Cisne Enterprises, Inc.

MELANOMA CANCER TEST

Efficacy Evaluation of Antitumor Activity

of Alka Vita - Alkahydroxy

in the LOX-GFP Human Melanoma Model

Final Report by:
Anti-Cancer Lab – San Diego California

March 4, 2005

Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model

1.0 OBJECTIVE

The purpose of this study was to evaluate the antitumor activity of Alkahydroxy on tumor growth in LOX-GFP human melanoma model through intradermal injection of LOX cells in nude mice.

2.0 INVESTIGATORS

Sponsor: Cisne Enterprises Inc.

Sponsor Investigators: Michael D Radcliffe

AntiCancer, Inc.: Yunling Jing, M.D., Principal Investigator

Zhijian Yang, Study Director

Shigeo Yagi, Ph.D., General Manager

Robert M. Hoffman, Ph.D., President

3.0 STUDY PERIOD

This study was conducted between January 3, 2005 and February 18,2005.

4.0 MATERIALS AND METHODS

Animals: Male NCr nude mice between 5 and 6 weeks of age were used in this study. The animals were bred and maintained in a HEPA filtered environment with cages, food and bedding sterilized by autoclaving. The breeding pairs were obtained from Charles River Laboratories (Wilmington, MA). The animal diets were obtained from Harlan Teklad (Madison, WI). 0.008% (w/v) Ampicillin (Sigma, St Louis, MO) was added to the autoclaved drinking water. A total of 15 animals were used for this study.

Study drugs: Alkahydroxy A and Alkyhydroxy B were supplied by Cisne Enterprises Inc. (see protocol).

GFP expression vector. The pLEIN vector was purchased from Clontech (Palo Alto, CA). The vector expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message that contains an internal ribosome entry site.

Cell culture, vector production, transfection, and subcloning. PT67, an NIH3T3-derived packaging cell line, expressing the 10 A1 viral envelope, was purchased from Clontech. PT67 cells were cultured in DMEM supplemented with 10% fetal bovine serum. For vector production, packaging cells (PT67), at 70% confluence, were incubated with a precipitated mixture of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl
sulfate reagent and saturating amounts of pLEIN plasmid for 18h. Fresh medium was replenished at this time. The cells were examined by fluorecence microscopy 48h post-transfection. For selection, the cells were cultured in the presence of 200-1000 μg/ml G418 for 7 days.

Retroviral GFP Transduction of LOX Cells. For GFP gene transduction, LOX cells (National Cancer Institute, Bethesda, MD) at 25 % confluence were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 (Life Technologies, Inc.) containing 10 % fetal bovine serum (Gemini Bioproducts) for 72 h. Fresh medium was replenished at this time. Cells were harvested by trypsin-EDTA 72 h after transduction and subcultured at a ratio of 1:15 into selective medium that contained 200 μg/kg G418. The level of G418 was increased stepwise to 800 μg/kg for LOX cells. Clones expressing GFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) using trypsin-EDTA and then amplified and transferred by conventional culture methods.

Doubling Time of Stable GFP Clones. GFP or nontransduced cells were seeded at 1.5 x 10^4 in 35-mm culture dishes. The cells were harvested and counted every 24 h using a hemocytometer (Reichert Scientific Instruments, Buffalo, NY). The doubling time was calculated from the cell growth curve over 6 days.

Intradermal Injection of LOX. Fifteen 6-week-old male NCr mice were injected intradermally with a single dose of 1 x 10^6 LOX-GFP cells. Cells were first harvested by trypsinization and washed three times with cold serum-free medium and then injected in a total volume of 0.1 ml within 30 min of harvesting. Cells were inoculated into dorsal skin using a 30 G1/2 precision glide needle (Becton Dickinson) and a 1-ml latex-free syringe (Becton Dickinson).

Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. A Leica stereo fluorescence microscope model LZ2 equipped with a mercury

lamp power supply was used. Selective excitation of GFP was produced through a D425/60 band-pass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology, Brattleboro, VT) on a Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems, Bridgewater, NJ). Images were processed for contrast and brightness and analyzed with the help of Image Pro Plus 3.1 software (Media Cybernetics, Silver Spring, Maryland). High-resolution images were captured directly on the computer or continuously through video output on a high -resolution Sony VCR.

Analysis of metastases: Metastases were detected by direct GFP open imaging at necropsy. Locations of metastases were recorded for each test animal.

Study design: The intradermal (orthotopically) injected animals used for the study were divided into 3 groups 4 days after surgery. Groups for each of the cohort conditions were randomly chosed. Treatment began 4 days after implantation three times a day and lasted for six weeks (The concentration of Alkahydroxy B was increased to 25% on January 25th, 2005, lasting 10 days; and the concentration of Alkahydroxy B was increased again to 50% on February 4th, 2005, lasting two weeks). Table 1 shows the study design and compounds used in each group.

Table 1 Treatment protocol

Study endpoint: The experiment was terminated 47 days after tumor cell injection (6 weeks treatment) due to the poor health status of the test animals.

Data collection:

Group & Agent	***Schedule***	***Route of administration***	***Number of Mice***
1, Untreated control	-	-	5
2, Alkahydroxy A	*Three times a day for 6 weeks*	*Topical*	5
3, Alkahydroxy B	*Three times a day for 6 weeks*	*Topical*	5

GFP Imaging - GFP whole body images for each mouse were obtained once a week after initial treatment.

Tumor Measurement - Tumor measurements were determined by GFP whole body imaging.

Body weights - Body weight for each animal was measured once a week after initial treatment. An electronic balance was used for body weight measurement.

Final tumor weights - The final primary tumor weight for each animal were determined with an electronic balance.

GFP open imaging – At the end of the study, GFP open imaging was conducted. The primary tumor and major metastatic organs were explored under fluorescence microscopy at necropsy.

Statistical methods used in efficacy evaluation: Final primary tumor weights in each group were analyzed using the ANOVA test (with the Dunnett's two sided test) with an $\alpha = 0.05$. Tumor metastatic rates of all groups were analyzed with Fisher's exact test with an $\alpha = 0.05$.

5.0 RESULTS

Efficacy on primary tumor: At the end of the study, the final primary tumor weights in treated groups were compared to that of the untreated control group using the Dunnett's two-sided test. Statistical significant differences were obtained in Alkahydroxy A treated group ($p<0.05$), verses the untreated control group. The results are shown in Table 2.

Table 2. Efficacy of Alkahydroxy A & B on final primary tumor weight

Group	*Mean final tumor weight (g))*	*P-value**
1, Untreated control	*8.1*	-
2, Alkahydroxy A	*3.9*	*0.034*
3, Alkyhydroxy B	*4.7*	*0.091*

* All treated groups are versus the untreated control group with Dunnett's two-sided test.

Efficacy on metastasis rate: At the end of the study**,** all animals were opened and imaged to examine metastasis. Metastatic incidences in different organs were determined

by direct GFP open imaging. The metastases and p-value for each organ in treated groups were compared to that of the untreated control group by using Fisher's exact test (Table 3). No statistical significant difference was seen in either treatment compared to the untreated control. The results are shown in Table 3.

Table 3 **Efficacy on Metastatic rate**

	Group	# of tested animals	Superficial axillary L.N.	
			MI[a]	*P*[b]
1	*Untreated control*	5	0	-
2	*Alkahydroxy A*	5	1	1.000
3	*Alkyhydroxy B*	5	0	1.000

a MI Metastatic Incidence.
b All treated groups compared to the untreated control group by Fisher's exact test.

Body weight: No acute loss of body weight within the duration of the experiment was observed (see "Body weight graph").

6.0 CONCLUSION

The results showed that Alkahydroxy A significantly inhibited the growth of primary tumor ($p<0.05$), verses the untreated control group. No significant difference was seen regarding metastases for both treated groups compared with the untreated control group. There was no evidence of toxicity as shown in the body weight graph.

7.0 SIGNATURES

Yunling Jing, M.D., Principal Investigator Date:

Zhijian Yang, M.D., Study Director Date:

Shigeo Yagi, Ph.D., General Manager Date:

Robert M. Hoffman, Ph.D., President Date:

Signatures on Original

8.0 REFRENCES

Hoffman, R. M., Orthotopic metastatic mouse model for anticancer drug discovery evaluation: a bridge to the clinic. Investigational New Drugs, 17: 497-500, 1999.

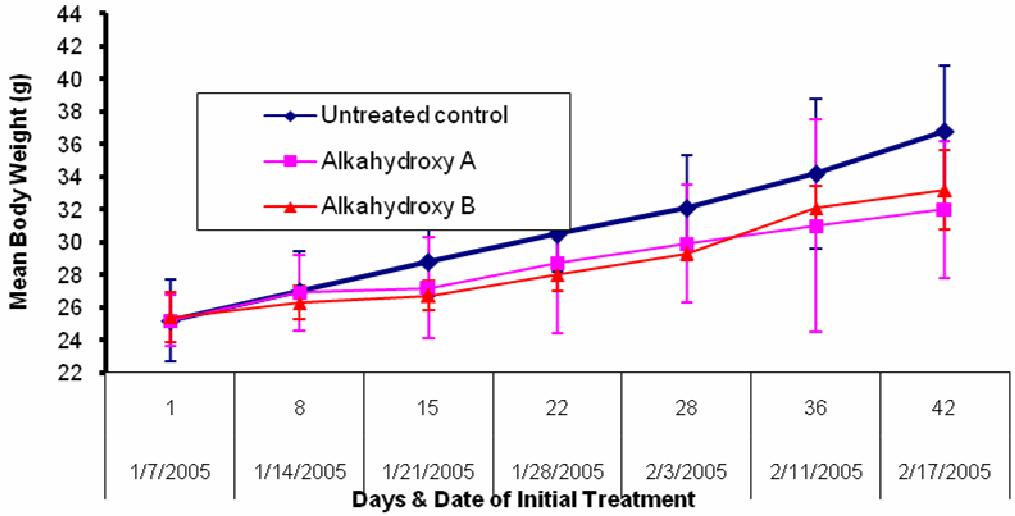
Yang, M., et al. Whole-body optical imaging of green fluorescent protein-expressing tumors and metastasis. Proc. Natl. Acad. Sci. USA 97, 1206-1211, 2000.

Yang M., et al. Genetically fluorescent melanoma bone and organ metastasis models. Clinical Cancer Research. **5** 3549-3559, 1999.

- **DATA (TABLES, GRAPHS AND PHOTOS)**

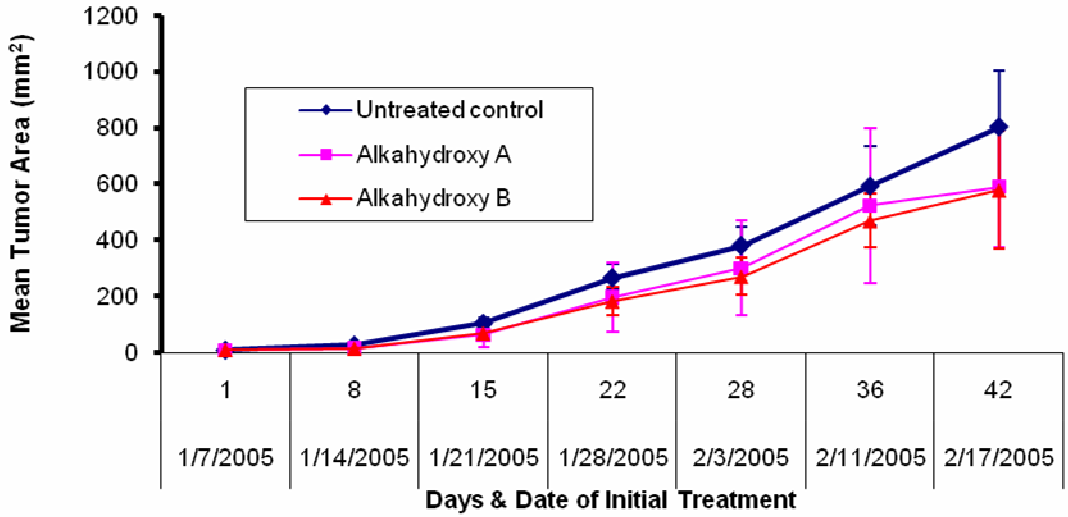
Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model

Mean Body Weight Graph



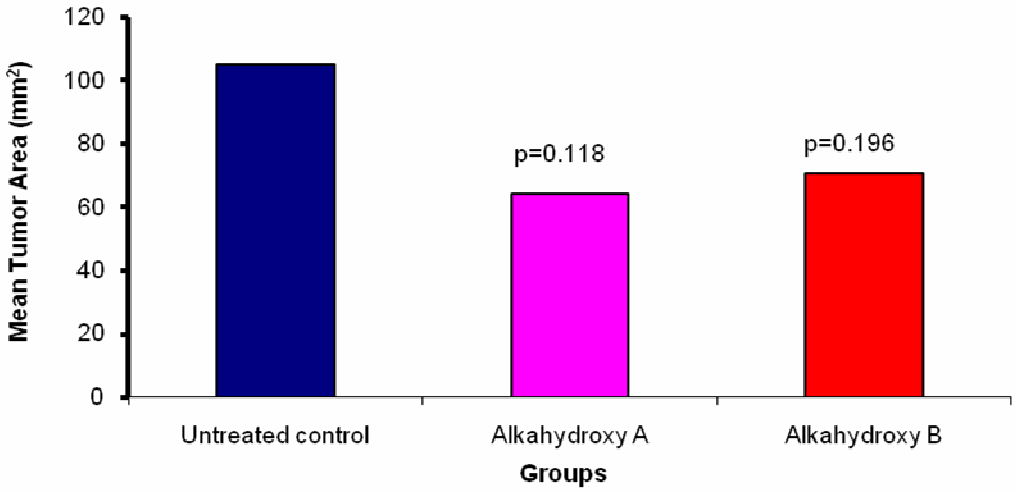
Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model

Mean Tumor Area Graph



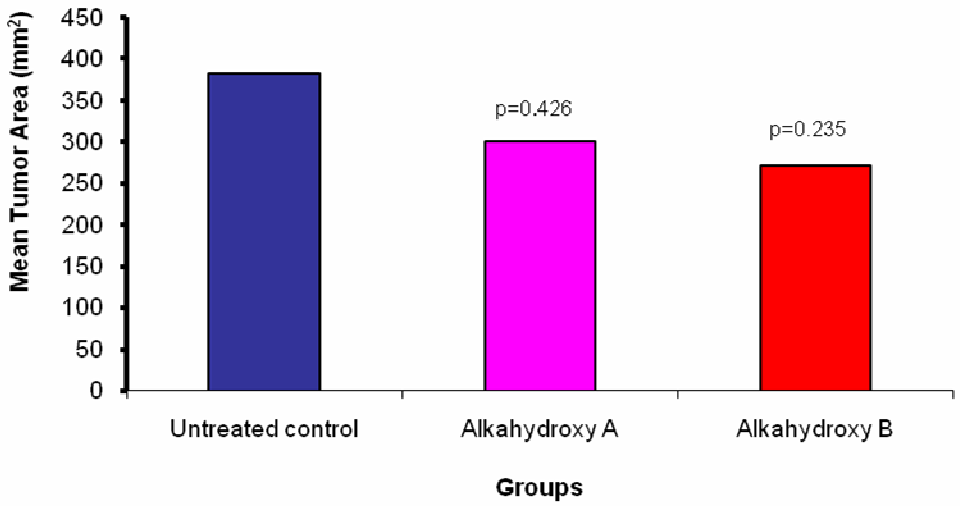
Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model

Mean Tumor Area Graph from FOTI (1-21-05)



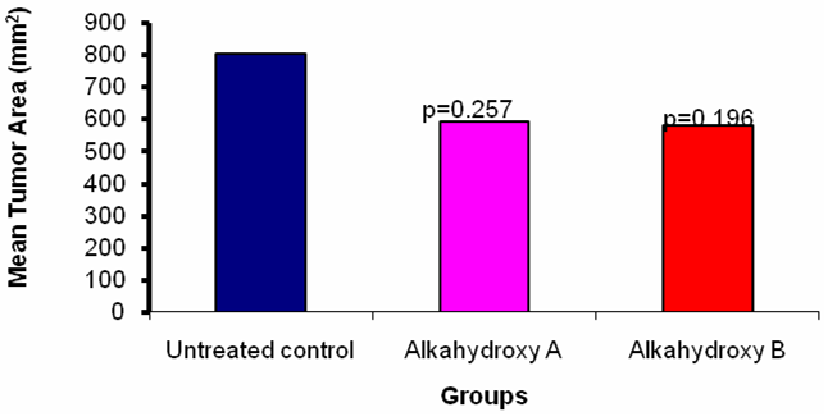
Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model

Mean Tumor Area Graph from FOTI (2-3-05)



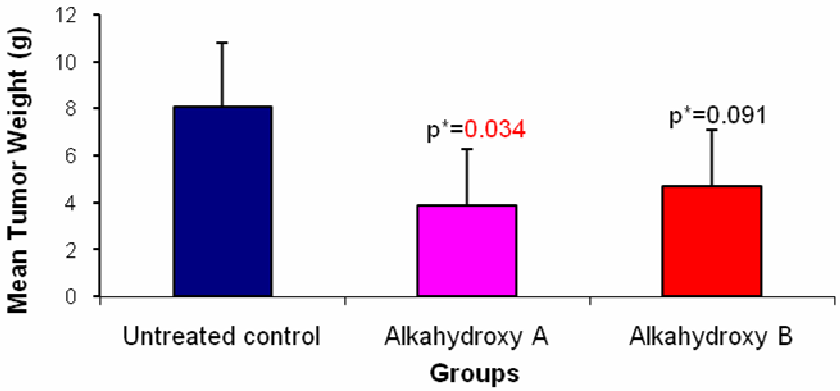
Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model

Mean Tumor Area Graph from FOTI (2-17-05)



Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model

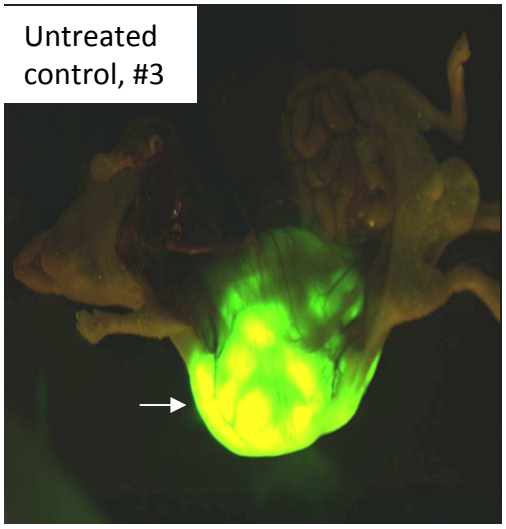
Mean Tumor Weight Graph (2-18-05)



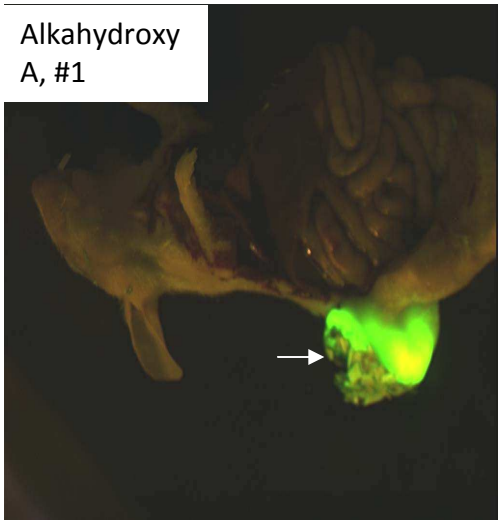
* Both treated groups were compared to the untreated control with Dunnett's two sided test.

Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model
GFP Open Images (2/18/05)

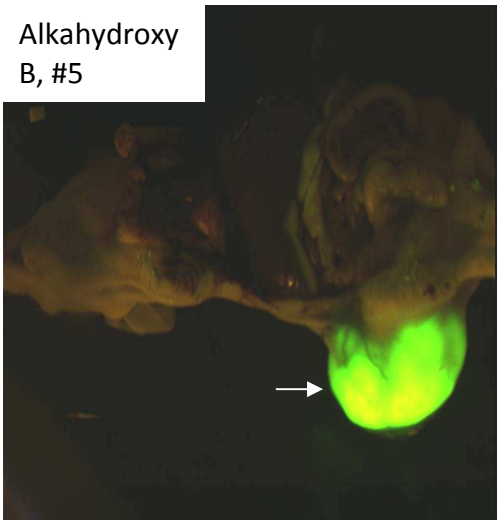
Untreated control, #3



Alkahydroxy A, #1



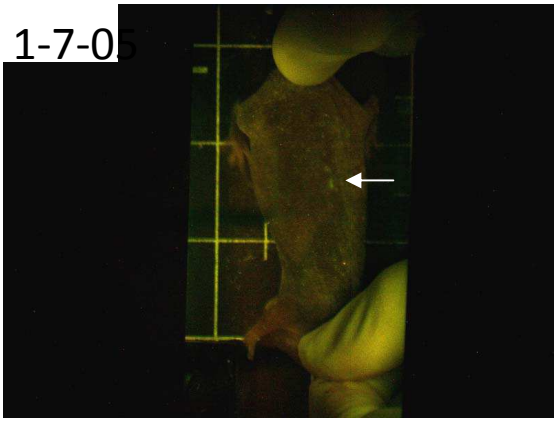
Alkahydroxy B, #5



White arrow: Primary tumor.

Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model Time-Course Whole Body Images (Alkahydroxy A, #1)

1-7-0



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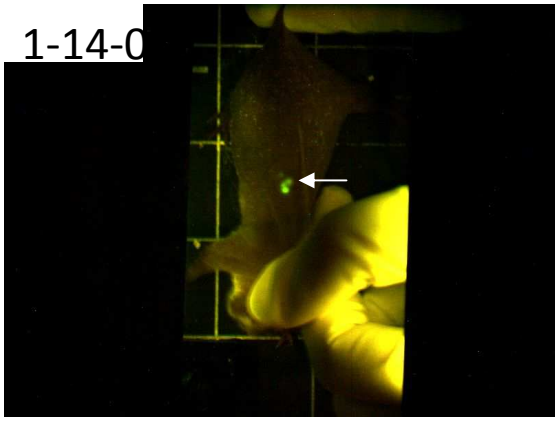

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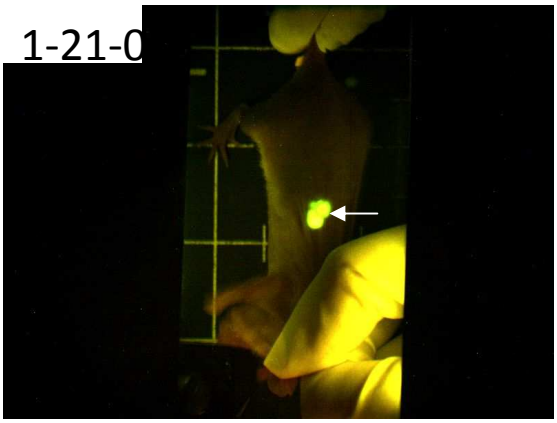

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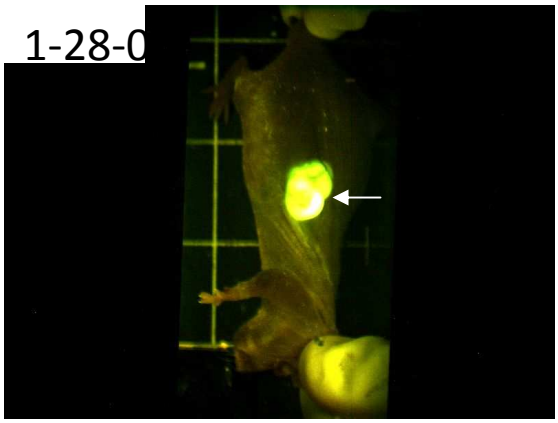

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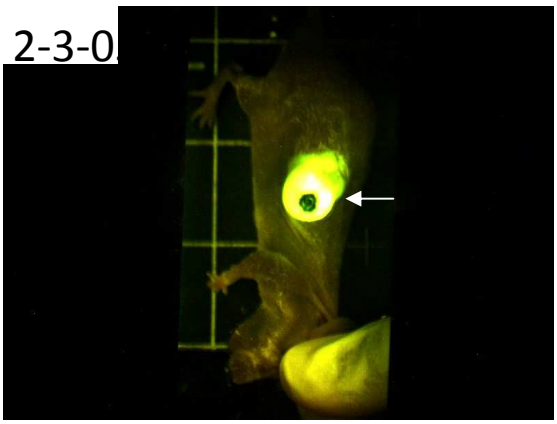

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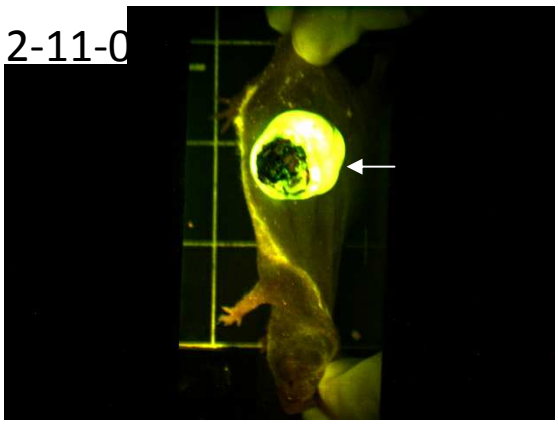

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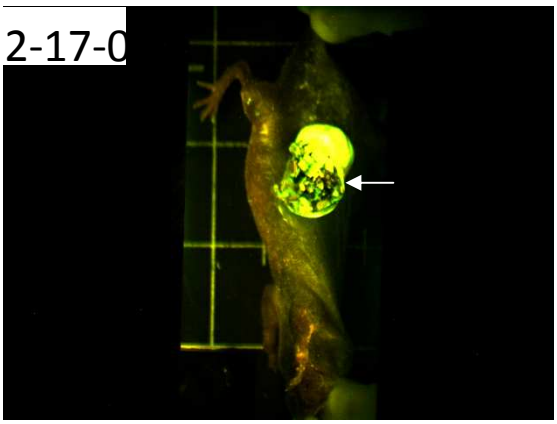

White arrow: Primary tumor.

Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model Time-Course Whole Body Images (Alkahydroxy B, #5)

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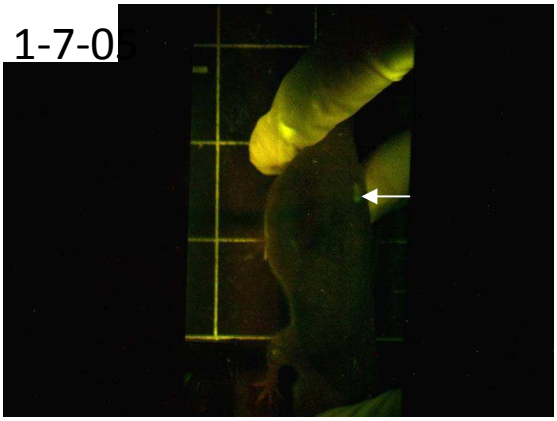

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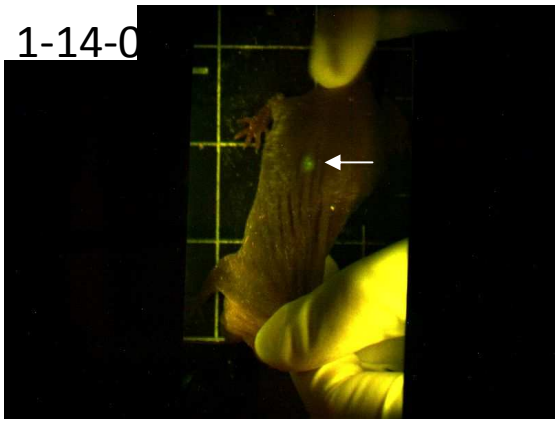

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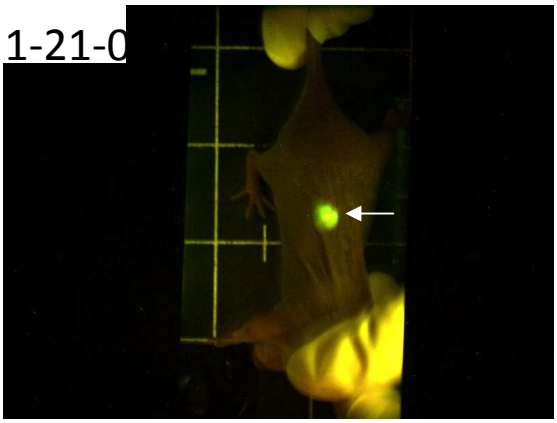

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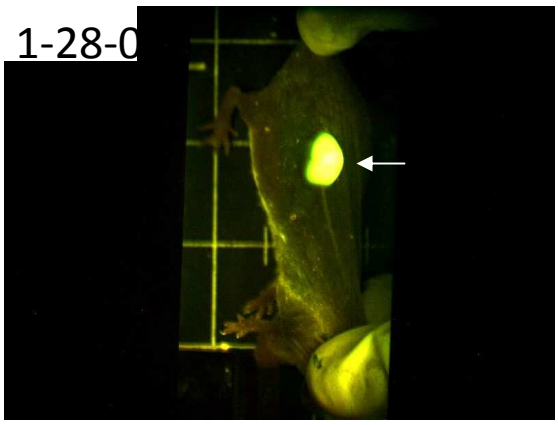

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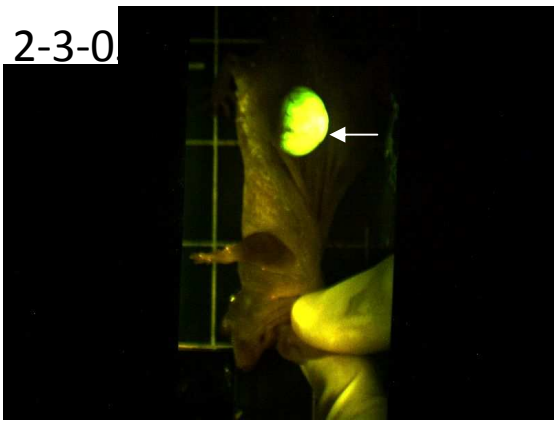

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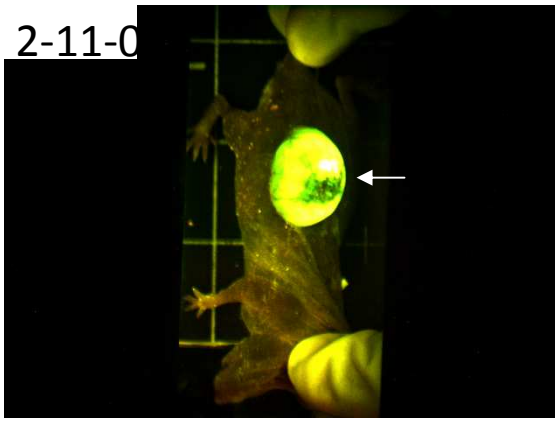

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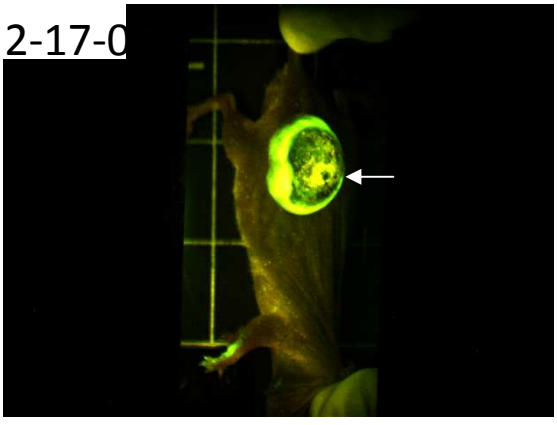
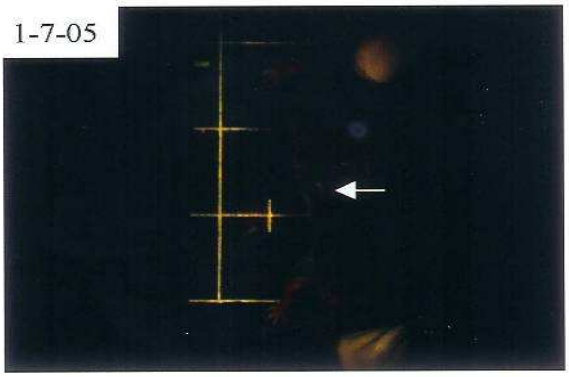


White arrow: Primary tumor.

Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model
Time-Course Whole Body Images
(Untreated control, #3)

1-7-05



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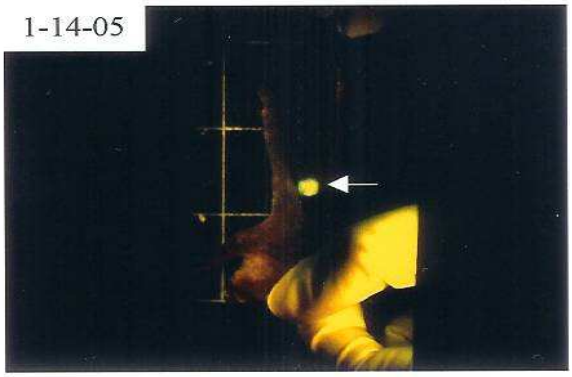

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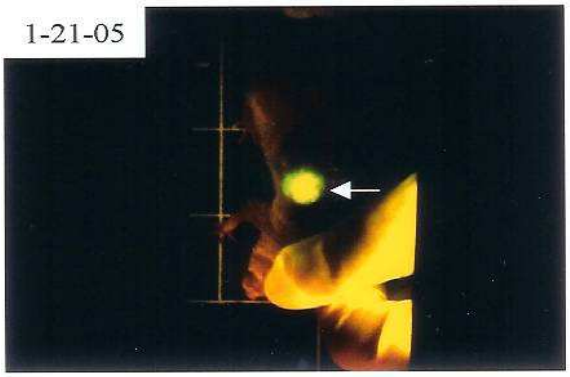

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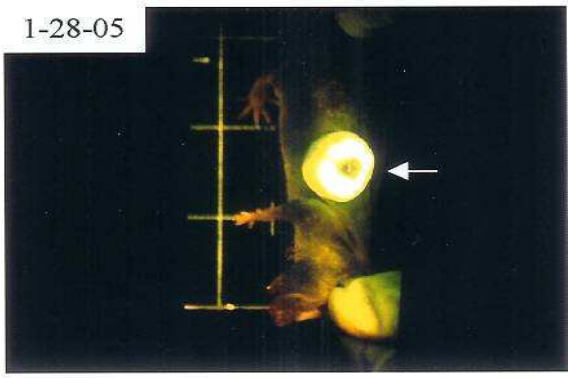

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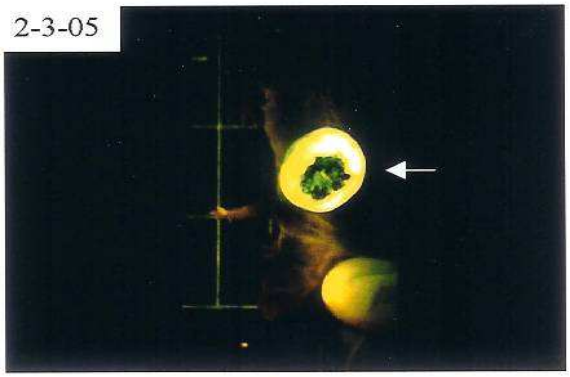

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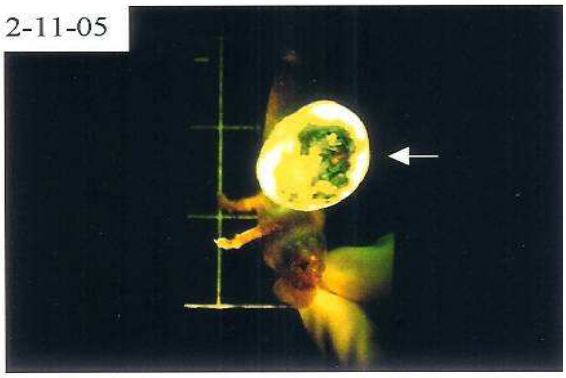

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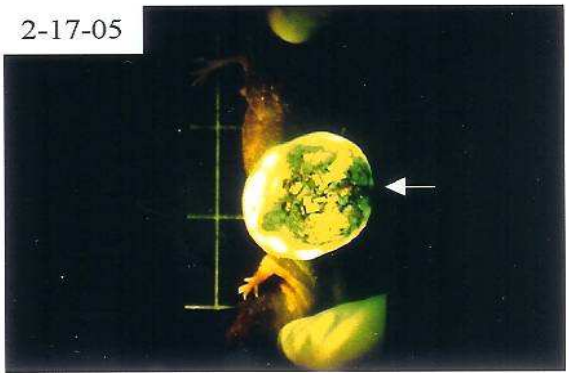
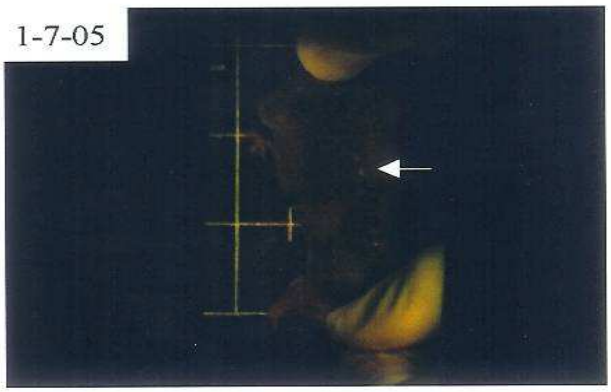
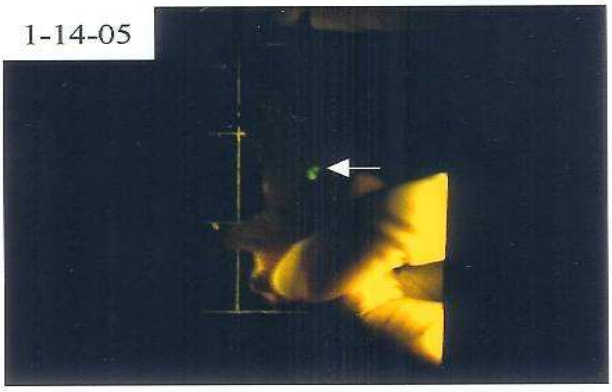
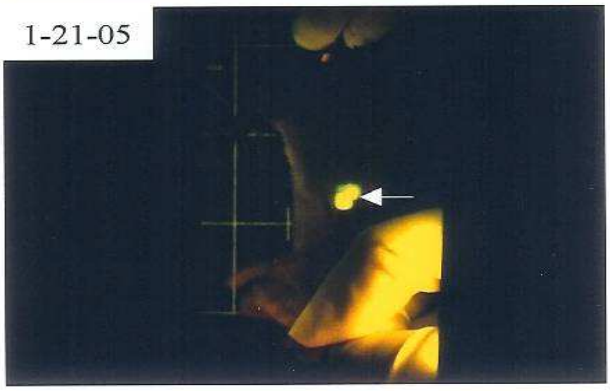


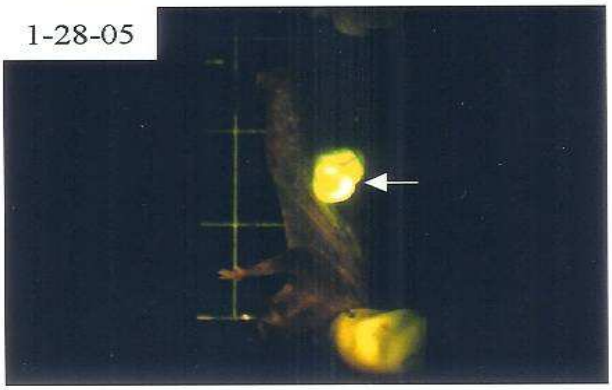
White arrow: Primary tumor.

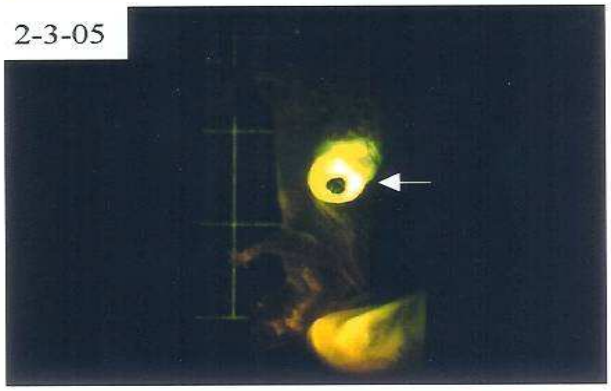
Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model
Time-Course Whole Body Images
(Alkahydroxy A, #1)

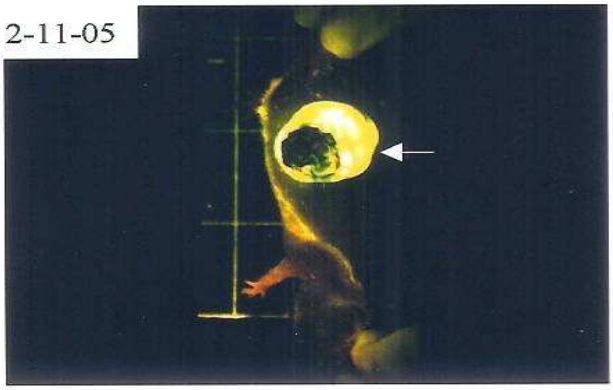


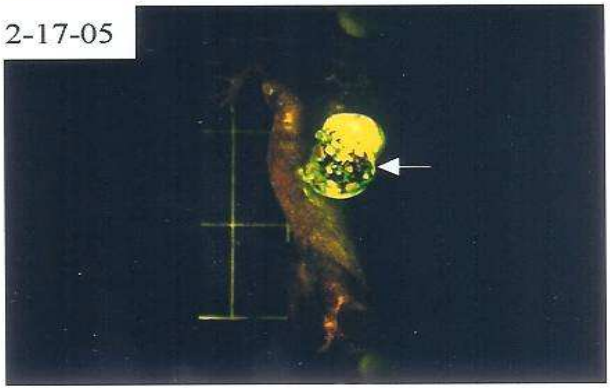








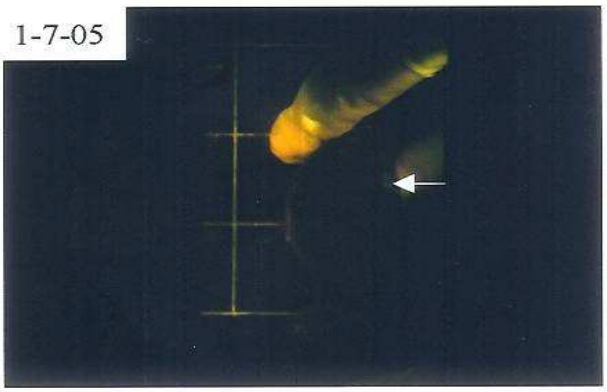




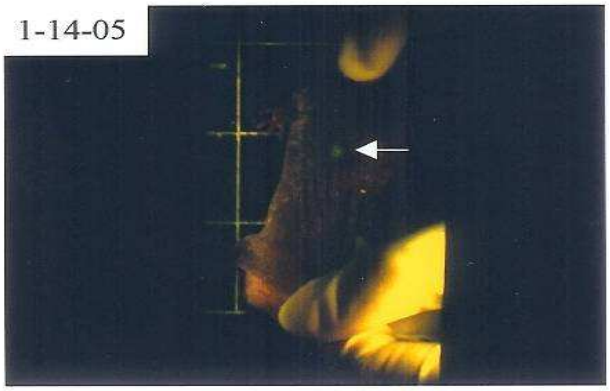
White arrow: Primary tumor.

Efficacy Evaluation of Antitumor Activity of Alkahydroxy in the LOX-GFP Human Melanoma Model
Time-Course Whole Body Images
(Alkahydroxy B, #5)

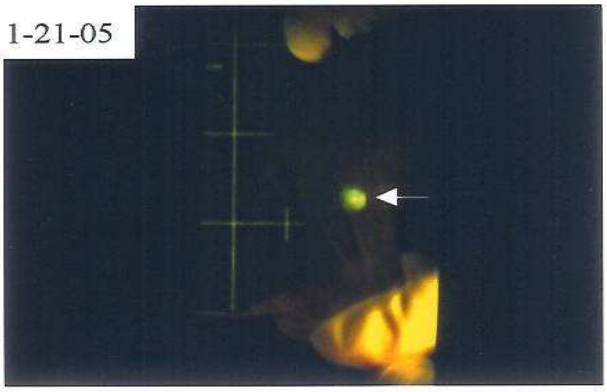
1-7-05



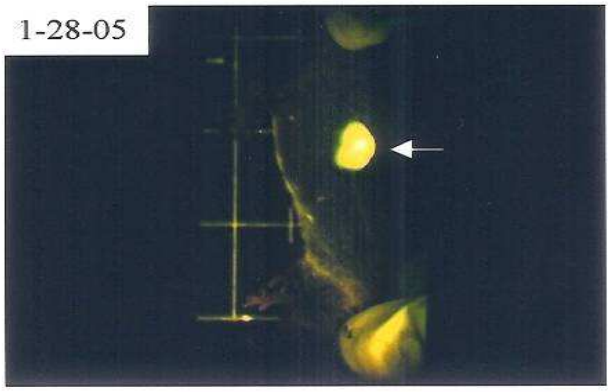
1-14-05



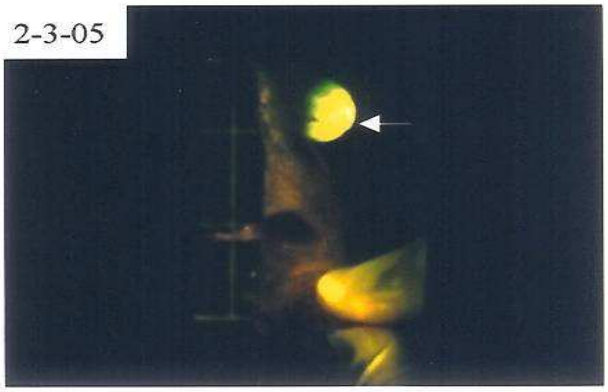
1-21-05



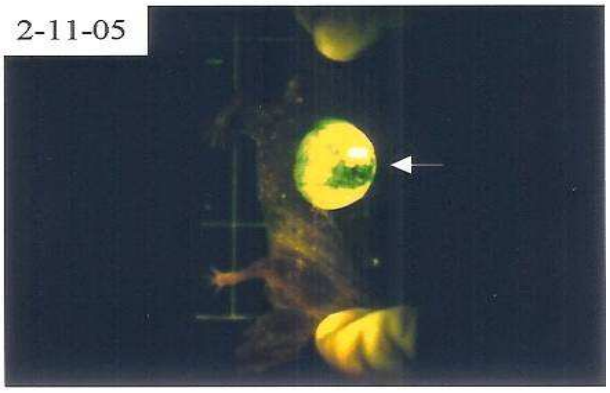
1-28-05



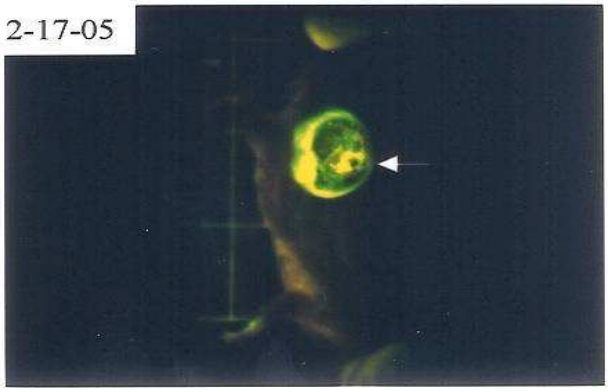
2-3-05



2-11-05



2-17-05



White arrow: Primary tumor.