PRELIMINARY TOXICITY STUDIES WITH THE DNA-BINDING ANTIBIOTIC, CC-1065†

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It was previously shown that the potent new DNA-binding antibiotic, CC-1065, prolonged life span, but was not curative, when administered to mice bearing a variety of transplantable tumors. In this paper we show results of preliminary studies indicating that CC-1065 caused lethal delayed hepatotoxicity at therapeutic antineoplastic doses.

In non-tumor-bearing mice toxic deaths were delayed *ca* 50 days after a single iv dose of 12.5 μg/kg and as much as 70 days after 10 μg/kg was given ip. Intravenous mouse LD_{50}'s were 9 μg/kg, single dose, and 0.3 μg/kg/day, five daily doses. Intraperitoneal LD_{50}'s were 0.53 ~ 6.90 μg/kg, single dose, and 0.14 μg/kg/day, five daily doses. Mice treated with high doses iv died within 12 days with frank hepatic necrosis, whereas delayed deaths at lower doses were associated with changes in hepatic mitochondrial morphology. This suggested that separate mechanisms of hepatotoxicity were operative at high and low dose ranges. Attempts to prevent the delayed toxicity of CC-1065 in the mouse by treatment with WR-2721, *N*-acetylcysteine, phenobarbital, Aroclor 1254, and 3-methylcholanthrene were unsuccessful; no effect on the LD_{50} or the times of death was observed.

Lethal doses in the rabbit were similar on a body surface area basis to those in the mouse; evidence of hepatotoxicity was also observed in the rabbit.

CC-1065 is an antibiotic of novel structure and unusually high potency which binds covalently to double-stranded mammalian DNA[1~5]. The agent inhibited DNA synthesis and killed tumor cells in culture at subnanogram/ml concentrations[6]. *In vivo*, CC-1065 prolonged life span, but was not curative when used to treat a variety of transplantable murine tumors[7]. In this paper we summarize more recent studies with CC-1065 in non-tumor-bearing mice and in rabbits which revealed an unusual delayed form of hepatotoxicity occurring at the doses which prolonged life in tumor-bearing mice. A preliminary report on the toxicity of CC-1065 has previously appeared[8].

Materials and Methods

Agents

CC-1065 was produced by microbial fermentation of *Streptomyces zelensis* and isolated by published methods at The Upjohn Company, Kalamazoo, Michigan[7]. CC-1065 was analytically pure by spectral and thin-layer chromatographic criteria. WR-2721** was obtained through the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, and used as received. *N*-

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** Abbreviations used are: WR-2721, *S*-2-(3-aminopropylamino)ethylphosphorothioic acid; DMA, dimethylacetamide; WBC, white blood cell; RBC, red blood cell; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase; BUN, blood urea nitrogen.

Acetyl-L-cysteine and 3-methylcholanthrene were obtained from Sigma Chemical Co., St. Louis. Aroclor 1254 was purchased from Analabs, Inc., North Haven, CT. Phenobarbital was purchased from Mallinkrodt, St. Louis. Emulphor EL-620P was obtained from GAF Corp., New York. DMA was purchased from Eastman Kodak, Rochester, New York or from Aldrich Chemical Co., Milwaukee. All commercially purchased chemicals were used as received. All other chemicals were reagent grade.

Formulations

A preliminary toxicity study of intravenous CC-1065 in non-tumor-bearing (normal) mice was conducted using a vehicle composed of 5% DMA, 10% propylene glycol, and 85% 0.1 M Na_2CO_3. This vehicle had a pH of *ca* 11. Dosing solutions were made up just prior to injection, kept on ice and protected from light until drawn into syringes. A stability study on this vehicle using *in vitro* L1210 leukemia cell growth inhibition for quantitation showed that *ca* 80% of bioactivity remained after one hour of incubation under the conditions used in the preliminary toxicity study. Because of limited stability and the fact that this vehicle was corrosive when injected into the mouse tail vein, a more physiologically compatible vehicle was developed for subsequent studies.

Intravenous and intraperitoneal toxicity studies in the mouse and an intravenous study in the rabbit were conducted using a vehicle composed of 5% DMA, 10% Emulphor, 85% distilled water. In the oral lethality study, 10% DMA, 10% Emulphor, 80% water was used. Preliminary stability studies using L1210 leukemia growth inhibition *in vitro* for quantitation indicated that CC-1065 was stable indefinitely when stored at −20°C as a stock solution in neat DMA or DMSO and stable (>90%) for at least 96 hours when stored at 4°C in the DMA/Emulphor/water vehicle.

A percutaneous absorption study was conducted to determine if CC-1065 elicited toxic effects or deaths when applied to the skin of hairless mice. This study used a vehicle of DMA - acetone (6: 4).

In protection experiments, *N*-acetylcysteine and phenobarbital were given as solutions in saline and Aroclor 1254 and 3-methycholanthrene were administered as solutions in corn oil. WR-2721 was dissolved in distilled water.

Intraperitoneal, Intravenous, and Oral Mouse Toxicity Studies

Mice were male and of the $CD2F_1$ strain and weighed 20∼25 g when studies were initiated. Injection volumes were 0.2 ml in iv and ip studies and 0.5 ml in po studies. Po doses were administered by gavage. The first day of dosing was defined to be day 1.

In a preliminary study, 8∼10 mice/dose level were treated iv with CC-1065 over a range of 25∼800 μg/kg, single dose, and 6∼200 μg/kg/day, five daily doses. Mice were weighed on days 1 and 8 and dead animals were recorded daily for 38 days, at which point all mice but controls had died. On days 4 (800 μg/kg, single dose; 50 and 200 μg/kg/day, five daily dose) and day 30 (all other groups), one or two moribund mice from each group were sacrificed and necropsied, gross observations were recorded, and tissues were preserved in buffered formaldehyde solution. All preserved tissues were examined by light microscopy; in addition selected liver sections were examined by electron microscopy. In a more extensive follow-up study, 10 animals/dose level were administered ip, iv, or po doses of CC-1065 on single dose or five daily dose schedules. Animals were weighed on days 1 and 5 and then weekly through day 79 (through day 120 for ip-treated survivors). Dead animals were recorded daily for 94 days (po and iv groups) or 120 days (ip groups). At selected dose levels two moribund mice were anesthetized with Metofane and terminal blood samples were taken from the inferior vena cava for hematological studies. Tissues were excised, preserved, and examined as described above. Hematological measurements included RBC and WBC counts (total and differential), hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet count. The studies by the ip route were subsequently repeated at lower doses because a nonlethal dose was not achieved in the first ip study. Histopathology and hematology studies were not done in the repeat ip studies.

Percutaneous Absorption Study in the Mouse

Fifty μl of a solution of CC-1065 at various concentrations in DMA - acetone (6: 4) was applied with a piston-actuated micropipetting device to the flanks of female HRS/J hairless mice (Jackson Laboratories, Bar Harbour, ME). Ten mice were treated at each dose level (14∼220 μg total dose/mouse plus

vehicle controls). Mice were weighed on days 1, 3, 5, 9, once weekly through day 58, and on days 77 and 122. Two mice from the highest dose group were necropsied and gross observations were recorded. No histopathological or hematological studies were conducted.

CC-1065 Toxicity Protection Experiments in the Mouse

Several substances were used in attempts to prevent the acute or delayed toxicities of CC-1065. All experiments were conducted using non-tumor-bearing male $CD2F_1$ mice (6~10/dose level). Test animals received a single iv treatment of CC-1065 at dose levels of 3~800 μg/kg along with the antidotal material. Control animals received CC-1065 alone, the antidote alone, or treatment with the two vehicles. Surviving mice were counted daily for at least 60 days. *N*-Acetylcysteine was used with two different protocols: a) given ip at 250 mg/kg/injection at 30 minutes prior to and 6 hours subsequent to CC-1065 administration, then twice weekly for 4 weeks; b) given at 2,000 mg/kg/injection 30 minutes prior to CC-1065, then weekly for 5 weeks. The microsomal-inducing agents, Aroclor 1254, phenobarbital, and 3-methylcholanthrene were also tested. Aroclor was given once ip 4 days prior to CC-1065. Phenobarbital and 3-methylcholanthrene were administered ip once daily for 3 days prior to CC-1065. The radio- and chemoprotective agent WR-2721 was given ip at 500 or 250 mg/kg once 30 minutes prior to CC-1065.

Rabbit Lethality Study

New Zealand white rabbits weighing 2.4~3.2 kg were treated iv with single doses of CC-1065 at 0.1, 0.3, 1, 3, 10, 30, 100, or 300 μg/kg, one animal/sex/dose level. CC-1065 was administered as an iv infusion (Harvard Model 940 infusion pump) into the marginal ear vein of restrained rabbits over 8~10 minutes. Blood samples were drawn pre-treatment, and, in surviving animals, on day 5 and weekly thereafter through day 72 for complete hematology and blood chemistry studies. Hematological measurements included all those listed above for the mouse toxicity studies. Blood chemistry measurements were carried out on a Technicon Auto Analyzer SMA 12/60 and included analyses for calcium, inorganic phosphate, glucose, BUN, uric acid, cholesterol, albumin, total protein, total bilirubin, alkaline phosphatase, SGOT and lactic dehydrogenase. Manual blood chemistry analyses were performed for SGPT, sodium, potassium, chloride, and on terminal samples only, creatine phosphokinase. Animals were observed daily. The study was terminated on day 73, necropsies were performed on all animals and gross observations were recorded. Limited histological studies were performed on preserved tissues.

Results

Toxicity in the Mouse

Toxicity by ip and iv Routes

A preliminary iv study in non-tumor-bearing mice revealed that groups of mice receiving "high" doses of CC-1065 (400~800 μg/kg, single dose or 50~200 μg/kg/day, five daily doses) died uniformly by day 9, as expected based on the dose-response data from most chemotherapy studies. Very few mice died at lower doses between days 6 and 30 at which point the study was scheduled to terminate. The period of observation was extended when, on day 30, extensive weight loss, a hunched posture, and ruffled fur were noticed in surviving animals. The remaining animals at all dose levels died (or were necropsied when moribund) between days 32 and 38. Observations of pale livers and kidneys were made at necropsy of high-dose animals (*ie*, moribund on or before day 9). At lower doses, ascites and lack of body fat were noted in drug-treated animals. Histological studies showed, at higher doses (200~800 μg/kg, single dose), disseminated degenerative changes in hepatocytes that ranged from acute diffuse cytoplasmic swelling to focal coagulation necrosis involving small groups of hepatocytes in a random pattern. At 100 μg/kg, single dose, the areas of necrosis were smaller generally involving single cells. Multifocal degeneration with necrosis of isolated solitary hepatocytes was observed with 50 μg/kg, five daily dose

treatment.

Lesions were similar in the 50, 25, and 12.5 μg/kg, five daily dose groups, with a moderate reduction in severity as dose was decreased. Ultrastructural changes at 12.5 μg/kg, five daily dose, involved a loss of endoplasmic reticulum and marked morphologic changes in the mitochondria. Mitochondrial changes involved swelling and derangement and hypersegmentation of cristae. Numerous mitochondria had electron dense foci typical of Ca^{++} accumulation. Foci of mineralization were also present in the cytoplasm. In the nucleus there was irregular dilatation of nuclear envelope. Findings in tissues other than liver were normal.

Subsequent studies were carried out with both iv and ip administration over a greater range

Table 1. Lethal doses of CC-1065 in the $CD2F_1$ male mouse.

Route	Schedule	Dose range (μg/kg/day)	LD_{50}[b] (μg/kg/day)
iv	Day 1 only	0.8~400	5.50~9.00
iv	Days 1~5	0.1~12	0.30
ip	Day 1 only	0.03~400	0.53~6.90
ip	Days 1~5	0.012~100	0.14
po	Day 1 only	250~8,000	>8,000[c]
po	Days 1~5	63~2,000	>2,000
Percutaneous	Day 1 only	14~220[a]	>220[a,c]

[a] Dose units are μg/mouse.
[b] Range of LD_{50} values in 1~3 experiments estimated by Probit method.
[c] No deaths were observed at the highest dose tested.

Table 2. Time of death in mice treated with CC-1065.

Intravenous administration			Intraperitoneal administration		
Schedule	Dose (μg/kg/day)	Median day of death[a]	Schedule	Dose (μg/kg/day)	Median day of death[d]
Day 1 only	400	4 ~ 8[b]	Day 1 only	300	8 ~ 31
	200	5.5~ 9		100	34.5~ 40
	100	27.5~34.5		30	72 ~ 65
	50	31.5~38		10	68.5~ 73
	25	37 ~42.5		3	117.5~no deaths
	12.5	43 ~54		1	No deaths~no deaths
	6.25	No deaths~[e]		0.3	No deaths~no deaths
	3.13	No deaths~[e]		0.1	No deaths~no deaths
				0.03	No deaths~no deaths
Days 1~5	200	5[c]	Days 1~5	100	6 ~ 8
	100	6		50	11 ~ 19.5
	50	9		25	33 ~ 36
	25	32		12.5	36 ~ 40
	12.5	31 ~10		6.25	39.5~ 81.5
	6.3	33 ~32		3.13	111 ~101
	3.13	~32		1.56	100 ~ 74.5
	1.56	~32		0.78	69 ~ 73.5
	0.78	~34		0.39	50 ~ 51
	0.39	~42		0.19	51 ~ 68
	0.19	~No deaths		0.098	[e]~no deaths
	0.098	~No deaths		0.048	[e]~no deaths
				0.024	[e]~no deaths
				0.012	[e]~no deaths

[a] Dead animals were counted for 75 days.
[b] Range of values in three experiments on the single dose schedule.
[c] Two experiments were conducted on the five daily dose schedule covering dose ranges of 6.3~200 μg/kg/day and 0.1~12.5 μg/kg/day. In the former study, a vehicle consisting of 5% DMA, 10% propylene glycol, and 85% distilled water was used (see Materials and Methods section).
[d] Range of values in two experiments; dead animals were counted for 150 days.
[e] Not tested in a given experiment.

Table 3. Hematological findings in CC-1065-treated mice.[h,i]

	Controls		Intravenous				Intraperitoneal									
			Day 1 only		Days 1~5		Day 1 only				Days 1~5					
			12[g]		0.4		25		1.6		12		0.8		0.4	
WBC[a]	5.1	4.9	3.6	3.5	4.1	3.9	1.2	1.0	5.3	8.1	1.2	0.3	10	5.8	4.0	4.7
RBC[b]	10	10	9.2	9.4	8.8	10	10	10	10	10	10	4.2	10	10	10	10
Hgb[c]	15.9	15.9	12.6	13.0	12.5	14.3	17.3	16.4	18.6	18.6	15.7	6.1	15.9	20.4	16.9	17.4
Hct[d]	47.8	47.0	38.2	38.2	37.5	42.9	50.1	48.1	52.2	52.9	46.5	16.7	44.7	58.4	50.0	50.5
Plate[e]	792	912	206	176	138	192	717	1,000	690	962	1,000	417	0	821	787	954
Non-Seg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Seg %	10	16	65	79	66	73	70	56	61	69	54	88	0	70	59	78
Lympho	85	80	34	21	32	26	28	40	39	31	46	12	99	30	39	21
Mono	3	3	1	0	0	1	2	4	0	0	0	0	1	0	2	1
Eosino	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Baso	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Cells/μl $\times 10^{-3}$.
[b] Cells/μl $\times 10^{-6}$.
[c] g %.
[d] %.
[e] Platelets/μl $\times 10^{-3}$.
[g] Dose units are μg/kg, day 1 only, and μg/kg/day, days 1~5.
[h] No significant differences from control animals were noted in measurements of MCV, MCH, or MCHC.
[i] Data obtained from moribund mice sacrificed between days 38 and 52. Two mice were studied at each dose level.

of doses. Table 1 lists LD_{50}'s by the iv and ip routes on both single dose and five daily dose schedules. Given iv, cumulative toxicity was observed on the multiple dosing schedule; a total dose of 1.5 μg/kg (0.3 μg/kg/day $\times$ 5) was the LD_{50} on the five daily dose schedule compared to 9 μg/kg as a single dose. This was also true with ip treatment although greater variability between experiments was noted.

Delayed deaths were observed with iv and ip administration on both single and multiple dose schedules (Table 2). With iv treatment times of death were quite uniform within a given dose level and between experiments. As noted above, more variability was seen between experiments with ip administration. There was a distinct "break" between the acute and delayed deaths.

Weight change data showed similar trends in both iv and ip-treated mice (data not presented). At higher doses mice lost weight progressively from dosing until death. At lower doses causing delayed deaths, mice gained weight initially after dosing but then lagged behind control animals and, in many cases, showed weight loss for a week or two prior to death. Mice receiving sublethal doses given iv gained weight at approximately the same rate as controls.

Gross observations at necropsy in moribund iv-treated mice and in higher-dose ip-treated mice were similar to those described above. In lower dose ip-treated mice (moribund at day 30), extensive visceral adhesions involving the alimentary canal and liver were observed in addition to pale kidneys. The adhesions produced restrictions in the small and large intestine. The adhesions were apparently progressive; mice posted at later times had more severe lesions.

Hematological studies in selected dose groups of mice (Table 3) which were moribund between days 38~52 revealed different drug effects depending on the route of administration. Depression of WBC count (*ca* 80%) was the major finding in ip-treated animals receiving the higher doses of those groups studied. In iv-treated animals, platelet counts were depressed *ca* 75%. However, in both iv and ip-treated animals, differential WBC counts revealed an increase in percent segmented cells and a corresponding decrease in lymphocytes.

Histological studies on iv-treated mice showed hepatic lesions similar to those reported above. Hepatocellular necrosis and erythroid hypoplasia of the bone marrow were noted in mice receiving higher doses ip.

Toxicity by po and Percutaneous Routes

No drug-related deaths or toxic effects were observed at any dose tested when CC-1065 was applied to the skin of hairless mice (highest dose tested was 220 μg/mouse). No drug-related deaths were observed at any dose of CC-1065 to 8 mg/kg, single dose, given orally. A few mice died between days 3 and 10 with five daily dose oral treatment; since 2/10 control animals also died during this period, the deaths may have been caused by the trauma of daily gavage. Gross observations at necropsy of selected mice treated orally or percutaneously were unremarkable.

Table 4. Acute toxicity of intravenous CC-1065 in the rabbit.

Dose[a] (μg/kg)	Day 60 Survivors/total	Day of death
300	0/2	1, 1
100	0/2	2, 2
30	0/2	5, 13
10	1/2	22
3	2/2	—
1	1/2	28
0.3	2/2	—
0.1	2/2	—

[a] Day 1 only schedule; drug was infused over *ca* 10 minutes into the marginal ear vein.

Attempts to Protect from CC-1065 Toxicity

Neither *N*-acetylcysteine, WR-2721, phenobarbital, 3-methylcholanthrene, nor Aroclor 1254 had any significant effect on the LD_{50} of intra-

venous CC-1065 nor the pattern of acute or delayed death times.

Rabbit Lethality Study

CC-1065 was also lethal at very low doses given iv to rabbits and caused delayed deaths (Table 4). The intravenous single dose LD_{50} in the mouse was 9 μg/kg or 27 mg/m^2, which is the body surface area dose equivalent of *ca* 3 μg/kg in the rabbit. Although a precise LD_{50} cannot be estimated with the limited number of rabbits treated, the data suggest that CC-1065 is equipotent in its toxicity to both mouse and rabbit. As in the mouse, high doses killed rabbits in a relatively short time and deaths at lower doses were delayed. Hepatocellular degeneration and multifocal necrosis were prominent microscopic lesions in the rabbit. Toxic deaths at 300 and 100 μg/kg levels occurred before terminal blood samples could be obtained. At 30 and 10 μg/kg, elevation of serum SGOT and cholesterol was noted compared to controls. SGPT, creatinine and BUN levels were also elevated in one of two rabbits at the 30 μg/kg dose level. Hematologic findings were unremarkable.

Discussion

Previous studies showed that CC-1065 given ip prolonged the lifespan of, but failed to cure, mice bearing a variety of transplanted tumors[7]. The data presented here indicates that therapeutic antitumor doses caused delayed deaths in non-tumor-bearing mice, apparently from hepatotoxicity and peritonitis. Lethal delayed hepatotoxicity was seen with both iv and ip administration. Bone marrow depression was observed but was of lesser severity than the hepatotoxicity. High doses of CC-1065 (above the optimum antitumor dose) were also hepatotoxic but deaths occurred much sooner after dosing than at lower doses; hepatic necrosis was evident by light microscopic examination. Preliminary histological studies summarized in this paper and a subsequent detailed evaluation[9] suggested that different mechanisms mediated the acute and delayed dose-related toxicities of iv-administered CC-1065. Alterations in mitochondrial structure and function, possibly as a result of drug binding to mitochondrial DNA, have been implicated in the delayed toxicity[9]. Unfortunately doses of CC-1065 which were not lethal in the mouse were only marginally antitumor active.

Delayed deaths at very low doses were also seen in the rabbit, suggesting that the toxicity was not peculiar to the mouse. The deaths in rabbits were associated with gross and microscopic lesions in the liver and kidneys.

Delayed toxicity in the mouse was previously reported for the clinically useful podophyllotoxin, VM-26[10]. VM-26 given ip caused peritonitis and morphologically atypical livers with reductions in hepatic cytoplasmic volume and degenerative nuclear changes. Deaths were delayed to *ca* 100 days after ip administration on a days 1, 4, 7 schedule. Studies of toxicity by the iv route were not reported in the mouse. When doses of VM-26 were reduced to levels that were non-lethal from delayed toxicity, significant antitumor activity was maintained against the murine L1210 and L5178Y leukemias.

Delayed deaths were also observed to occur in mice treated ip with certain antileukemic phthalanilide derivatives[11]. Hepatic and renal toxicities were implicated as possible causes of these delayed deaths.

Various studies have demonstrated the ability of WR-2721 and *N*-acetyl-L-cysteine to protect mice from the lethal effects of DNA-reactive antitumor agents[12,13]. These agents were shown in our studies to have no effect on either the LD_{50} or the time of death after iv administration of CC-1065. Likewise, pretreatment of mice with the microsomal inducers, phenobarbital, 3-methylcholanthrene, and Aroclor 1254 had no effect on CC-1065 lethality. The latter result suggests that enzyme activation by cytochromes is not involved in CC-1065 toxicity.

In view of the antitumor efficacy of CC-1065 in the mouse and the previously reported activity against a broad spectrum of tumor types in the human tumor cloning assay[6], the occurrence of lethal delayed hepatotoxicity is unfortunate. Efforts to prepare analogs lacking this toxicity but retaining antitumor efficacy are underway in our laboratories[14].

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