ViroMED
LABORATORIES, INC.

10 August 1992

Sudhakar Wagle, Ph.D.
Senior Vice President
Research & Development
Schwarz Pharma
5600 West County Line Road
Mequon, Wisconsin 53092

Dear Dr. Wagle;

Enclosed are the **results of the study using ViroMED protocol ARTS-1001 to determine the in vivo anti-HIV efficacy of KU 10280.**

If you have any questions, comments, or would like to discuss any of the particulars regarding this report, or any other areas of ViroMED's services, please do not hesitate to contact me directly. Thank you again for allowing ViroMED's Scientist's to be of service to you and your Company!

Sincerely yours,

Neal T. Wetherall, Ph.D.
Scientific Director

5500 Feltl Road
Minnetonka, MN 55343-9806
(800) 582-0077
(612) 931-0077
FAX (612) 931-0537

ViroMED
LABORATORIES, INC.

REPORT TITLE

Evaluation of KU10280 in the HIV-1 Xenotransplanted Nude Mouse Animal Model System

COMPOUND IDENTITY

SCHWARZ PHARMA KU10280
AZT - Lot No.: 9Z1573 (Burroughs Wellcome Co.)

AUTHORS

Xin Qin Li, M.D., Sc.D.
Neal T. Wetherall, Ph.D.

STUDY COMPLETION

11 August 1992

PERFORMING LABORATORY

ViroMED Laboratories, Inc.
5500 Feltl Road
Minneapolis, Minnesota 55343

SPONSOR

SCHWARZ PHARMA
5600 West County Line Road
Mequon, WI 53092

5500 Feltl Road
Minnetonka, MN 55343-9806
(800) 582-0077
(612) 931-0077
FAX (612) 931-0537

GOOD LABORATORY PRACTICE STATEMENT

The study referenced in this report was conducted in compliance with U.S. Food and Drug Administration Good Laboratory Practice (GLP) regulations set forth in 21 CFR part 58.

The studies not performed by or under the direction of ViroMED Laboratories, Inc. are exempt from this Good Laboratory Practice statement and include: characterization of the compounds.

Study Director: _____ 11 AUG 1992
Neal T. Wetherall, Ph.D. Date

Submitter: _____ _____
Date

Sponsor: _____ _____
Date

QUALITY ASSURANCE UNIT SUMMARY

Study: HIV Animal Model System

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of nonclinical laboratory studies. These studies have been performed under Good Laboratory Practice regulations (21 CFR part 58) and in accordance to standard operating procedures and standard protocols. The Quality Assurance Unit maintains copies of study protocols and standard operating procedures and has inspected this study on the dates listed below. Studies are inspected at time intervals to assure the integrity of the study. The findings of these inspections have been reported to management and the Study Director. All materials and data pertinent to this study will be stored at ViroMED Laboratories, Inc., 5500 Feltl Road, Minneapolis, Minnesota 55343.

Phase Inspected	Date
Project Set-up	28 May 1992
In Progress	6 July 1992
Terminal Measurement	15 July 1992
Final Report	11 Aug. 1992

Professional personnel involved:

Neal T. Wetherall, Ph.D.	-	Study Director
Xin Qin Li, M.D., Sc.D.	-	Staff Scientist
Jeffrey A. Schrager, M.D.	-	Staff Scientist
Sheila Sullivan, M.T.	-	Quality Assurance Director

This study meets the requirements for 21 CFR part 58.

Quality Assurance Director: Sheila Sullivan Date: 8-11-92

Study Director: [signature] Date: 11 Aug 1992

FINAL REPORT

Evaluation of KU10280 in the HIV-1 Xenotransplanted Nude Mouse Animal Model System

SPONSOR:	BIOCHEM PHARMA
COMPOUND NAME OR CODE:	KU10280 AZT - Lot No.: 9Z1573 (Burroughs Wellcome Co.)
DATE COMPOUND RECEIVED:	11 May 1992
TEST FACILITY:	ViroMED Laboratories, Inc. 5500 Feltl Road Minneapolis, Minnesota 55343
TEST START DATE:	11 May 1992
TEST COMPLETION DATE:	11 August 1992
VIRUS:	Human Immunodeficiency Virus Type 1, Strain III_B
TEST OBJECTIVE:	To determine the <u>in vivo</u> anti-HIV efficacy of KU10280 using ViroMED's protocol ART - 1001 with one drug dose

EXPERIMENTAL METHODS:

Cell Line and HIV Culture. CCRF-CEM or CEM is a well characterized[1] tumorigenic[2] and HIV permissive[3] cell line, was acquired from the American Type Culture Collection, Rockville, Md. (ATCC CCL 119) and maintained in RPMI 1640 medium with 15% heat inactivated fetal calf serum and 50 μg/ml gentamicin as described previously[4]. The cells were propagated at 37^{0}C in a humid 5% CO_2 atmosphere. Stocks of the HIV-1 isolates HTLV-III$_B$ were acquired from the NIH AIDS Research and Reference Reagent Program (catalog no. 398), and were harvested from cultures of chronically-infected CCRF-CEM cells. For routine propagation, virus containing culture fluids were clarified of cells by low speed centrifugation and passed through 0.45 μm filters. Infectious virions were quantitated on MT-2 cells in microculture using cytopathic effect (CPE) as end point for infection[5]. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated by the method of Reed and Muench[6]. CCRF-CEM cells used for virus xenotransplantation were acutely infected with stock dilution of HIV-1 at a MOI (input multiciplicity of infection) of 0.1 followed by adsorbtion for 2 hours at 37^{0}C. All HIV-1 procedures are performed within a BioSafety Level 3 (BSL-3) facility, and all procedures adhere to BSL-3 guidelines[7].

Animals, Diet, Environment, and Cell/HIV Transplantation. As previously described[4,8], Outbred, athymic, 27$\pm$2 days old female nude (nu/nu) mice were be purchased from Harlyn Sprague Dawley, Inc. The athymic mice were housed at 23$\pm$1^{o}C, without antibiotic coverage, in a specific-pathogen-free room under laminar-flow HEPA-filtered air. All bedding, cages, water, and other material coming in direct contact with the mice were autoclaved before use. Animals were permitted access to food and water ad libitum. The mice were fed a diet of pelleted chow which contains elevated levels of heat-sensitive nutrients (Purina autoclavable rodent laboratory chow #5010). The solid-wall and -bottom cages are covered by microisolators, and the litter is changed twice a week. The BSL-3 guidelines as outlined by the CDC are followed[7,9]. A maximum of ten mice were housed in large cages for the experimental period.

CEM cell cultures, both HIV infected or uninfected, were harvested and washed with serum free media and reharvested. Inocula were standardized by counting the cells with a hemocytometer, and adjusting the cell suspension with serum free medium. Cells suspended in 0.2 ml media were injected subcutaneously (s.c.) into the intrascapular region using a syringe attached by luer lock to a 22ga. teflon pediatric angio-catheter. This catheter was first tunneled through the subcutaneous tissues to eliminate leakage of the inoculum. Aseptic procedures were used throughout.

Animals were delivered to ViroMED on the Monday of the initiation of the experiment, and were allowed two days to acclimate to the new environment. The next Wednesday, the animals were exposed to 500 Rad's of ^{137}Cs irradiation (University of Minnesota Medical School's J.L.Shepherd Mark I irradiator). This dose reduces natural killer cell activity, and usually no morbidity is apparent. On Thursday the drug dosing was started, and on Friday of this week the animals were inoculated as described above. The mice were observed the following day and at least three times a week for the duration of the experiment. At each of these time points the animal group weight is determined, and the inoculation site is gently palpated to determine the date of gross tumor onset, or the tumor size which was measured in two dimensions via calipers. The tumor volume was calculated from measurements in two dimensions using the formula for a prolate ellipsoid, $\pi/6\ LW^2$ [10]. Significance of differences between groups was evaluated by the unpaired Student's t-test, and probability values were specified in two tails.

At the termination of an experiment, the animals were placed under Methoxyflurane anesthesia. After the pain responses were absent, the animals were bled by cardiac puncture until dead. If the animals were not dead by this means, cervical dislocation was used. These methods are consistant with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. After death the animals were necropsied and tumor tissue was obtained aseptically for CEM cell examination and for HIV antigen expression using an indirect immunofluorescent assay[11].

In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

The rights for the animal model using the above described procedures are protected under pending U.S. Patent Application Serial No. 07/805,484 "Animal Model for Human Virus Infection".

p24 Enzyme Immunoassay. The p24 enzyme immunoassay (EIA) used was the unmodified procedure commercially available from Coulter Corporation (Hialeah, Fl), which uses a murine monoclonal antibody to the HIV core protein coated onto microwell strips. The assay detects p24 gag antigen in culture supernatants, plasma, and serum. Non-specific cross reactions with mouse serum are not seen with this assay.

Preparation and Administration of Compounds. Doses of KU10280 were freshly prepared for each day's drug administration. Ampules for daily injection was provided by the sponsor. Each ampule was reconstituted with 1.2 ml of sterile distilled water, and 0.1 ml was injected i.m. (intramuscular), day -1 to day 11, and s.c., day 19 to day 38 for daily drug administration, unless noted. Control

animals received an injection of 0.1 ml water (placebo).

AZT was procured as over-the-counter RETROVIR^{R} 100mg capsules. The contents from each capsule was disolved in 200 ml of sterile distilled water by mixing on a stir plate for one hour. The inactive filler component of the capsule contents was removed by centrifugation at 3,000 rpm for 10 minutes. The supernatant was adjusted with additional sterile distilled water to achieve a final concentration of .125 mg/ml. The animals were monitored daily to assure an average consumption of 4ml/day/mouse, which was achieved. This method delivers .5 mg AZT/day/mouse or 20 mg AZT/kg/day. The prepared dilutions of the KU10280 and AZT were soluble when used, and AZT was prepared fresh on a weekly basis to maintain stability.

Literature Cited.

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2. Graham BS, and Wetherall NT: Growth of human cell lines in BALB/c mice. Cancer Res 1990;50:5943-5946.
3. Dalgeish AG, Beverly PCL, Clapham PR, Crawford DH, Greaves MF, and Weiss RA: The CD4(T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 1984;312:736-767.
4. Wetherall NT: The development of HIV-1 p24 antigenemia in the athymic "nude" mouse. In: Animal Models in AIDS. Schellekens H and Horzinek MC (eds.). Elsevier; Amsterdam, 1990, pp. 291-302.
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7. Centers for Disease Control (CDC): Agent summary statement for human immunodeficiency virus and report on laboratory-acquired infection with human immunodeficiency virus. MMWR 1988;37(S-4): 11-15.
8. Johnson MD, Davis BW, and Wetherall NT: Production of proto-oncogene expressing control tissues for in situ hybridization and immuno-histochemical studies. J Environ Path Tox and Onc 1989;9:171-190
9. Milman G, and D'Souza P: HIV infections in SCID mice: safety considerations. ASM News 1990;56:639-642.

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EXPERIMENTAL DESIGN

The in vivo assay using this model consisted of testing the KU10280 in a prophylactic manner, whereby a single dose of agent is administered to a group of mice one day prior to HIV challenge. The test and control animals were followed for a period of 39 days with washout. The specific experimental design consisted of the comparisons of the following groups:

Cell control - (Group No.1)	12 mice transplanted with human CCRF-CEM cells only
Virus control - (Group No.2)	12 mice transplanted with CCRF-CEM infected with HIV at a M.O.I. of 0.1, placebo treated
Drug control - (Group No.3)	6 mice treated with KU10280, at 8.3 mg/mouse/day administered i.m. or s.c. daily, transplanted with CCRF-CEM Cells (without virus)
AZT control - (Group No.4)	7 mice treated with AZT (0.5 mg/day/mouse or 20mg/kg/day), challenged with HIV at the same M.O.I. as the virus control
Test Drug - (Group No.5)	6 mice treated with KU10280, at 8.3 mg/mouse/day administered i.m. or s.c. daily, challenged with HIV at the same MOI as the virus control

Upon the experiment termination and animal sacrifice, serum p24 antigen levels were determined as the experimental endpoint.

RESULTS

Tables 1 and 2 outline the overall course of the experiment. Due to irradiation problems (apparent overdose), morbidity was readily apparent by day 11, causing the cessation of all treatments. All mice exhibited cutaneous petechiae to varying degrees which was due to irradiation induced thrombocytopenia, especially excerbated in the AZT treated group. Mortality appeared in all groups (days 11-20, with the loss of AZT treated animal the day prior to the experiment's end), but fortunately ViroMED scientists had increased the number of animals in all populations at the initiation of the experiment, minimizing the negative impact of the deaths. It should be noted that the % survival was equal or better in the KU10280 treated animals suggesting some protection from the irradiation. The mice recovered from the apparent irradiation poisoning to restart treatment on day 19. The initial tumor formation occurred on day 11 (groups 1 & 3), day 15 (group 4), or day 18 (groups 2 & 5) and progressed until the termination of the experiment at day 39. The total period of drug treatment was 31 days (1 day prior to xenotransplantation through day 10, and days 19 - 39).

TABLE 1: COURSE OF EXPERIMENT

Group No.	Number of Mice at Day 0	Number of Mice at Day 39	% Survival
1	12	7	58.3
2	13	9	69.2
3	6	6	100.0
4	7	3	42.9
5	6	4	66.6

TABLE 2: COURSE OF EXPERIMENT (CONT.)

Group No.	Treatment Ceased	Mortality No. Mice	Treatment Restarted
1	Day 11	3 x Day 15 1 x Day 19 1 x Day 20	Day 19
2	Day 11	1 x Day 11 2 x Day 15 1 x Day 16	Day 19
3	Day 11		Day 19
4	Day 11	1 x Day 13 2 x Day 14 1 x Day 38	Day 19
5	Day 11	1 x Day 15 1 x Day 20	Day 19

Note: By Day 11, 100% of Mice exhibited cutaneous petechaiae, indicating Thrombocytopenia due to irradiation poisoning. Mice recovered by Day 19.

At the termination of the experiment, the group body weights, group tumor volumes, and the serum p24 levels were determined, displayed in tables 3, 4, and 5, and figures 1, 2, and 3. Levels of significance (p values) comparing the various groups are listed in table 6.

TABLE 3: TERMINAL MEASUREMENTS

Mean Group Body Weight (grams)

Group No.	MOUSE No. 1	2	3	4	5	6	7	8	9	Mean	S.D.
1	34	30	36	34	28	35	26			32	3.8
2	24	21	19	25	23	20	18	18	22	21	2.6
3	28	25	35	27	34	23				29	4.8
4	21	21	16							19	2.9
5	26	22	22	22						23	2.0

TABLE 4: Mean Group Tumor Volume (CM^3)

Group No.	MOUSE No. 1	2	3	4	5	6	7	8	9	Mean	S.D.
1	5.94	6.11	8.54	9.63	5.77	9.01	3.35			6.9	2.24
2	2.68	1.15	1.33	0.94	2.47	3.18	0.53	2.55	0.70	1.7	0.99
3	6.81	4.58	12.4	7.86	15.6	2.68				8.3	4.86
4	1.5	1.85	0.73							1.4	0.57
5	1.42	2.28	0.89	.004						1.1	0.95

TABLE 5: Mean Group Serum p24 (ng/ml)

Group No.	MOUSE No. 1	2	3	4	5	6	7	8	9	Mean	S.D.
1	0	0	0	0	0	0	0			0	0
2	1.213	0.022	1.048	0.978	0.727	0.07	1.193	1.008	1.384	0.849	0.49
3	0	0	0	0	0	0				0	0
4	0.156	0.280	0.269							0.235	0.068
5	0.067	0.042	0.124	0.028						0.065	0.042

TABLE 6: UNPAIRED STUDENTS' t-TEST (P-VALUE)

Group Comparison	Body Weight	Tumor Volume	Serum p24 Antigen
1 vs 2	<0.0001	<0.0001	
1 vs 3	0.2123	0.5041	
1 vs 4	0.0011	0.0034	
1 vs 5	0.0022	0.0010	
2 vs 3	0.0016	0.0014	
2 vs 4	0.3361	0.5673	0.0626
2 vs 5	0.2222	0.3544	0.0098
3 vs 4	0.0195	0.0480	
3 vs 5	0.0605	0.0212	
4 vs 5	0.1013	0.7506	0.0095

CONCLUSIONS

Although this experiment did exhibit an unusually high level of mortality, and treatments were stopped for several days, we believe that the experimental results are still valid based upon the sufficient number of healthy (due to weight gain) surviving animals, and the anticipated p24 supression found in the AZT treated group when compared to the untreated virus control. The change from i.m. dosing to the s.c. route did not appear to negatively impact this experiment since the KU10280 antiviral effect was greater than AZT in vivo, without exhibiting any antineoplastic effect on the transplanted CEM cells. This dose of KU10280 effectiveness is particually important when considering additional experiments, especially since no toxicity nor significant weight loss was evident in any of these animals. Therefore, it can be anticipated that higher, or perhaps oral doses would also yield significant reduction in p24 levels when compared to the untreated virus controls.

FOOTNOTE: We regret the problems that we suffered with the ^{137}Cs irradiator, unfortunately this is the only instrument that we subcontract out the use of. We believe that we have corrected the problem, and subsequent quality control experiments performed since your data was produced have determined that the morbidity and mortality using this irradiator has been reduced to zero (this data is available to you upon request).

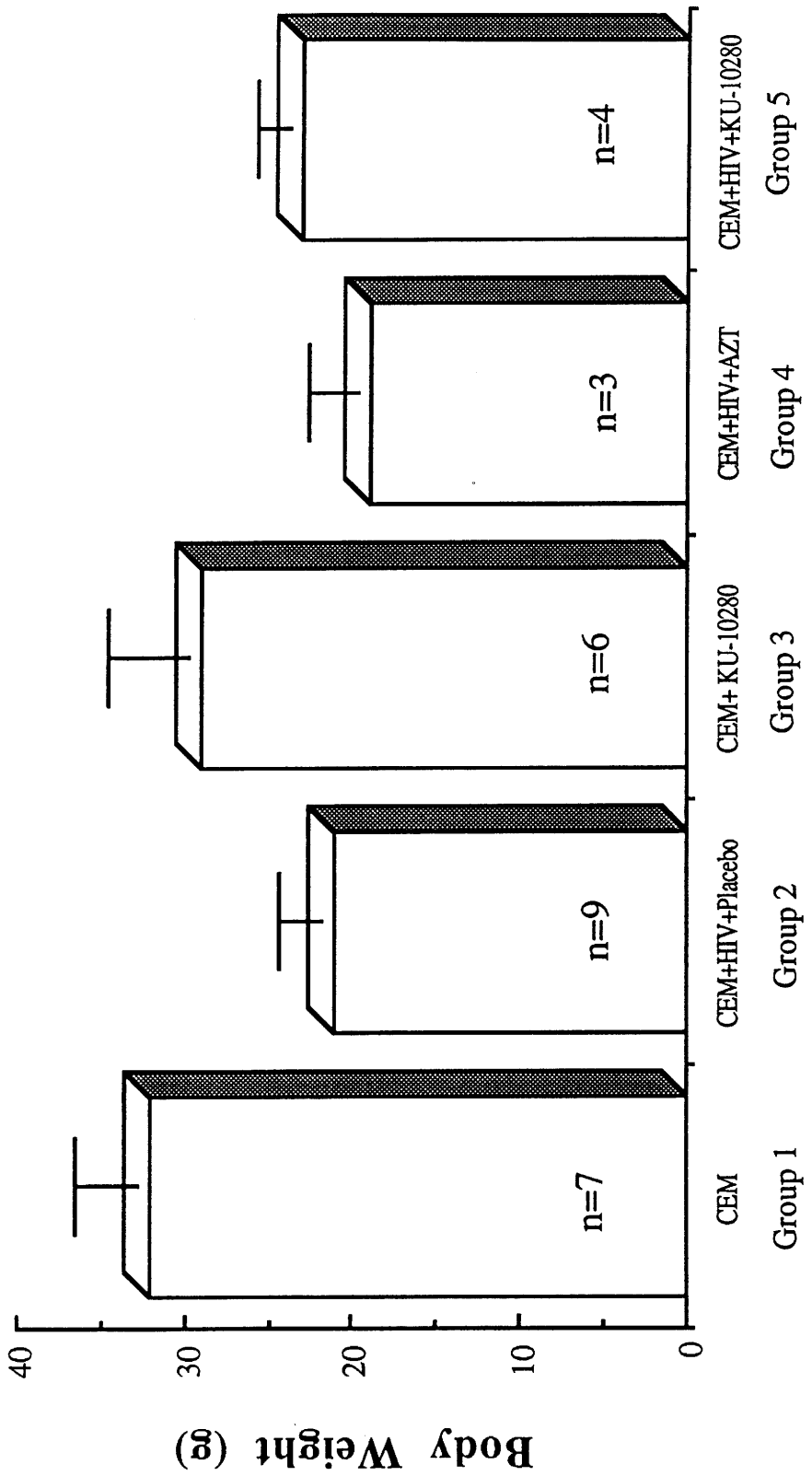
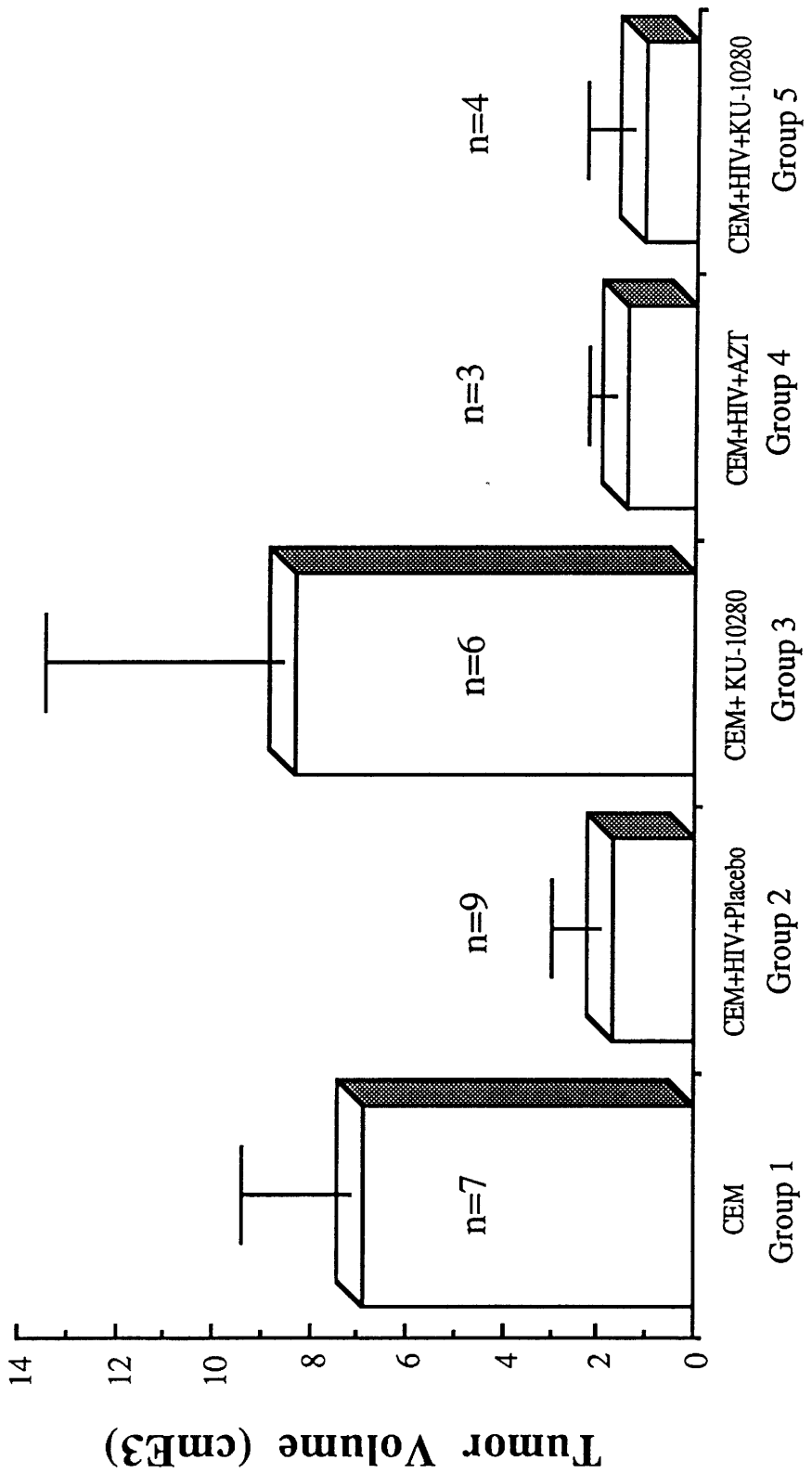

Body Weight (g)
0
10
20
30
40
n=7
n=9
n=6
n=3
n=4
CEM
CEM+HIV+Placebo
CEM+ KU-10280
CEM+HIV+AZT
CEM+HIV+KU-10280
Group 1
Group 2
Group 3
Group 4
Group 5
HIV Infection and Treatment

Figure 1



HIV Infection and Treatment

Figure 2

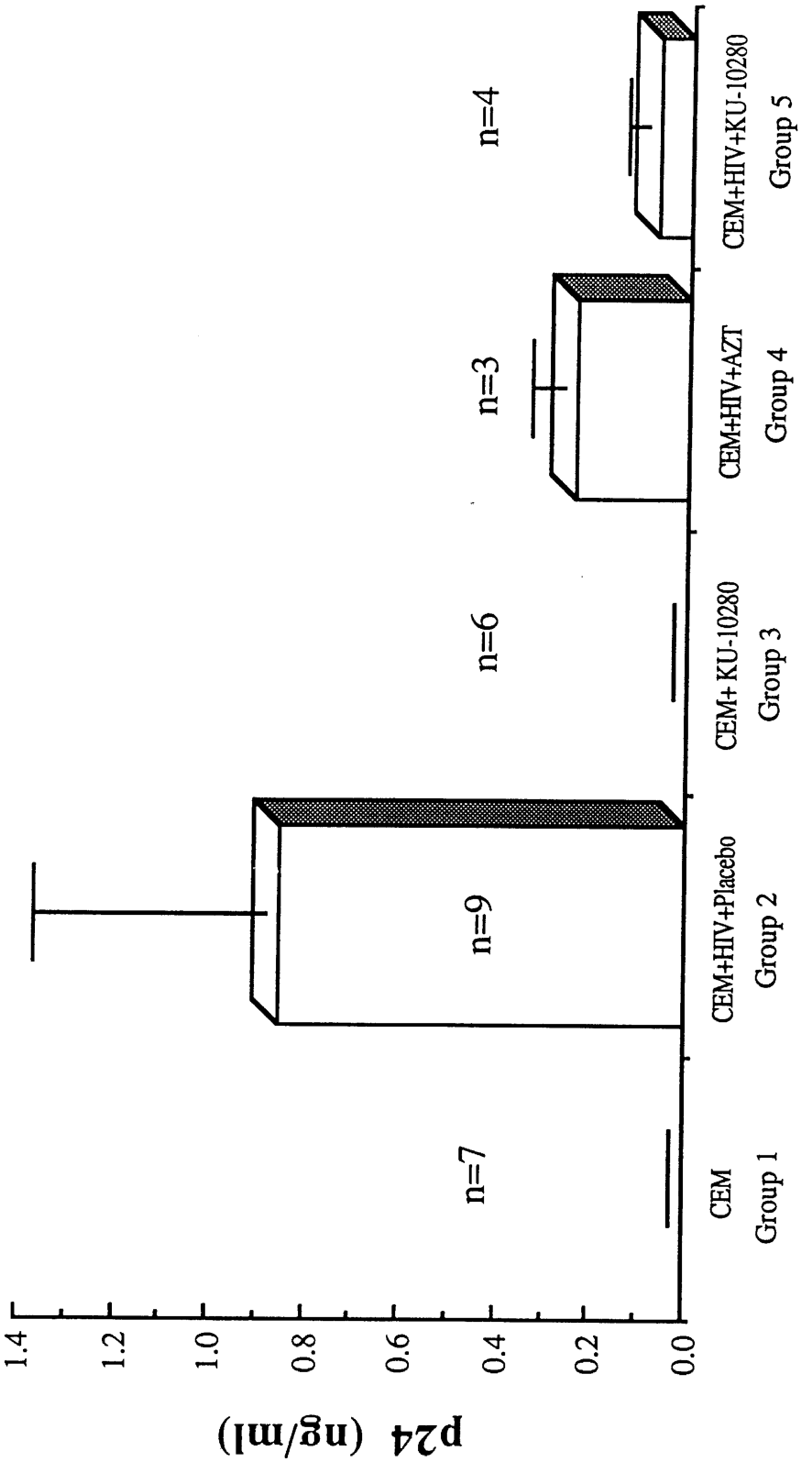

HIV Infection and Treatment
p24 (ng/ml)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
n=7
n=9
n=6
n=3
n=4
CEM
Group 1
CEM+HIV+Placebo
Group 2
CEM+ KU-10280
Group 3
CEM+HIV+AZT
Group 4
CEM+HIV+KU-10280
Group 5

Figure 3

REPORT SUBMITTED BY

Viro MED Laboratories, Inc.
5500 Feltl Road, Inc.
Minneapolis, Minnesota 55343

Neal T. Wetherall, Ph.D.
Study Director

Xin Qin Li, M.D., Sc.D.
Staff Scientist