not included
Incubation and sampling times:
Lac$^+$ reversion assay:
- Incubation time: 24 hours
- Sampling time: 36 and 48 hours after plating on lactose minimal medium, the mutant colonies were counted.

Study design: Preincubation method
Full growth plates: viable titer plated on glucose minimal medium or LB medium

Analysis:
No. of replicates: average of several determinations.
Counting method: 36-48 h after incubation. Method: not indicated.

Criteria for positive results: not defined in the article.

Summary of individual study findings:

Study validity:
- Not provided. The pre-incubation culture before plating was at density of 10^8 and 10^9 cells/ml.
- Spontaneous control data: Table 1.

Study outcome:
- 5-Azacytidine at 1-10 μg/plate induced a dose-related increase in transversions in CC103.
- pGW1700 (*mucAB*) did not affect revertant frequency 5-azacytidine in CC103. An increase in Lac$^+$ revertants was observed in CC102, CC104 and CC105 only in the presence of this plasmid.

TABLE 7

BASE SUBSTITUTION INDUCED BY 5-AZC IN STRAINS WITH AND WITHOUT PLASMID

Strain	5-AZC (μg/plate)	Number of Lac+ revertants/plate			
		no plasmid	pSE117	pGW1700	pYG8011
CC101	0	1	2	3	1
	5	1	0	1	0
	10	0	1	2	0
	15	0	0	1	0
CC102	0	2	4	2	2
	5	0	2	7	2
	10	2	1	7	0
	15	1	4	12	0
CC103	0	4	4	2	2
	1	62	36	72	40
	5	174	105	170	69
	10	299	145	277	73
CC104	0	1	1	4	1
	5	1	2	25	2
	10	3	4	39	1
	15	3	7	46	7
CC105	0	2	1	1	2
	5	1	1	145	1
	10	2	0	221	0
	15	0	0	215	0
CC106	0	0	0	0	0
	5	1	0	0	0
	10	0	0	0	0
	15	0	0	0	0

All values are the average of two plates.

TABLE 8

ENHANCING EFFECT OF *mucAB* AND *samAB* GENES OF TRANSITION AND TRANSVERSION

Strain	4-NQO	AF-2	9-AA	ICR-191	MMC	MNNG	4-AMC	5-AZC
mucAB (pGW1700)								
CC101	+++	+++	±	++	±	+++	±	±
CC102	+	++	±	+	±	−	−	++
CC103	−	++	±	±	±	++	±	−
CC104	+++	+++	++	+++	+++	+++	+++	+++
CC105	+++	+++	±	+++	+	+++	+++	+++
CC106	±	±	±	±	±	+	−	±
umuDC (pSE117)								
CC101	±	±	±	±	±	±	±	±
CC102	+	−	±	−	±	−	−	±
CC103	−	−	±	±	±	±	±	−
CC104	++	+++	+	+++	++	++	++	−
CC105	+	−	±	++	+	++	±	±
CC106	±	±	±	−	±	−	−	±
samAB (pYG8011)								
CC101	±	±	±	±	±	±	±	±
CC102	−	−	±	−	±	−	−	±
CC103	−	±	±	±	±	±	±	−
CC104	++	++	+	+++	++	++	++	−
CC105	−	−	±	±	±	−	−	±
CC106	±	±	±	±	±	−	−	±

Mutation enhancement by *mucAB* or *umuDC* was more than 10-fold (+++), 3–10-fold (++), 2–3-fold (+), less than 2-fold (−). ±, no mutation induction.

Study title: A comparison of mutation spectra detected by the *Escherichia coli* Lac$^+$ reversion assay and the *Salmonella typhimurium* His$^+$ reversion assay. Mutagenesis, 15(4): 317-323, 2000 (Ohta *et al.*). (Module 4.3, References)

Key study findings:

- 5-Azacytidine induced predominantly GC→CG transversion and also GC→TA transversion.
- 5-Azacytidine induced GC→A.T transition and GC→TA transversion was detected in the *E. coli* strains but not in the *S. typhimurium* strains.

Note: data tables are from the article.

Study no.: Not applicable
Study type: bacterial mutagenesis
Volume #, and page #: Module 4
Conducting laboratory and location: published article
Date of study initiation: published 2000
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: Not reported

Formula/vehicle: DMSO

Methods:

Strains/species/cell line:

E. coli tester strains: WP3101P-WP3106P (F' plasmids from CC101-106 (see study above) transferred to E. coli strain WP2*uvrA*).

S. typhimurium tester strains: TA7002, TA7004, TA7005.

The reversion event in each tester strain of *E. coli* and *S. typhimurium* tester is presented in the following table:

Table 1. Reversion event in tester strains of *E.coli* and *S.typhimurium*

Strain	Target gene	Base substitution for reverse mutation[a]	Amino acid change by reversion	Detected base substitution
Escherichia coli				
WP3101P	*lacZ*	aat Tag tca→aat Gag tca	Stop→Glu461	A·T→C·G transversion
WP3102P	*lacZ*	aat gGg tca→aat gAg tca	Gly→Glu461	G·C→A·T transition
WP3103P	*lacZ*	aat Cag tca→aat Gag tca	Gln→Glu461	G·C→C·G transversion
WP3104P	*lacZ*	aat gCg tca→aat gAg tca	Ala→Glu461	G·C→T·A transversion
WP3105P	*lacZ*	aat gTg tca→aat gAg tca	Val→Glu461	A·T→T·A transversion
WP3106P	*lacZ*	aat Aag tca→aat Gag tca	Lys→Glu461	A·T→G·C transition
Salmonella typhimurium				
TA7002	*hisC*	tcg aTa gct→tcg aAa gct	Ile→Lys217	A·T→T·A transversion
TA7004	*hisG*	tgc gGg ttg→tgc gAg ttg	Gly→Glu169	G·C→A·T transition
TA7005	*hisG*	tgc gCg ttg→tgc gAg ttg	Ala→Glu169	G·C→T·A transversion

[a]Capital letters denote the target base

Dose selection criteria:

Basis of dose selection: Not defined.

Doses used in definitive study:

- Lac$^+$ reversion test: 0-5 µg/plate (see figure below)
- His$^+$ reversion test: 0-5 µg/plate (see figure below)

Test agent stability: Not reported.
Metabolic activation system: none
Controls:
Negative control: vehicle control DMSO
Positive controls: not included
Incubation and sampling times:
Lac^+ reversion assay:
- Strains WP3101P and WP3106P were pre-incubated 20 min, and revertant colonies were scored 48 hr after plating.

His^+ reversion assay:
- *S. typhimurium* TA7002, TA7004 and TA7005 were pre-incubated 20 min, and revertant colonies were scored 48 hr after plating.

Study design:
- Preincubation method.

Analysis:
No. of replicates: single plate for each dose mutagen and duplicate plates for the solvent control. Assays were performed at 3-5 times to confirm reproducibility.
Counting method: the plates were counted 48 h after incubation.
Criteria for positive results: not defined.

Summary of individual study findings:
Study validity:
- No information provided
- Spontaneous control data:
  - The ranges of spontaneous Lac^+ per plate: WP3101P: 1-3, WP3102P: 7-15, WP3103P: 1-3, WP3104P: 6-11, WP3105P: 3-9, and WP3106P: 1-3.
  - The ranges of spontaneous His^+ per plate: TA7002: 4-10, TA7004: 15-29, and TA7005: 20-39.

Study outcome:
- Mutations were observed in WP3103P (89%) and WP3104P (11%).
- In *S. typhimurium* strains neither GC→AT transitions nor GC→TA transversions were detected.

Study title: Induction of malignant transformation and mutagenesis in cell cultures by cancer chemotherapeutic agents. Marquardt and Marquardt, Cancer (suppl.) 40: 1930-1934, 1977.

- The authors found 5-azacytidine to be mutagenic in *S. typhimurium* as well as in V79 CHO cells.

TABLE 3. Mutagenicity of 5-Azacytidine and Pseudoisocytidine

Compound	Mutagenicity test in Salmonella typhimurium (TA 100) µg/plate	% Survivors	his+ revertants/ 10^8 survivors	Mutagenicity test in V79 Chinese hamster cells µg/ml	% Survivors	Ag^r colonies/ 10^5 survivors
Control		100	2		100	2
N-methyl-N'-nitro-N-nitrosoguanidine	1	86	5	0.2	60	44
	10	18	168	0.4	44	85
	20	4	687	1	17	334
5-azacytidine	1	92	3	1	83	8
	10	67	5	5	72	14
	20	49	10	10	61	15
	40	32	18	20	49	19
	60	19	26			
	80	0	—			
pseudoisocytidine	1	90	3	1	90	11
	10	82	7	5	85	14
	20	38	19	10	76	15
	40	7	34	20	56	24
	60	0	—			

- The dose levels of 1 and 10 µg/plate are considered nontoxic (≤ 50% reduction in the background lawn) and 20 µg/plate were borderline toxic in the TA100 mutagenesis assay.

Study title: Mutagenicity of 5-azacytidine in *Salmonella typhimurium*. Podger, Mutation Research, 121 (1): 1-6, 1983. Module #4.2.3.3.1.3

- The Ames mutagenicity test used the plate incorporation method with incubation time 3-4 days, and revertant colonies were counted with an Artek 800 automatic counter. All experiments were repeated at least 3 times.
- 5-Azacytidine at 1-10 µg/plate was not mutagenic in TA98 and TA100.
- 5-Azacitidine induces revertants in the *trpE8* strains of *S. typhimurium*.

Tables are from the article.

TABLE 1

MUTAGENICITY TEST WITH 5-AZACYTIDINE IN STRAINS TA98 AND TA100

5-Azacytidine (µg/plate)[b]	Number of His+ colonies per plate[a] TA98	TA100
	36	149
1	41 (5)	152 (3)
2	40 (4)	146 (−)
4	32 (−)	147 (−)
6	37 (1)	144 (−)
8	29 (−)	155 (6)
10	26 (−)	153 (4)

[a] First numbers are the means of colonies appearing on the selection plates. Numbers in brackets have been corrected for the spontaneous mutation frequency and are therefore the numbers of mutants induced by each dose.

[b] Maximum volume of 5-azacytidine added to any plate was 100 µl.

TABLE 2

5-AZACYTIDINE-INDUCED BACK-MUTATION TO PROTOTROPHY OF DIFFERENT DERIVATIVES OF *Salmonella typhimurium* STRAIN *trpE8*

5-Azacytidine (μg/plate)[b]	Number of Trp$^+$ colonies per plate[a]			
	trpE8	*trpE8 uvr*	*trpE8 recA*	*trpE8*/pKM101
0	23	44	16	166
1	66 (43)	88 (44)	40 (24)	246 (80)
2	89 (66)	123 (79)	34 (18)	283 (117)
4	169 (146)	205 (161)	15 (0)	449 (283)
6	263 (240)	303 (259)	13 (0)	589 (423)
8	327 (304)	411 (367)	10 (0)	762 (596)
10	442 (419)	558 (514)	9 (0)	906 (740)

[a] First numbers are the means of colonies appearing on the selection plates. Numbers in brackets have been corrected for the spontaneous mutation frequency and are therefore the numbers of mutants induced by each dose.

[b] The maximum volume of 5-azacytidine added to any plate was 100 μl.

Study title: The mutagenicity and other inhibitor of replicative DNA synthesis in the L5178Y mouse lymphoma cell. Mutation Research, 176(1): 123-31, 1987 (Amacher and Turner).

Key study findings: 5-Azacytidine was mutagenic in L5178Y mouse lymphoma cells.

Study no.: Not applicable
Volume #, and page #: Module 4.2.3.3.1.1
Conducting laboratory and location: published article
Date of study initiation: published 1987
GLP compliance: No
QA reports: No
Drug, lot #, and % purity: Not reported

Formula/vehicle: DMSO

Methods:
Strains/species/cell line: mouse lymphoma L5178Y cells:
Concentration selection criteria:
Basis of concentration selection: Not defined
Concentrations used in definitive study:

- Mutagenicity and cytotoxicity test: 0-approximately 1200 ng/ml
- 5-Azacytidine: 51 ng/ml and 69 ng/ml
  Cytidine: 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 x 10^{-4} M.

Test agent stability: Not reported.
Metabolic activation system: rat liver S9

Controls:

Negative control: vehicle control DMSO

Positive controls: ethyl methanesulfonate (EMS) (621 µg/ml)

Incubation and sampling times:

- Incubation time: 3 h
- Expression period: 48 h
- Selection period: 8 days

Study design:

Plate method

Analysis:

No. of replicates: not provided.

Counting method: automatic counter.

Criteria for positive results: not defined

Summary of individual study findings:

Study validity: Not specified

Study outcome:

- 5-Azacytidine induced a dose-related increase in the TFT^{res} mutant frequency as cell survival decreased exponentially.
- The mutagenic effect was decreased in the presence of either S9 activation or heat-inactivated S9 mix. The authors suggest this may be due to the presence of endogenous cytidine in the S9 mix.
- An inhibition of 5-azacytidine mutagenicity was observed with supplemental cytidine. The authors suggest that the findings may be related to the higher affinity of cytidine for uridine kinase, the enzyme that catalyzes the phosphorylation of uridine and cyditine.

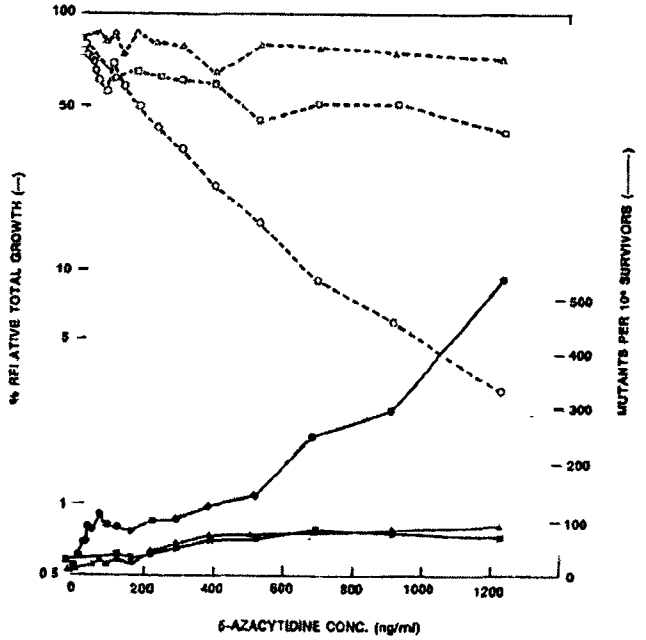


Fig. 1 Mutagenicity and cytotoxicity of 5-azacytidine in 3.7.2C L5178Y cells. Results are shown for 3 h treatments with 5-azacytidine (circles); 5-azacytidine + enzymatically active S9 mix (triangles), and 5-azacytidine + heat-denatured S9 mix (squares). Final S9 mix concentration was 20 µl/ml.