AD_____

Award Number: DAMD17-02-1-0001

TITLE: Genetic Radiotherapy of Prostate Cancer

PRINCIPAL INVESTIGATOR: Donald J. Buchsbaum, Ph.D.

CONTRACTING ORGANIZATION: The University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

REPORT DATE: December 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2002	Annual (1 Dec 01 - 30 Nov 02)

4. TITLE AND SUBTITLE
Genetic Radiotherapy of Prostate Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0001

6. AUTHOR(S) :
Donald J. Buchsbaum, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

E-Mail: djb@uab.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates; all DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

A goal of this proposal is to achieve a high intratumoral concentration of 5-fluorouracil (5-FU) *via* molecular chemotherapy employing genetically modified adenoviral (Ad) vectors encoding the genes for somatostatin receptor subtype 2 (SSTr2) and cytosine deaminase (CD) which converts the prodrug 5-fluorocytosine (5-FC) to 5-FU under control of the cyclooxygenase-2 (COX-2) tumor-specific promoter. The purpose of Specific Aim 1 was to develop, validate, and evaluate genetically modified Ad vectors that will increase expression levels of both SSTr2 and CD. We proposed to initially evaluate two novel two-gene Ad vectors: (1) a native fiber Ad (AdCMVCDSSTr2) and (2) Ad under control of the COX-2 promoter expressing CD and SSTr2. We have produced several new vectors including AdCOX-2LCDCOX-2LSSTr2, AdCOX-2LSSTr2COX-2LCD, AdCOX-2MSSTr2COX-2MCD, AdCOX-2MCDCOX-2MSSTr2 and AdRGDCOX-2LCDCOX-2LSSTr2, as well as AdCOX-2LCDIRESSSTR2. The vectors we developed were tested for magnitude of SSTr2 expression employing membrane receptor binding *in vitro* with ^{99m}Tc-P2045 that binds to SSTr2, conversion of 5-FC to 5-FU, and cytotoxicity against Ad infected cells in the presence of 5-FC. The first two vectors look promising, and will be evaluated *in vivo* in the next year of the grant.

14. SUBJECT TERMS	15. NUMBER OF PAGES
prostate cancer, gene therapy, molecular chemotherapy, transcriptional targeting, cytosine deaminase, somatostatin receptor, brachytherapy	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The purpose of the research carried out in the first year of this project was to develop a system to express the therapeutic genes cytosine deaminease (CD) and somatostatin receptor (SSTr2) in localized and disseminated prostate cancer cells with sufficient selectivity for cancer gene therapy. The specificity of these vectors for prostate cancer will be improved by combining transductional targeting of Ad with the target cell-specific expression of the therapeutic gene. This will be achieved by utilizing the tumor-specific promoter, cyclooxygenase-2 (COX-2), to drive the transcription of the SSTr2 and CD genes. By employing this double targeting maneuver, we expect to achieve a much higher level of vector specificity: cell killing would only occur as a result of two independent events – cell infection and transgene expression. Although both events are not perfectly tumor-specific, their combination will lead to a desired synergistic effect of specificity. In other words, if the vector randomly infects non-target (normal) cells, the cell killing will not take place, since the SSTr2 and CD expression will be limited primarily to prostate tumor cells. The liver is the predominant site of Ad vector localization after systemic administration, and as a consequence is at risk when Ad vectors containing suicide genes such as CD ectopically localize to this site. Thus, a promoter with both tumor specificity and minimal transcriptional activity in hepatocytes would be ideal for cancer gene therapy. The hypothesis of the grant was that the COX-2 promoter is a promising candidate tumor-specific promoter for prostate cancer, since it reveals low hepatic activity and toxicity, and shows high tumor activity in established cell lines. Combined with the targeted Ad vectors described in the application, the COX-2 promoter may be an ideal candidate tumor-specific promoter for GRITS/molecular chemotherapy of disseminated hormone refractory prostate cancer.

BODY

Specific Aim 1 of the grant was to develop, validate, and evaluate genetically modified Ad vectors that will increase expression levels of both SSTr2 and CD in the context of localized and metastatic prostate cancer compared to our first generation AdSSTr2 and AdCD vectors. Thus, the goal of the first year of support was to develop a novel class of vectors for prostate cancer that embody design features predicating their superior utility for the achievement of effective delivery of SSTr2 for radioligand therapy and CD for molecular chemotherapy.

The vectors that we developed were tested for magnitude of expression employing membrane receptor binding *in vitro*. Binding studies were performed to demonstrate receptor expression on infected human tumor cell lines. Expression of CD in DU145 and PC-3 prostate cancer cells after infection with the genetically-modified Ad vectors was accomplished using 5-FC to 5-FU conversion assays as described in our publications.[1, 2]

Several new Ad vectors expressing CD and SSTr2 were produced using both long (L) and medium (M) length COX-2 promoters as follows: AdCOX-2MSSTr2COX-2MCD, AdCOX-2MCDCOX-2MSSTr2 AdCOX-2LCDCOX-2LSSTr2, AdCOX-2LSSTr2COX-2LCD, AdRGDCOX-2LCDCOX-2LSSTr2 and AdCOX-2LCDIRESSSTr2. In addition, the single gene vectors expressing SSTr2 (AdCOX-2LSSTr2 and AdCOX-2MSSTr2) and CD (AdCOX-2LCD, and AdCOX-2MCD) were produced. A control Ad expressing luciferase under control of the COX-2L promoter, AdCOX-2LLuc, was used as a control.

These new vectors expressing CD and SSTr2 were first tested for SSTr2 expression against MKN-289 gastric and MIA PaCa-2 pancreatic cell lines known to express COX-2 by our previously published internalization assay in which Ad infected cells are incubated with ^{99m}Tc-P2045 (P2045 is a peptide which binds to SSTr2) and imaged 5 min (**Figure 1**) after addition of the radiolabeled peptide and 20 min later after removing the excess peptide and stripping the SSTr2 bound to the surface.[3, 4] The Experimental Protocol appears on pages 7 to 12 of this report. As shown in **Figure 2**, MKN-28 and MIA PaCa-2 cells infected with AdCOX-2MSSTr2COX-2MCD and AdCOX-2LCDCOX-2LSSTr2 both resulted in internalization of ^{99m}Tc-P2045 similar to that observed with AdCOX-2LSSTr2 and AdCOX-2MSSTr2. Binding of ^{99m}Tc-P2045 was blocked by the addition of excess unlabeled sandostatin which binds to SSTr2.

The new vectors expressing CD and SSTr2 were next tested to determine their conversion of 5-FC to 5-FU following infection of DU145 and PC-3 human prostate cancer cell lines using the protocol presented on page 13 of this report. The results are presented in **Table 1**. They indicate that AdCOX-2LCDCOX-2LSSTr2 and AdCOX-2LSSTr2COX-2LCD produced a higher level of conversion of 5-FC to 5-FU in DU145 and PC-3 cells than AdCOX-2MSSTr2COX-2MCD. Furthermore, the two gene vectors AdCOX-2LCDCOX-2LSSTr2 and AdCOX-2LSSTr2AdCOX-2LCD produced levels of conversion that were similar to the single gene vectors AdCOX-2LCD and AdCOX-2MCD, but were somewhat lower than

AdCMVCDSSTr2 and substantially lower than AdCMVCD. These results support earlier ones that the CMV promoter is stronger than the COX-2 promoter, although less specific. The conversion results are plotted in **Figures 3** and **4**.

Table 1. Conversion of 5-FC to 5-FU in pmol/min/mg ± SD measured over a 1 h period after infection of DU145 and PC-3 human prostate cancer cell lines with 100 MOI of each virus.

Vector	DU145	PC-3
Uninfected	0.00	0.00
AdCOX-2LCDCOX-2LSSTr2	2.95 ± 0.18	1.21 ± 0.30
AdCOX-2LSSTr2COX-2LCD	2.51 ± 0.24	0.87 ± 0.06
AdCOX-2MSSTr2COX-2MCD	1.45 ± 0.14	0.68 ± 0.16
AdCMVCDSSTr2	7.53 ± 0.30	2.46 ± 0.24
AdCOX-2LCD	2.06 ± 0.16	0.98 ± 0.04
AdCOX-2MCD	2.10 ± 0.11	0.70 ± 0.15
AdCMVCD	25.64 ± 1.25	8.06 ± 0.42
AdCMVSSTr2	0.04 ± 0.97	-0.02 ± 0.04

In the next set of studies, we evaluated the cytotoxicity of the new vectors after infection of DU145 and PC-3 cells with 10 MOI (plaque forming units/cell) of virus. At 24 h after infection, the cells were seeded at 5,000 cells/well and exposed to varying concentrations of 5-FC. Cell viability was assessed by the MTS assay (Celltiter 96 AQ, Promega). The results are shown in **Figures 5-8**. The IC_{50} values for these cell lines are shown in **Table 2**. The results indicate that AdCOX-2LCDCOX-2LSSTr2 and AdCOX-2LSSTr2COX-2LCD produced greater cytotoxicity than AdCOX-2MSSTr2COX-2MCD. In addition, these two vectors under control of the COX-2L promoter produced greater cytotoxicity than the single gene vector AdCOX-2LCD. Furthermore, infection with 100 MOI of virus resulted in greater cytotoxicity, although the level of viral cytotoxicity without 5-FC also increased (data not shown). The cytotoxicity and conversion results presented above both show that the COX-2L vectors were more effective than the COX-2M vector.

Table 2. Cytotoxicity of Ad infected DU145 and PC-3 human prostate cancer cells (10 MOI) exposed to 5-FC expressed as IC_{50}.

Vector	DU145	PC-3
Uninfected	202.62	514.52
AdCMVCD	171.67	101.38
AdCOX-2LCD	45.99	122.42
AdCOX-2LCDCOX-2LSSTr2	13.05	24.47
AdCOX-2LSSTr2COX-2LCD	21.35	30.39
AdCOX-2MCD	107.45	200.90
AdCOX-2MCDCOX-2MSSTr2	ND	ND
AdCOX-2MSSTr2COX-2MCD	104.28	77.01

In the coming year, we will determine the capability of the newly derived vectors to achieve tumor receptor expression in a local *in vivo* prostate cancer tumor model after intratumoral delivery of the vector. The transduction of the tumor nodules will be evaluated by radiolabeled peptide uptake in the tumor and

other normal tissues. We will also begin to evaluate therapeutic efficacy using the newly derived vectors in combination with 5-FC and radiation therapy.

KEY RESEARCH ACCOMPLISHMENTS

- Produced several new Ad vectors expressing SSTr2 and CD under control of COX-2 promoters.
- Validated the expression of SSTr2 and CD in infected tumor cells.
- Determined the conversion of 5-FC to 5-FC in prostate cancer cells.
- Demonstrated cytotoxicity of prostate cancer cells infected with new Ad vectors expressing CD and exposed to 5-FC.

REPORTABLE OUTCOMES

Developed six new Ad vectors expressing SSTr2 and CD.

CONCLUSIONS

The results generated to date indicate that the new vectors developed result in the expression of SSTr2 and CD in infected cells, that prostate cancer cells infected with the new Ad vectors were killed following exposure to 5-FC, and that the COX-2L promoter results in higher SSTr2 and CD expression than the COX-2M promoter. These new vectors show potential for selective expression of SSTr2 and CD in prostate tumor xenografts in comparison to liver which is COX-2 negative. Animal model biodistribution studies with radiolabeled peptides that bind to SSTr2 will be carried out in localized and metastatic prostate cancer models infected with the most promising new Ad vectors in year 2 of the project. In addition, therapy studies in a localized prostate tumor model will be carried out with administration of 5-FC and external beam radiation in year 2 to demonstrate that the vectors are active *in vivo* and to select the best one for development against disseminated tumors in year 3.

ABBREVIATIONS

Ad	adenoviral
CD	cytosine deaminase
COX-2	cyclooxygenase-2
CMV	cytomegalovirus
5-FC	5-fluorocytosine
5-FU	5-fluorouracil
GRITS	Genetic Radio-Isotope Targeting Strategy
SSTr2	somatostatin receptor subtype 2

REFERENCES

1. Pederson LC, Buchsbaum DJ, Vickers SM, Kancharla SR, Mayo MS, Curiel DT, and Stackhouse MA. Molecular chemotherapy combined with radiation therapy enhances killing of cholangiocarcinoma cells *in vitro* and *in vivo*. *Cancer Res*, **57**: 4325-4332, 1997.
2. Miller CR, Gustin AN, Buchsbaum DJ, Vickers SM, Manne U, Grizzle WE, Cloud GA, Diasio RB, and Johnson MR. Quantitation of cytosine deaminase mRNA by real time reverse transcription polymerase chain reaction: A sensitive method for assessing 5-fluorocytosine toxicity *in vitro*. *Anal Biochem*, **301**: 189-199, 2002.
3. Zinn KR, Chaudhuri TR, Buchsbaum DJ, Mountz JM, and Rogers BE. Detection and measurement of *in vitro* gene transfer by gamma camera imaging. *Gene Ther*, **8**: 291-299, 2001.
4. Zinn KR, Chaudhuri TR, Buchsbaum DJ, Mountz JM, and Rogers BE. Simultaneous evaluation of dual gene transfer to adherent cells by gamma-ray imaging. *Nucl Med Biol*, **28**: 135-144, 2001.

Experimental Protocol

Step 1: Wash cell layers 2X with (room temp) HBSS (Hanks Balanced Salt Solution pH 7.2).
Step 2: Add internalization media (0.3 mL) to appropriate wells-see Figure.

Step 3: Add internalization media + unlabeled Sandostatin (0.3 mL) to appropriate wells-See Figures

Step 4: Add hot Tc-99m-P2045 to indicated wells (See Figure), pipette aliquot to tubes for 100% yield at the same time.
Step 5: Image plates with Tc-99m window **(INITIAL IMAGE-5 min)**.

Step 6: Incubate rocking for 3 hours at 37 C
Check with Dr. Zinn.
Step 7: Aspirate hot ambient media. Wash 2X with ice-cold HBSS (pH 7.2). Discard these rinses.
Step 8: Add 0.3 mL per well of 20mM NaAcetate-HBSS pH 4.0 to each well and wait 5 minutes. Collect NaAcetate rinse in vials (radioactive) . Rinse wells with 0.3 mL NaAcet (radioactive). This is the surface bound fraction.
Step 9: Add 0.3 mL 1 M NaOH to each well. **Image** all plates (20 min) after adding first 0.3 mL aliquot. Put NaOH solution in vials for counting. Rinse wells with 0.3 mL 1 M NaOH.

Mixing Reagents

Binding Assay with FOUR 12-well plates: MKN-28 #1,#2, and MiaPaca #1,#2.

Test: Tc-99m-Labeled P2045 binding to stable MKN-28 and MiaPaca cells infected with various Ad encoding hSSTr2 (cox2L, cox2M, etc.)

Plates will be prepared according to the attached Figures. The four plates will be incubated at 37 C for 3 hours

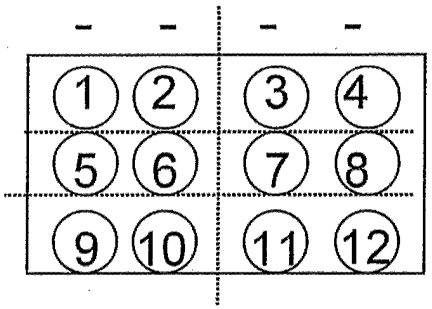
Preparation of unlabeled sandostatin: Use stock solution of 500 ug/mL. You will need 7.2 mL, therefore make 8.0 mL. Add 0.6 mL stock sandostatin to 7.4 mL internalization buffer. This stock is 37.5 ug/mL, adding 0.3 mL to a well will equal 11.25 ug.

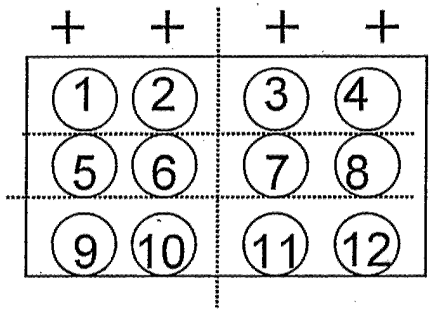
Preparation of Tc-99m-Labeled P2045 Prepare stock #1 at ~100 μCi/mL in Internalization buffer. Prepare 17 mL total, you will need 14.4 mL plus standards

No other stock solutions are needed

Summary of Cell Treatments in Plates (all in duplicate)

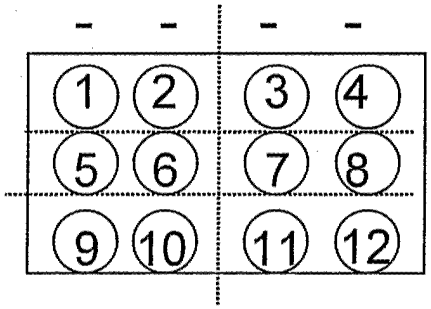
MKN-28: Plate 1

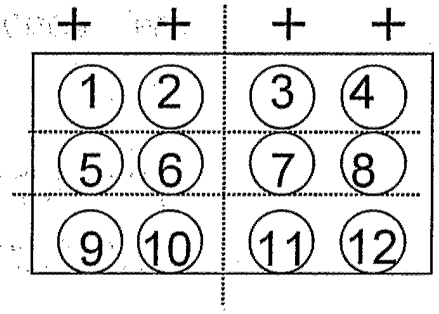




MKN-28: Plate 2

MiaPaca: Plate 1





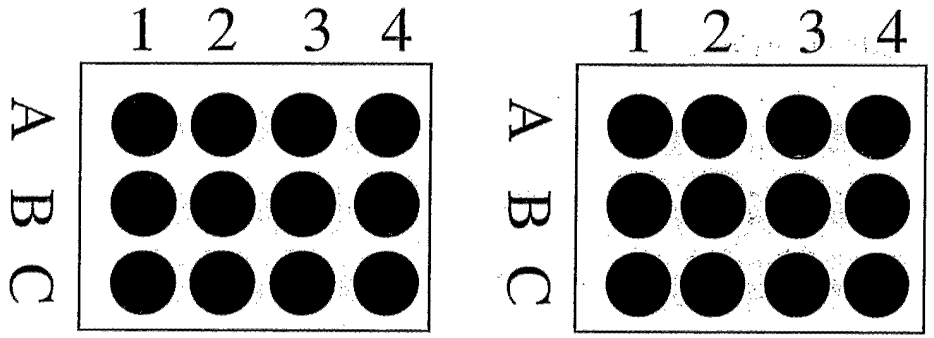
MiaPaca: Plate 2

1-2= Ad-cox2L-Luciferase
3-4=Ad-cox2M-hSSTr2-cox2M-CD
5-6=Ad-cox2L-hSSTr2
7-8=Ad-cox2L-CD-cox2L-hSSTr2
9-10=Ad-cox2M-hSSTr2
11-12=Ad-cox2L-CD-IRES-hSSTr2
Note: Cells infected on 7-8-02 with 3000 vp/cell (~450-500 moi); cell number was 100K/well

-/+ refers to addition of unlabeled P2045

Step 2:

Indicated Wells get 0.3 mL Intern. Media= ●

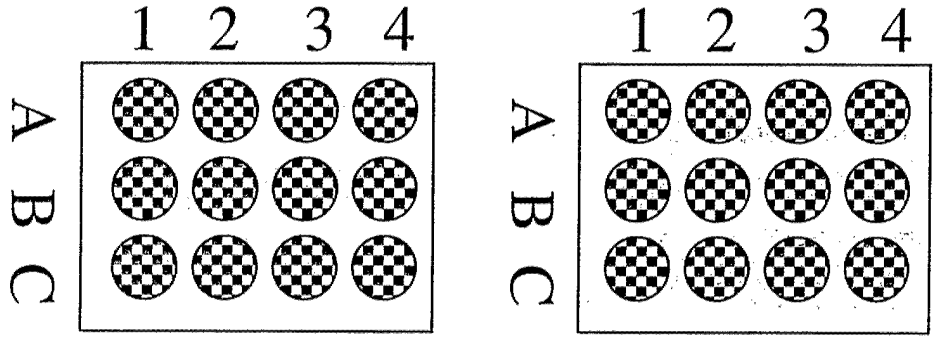


MKN -28
Plate 1

MiaPaca
Plate 1

Step 3:

Indicated Wells get 0.3 mL Intern. Media
+ unlabeled Sandostatin= ⊗

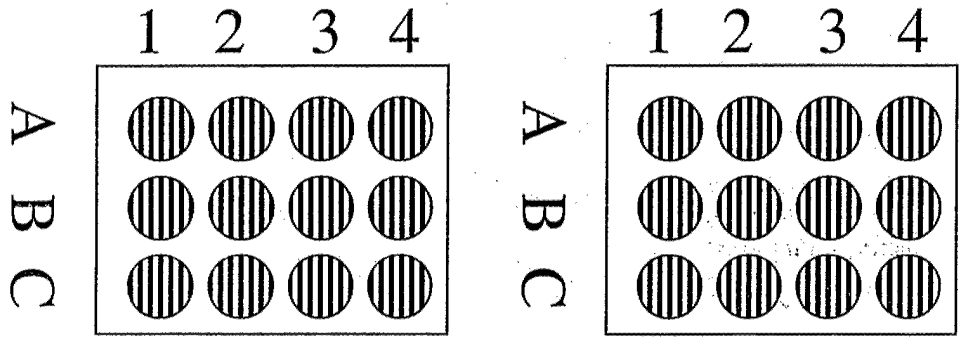


MKN -28
Plate 2

MiaPaca
Plate 2

Step 4:

Indicated Wells get 0.3 mL Tc-99m-P2045 Dil. 1= ◍



MKN -28
Plates 1 and 2

MiaPaca
Plates 1 and 2

CD conversion assay

Part 1:

- Infect cells with virus of interest (AdCD, AdCDSSTr2, AdE1aCD etc.) in a 25 cm^2 flask ($1.2e^6$ cells seeded day prior to infection) for 2 hrs, aspirate virus and feed back; also include uninfected cells
- After 48 hr (or desired time post-infection), wash with PBS, trypsinize and resuspend the cells in 50μL CD lysis buffer; freeze at –20°C

Part 2:

- Lyse the cells by freeze-thawing (thaw slow (RT)/freeze fast (dry ice/ETOH)) 8X
- Centrifuge at 14000 rpm @ 4°C for 5 min to pellet cell debris
- Put supernatant into a new tube and dilute some in PBS to run protein assay (1:25 dilution), freeze remainder at –20°C

Part 3:

- Warm up samples, CD lysis buffer; 5FC at 56°C
- make up 95% Ethanol
- Dilute [^{3}H] 5FC (1:20) in TE buffer (will need 140μL per sample)
- Set up tubes in duplicate with the desired amount of protein (10-50μg) for each time point: 0, 5, 10, 15, 30, 45, 60 min (this will take 2 TLC plates per sample)
- Add CD lysis buffer so the total volume of buffer and sample is 5μL
- Add 10μL of [^{3}H] 5FC (1:20)
- At each time point, add 10μL 95% ethanol to each tube to stop the reaction
- Make up 5FC/5FU mix:

  62μL 5FC (13mg/mL) + 38μL TE >1:1 mix = 4mg/mL of each
  16μL 5FU (50mg/mL) + 84μL TE
- Add 5μL 5FC/5FU mix to each sample; include a standard with 5FC/5FU alone (blank) and one with 10μL [^{3}H] 5FC (1:20) and 5μL 5FC/5FU (total counts)
- Spot total volume on plates (30μL), dry plates

Part 4:

- Make solvent: butanol:water 86:14 300mL needed per tank
- Run TLC plates until solvent reaches the top (this will take at least 6 hours)
- Dry plates overnight
- Cut out 5FC (bottom) and 5FU (top) bands
- Put into scintillation vials with fluid (thin vials = 4mL, big vials = 10 mL), let sit at least 2 hours
- Vortex samples 1min and count using program 13 (1min count)

Figure 1

Plate gamma camera image of Ad infected cells with addition of ^{99m}Tc-P2045

Initial Image (5 min)

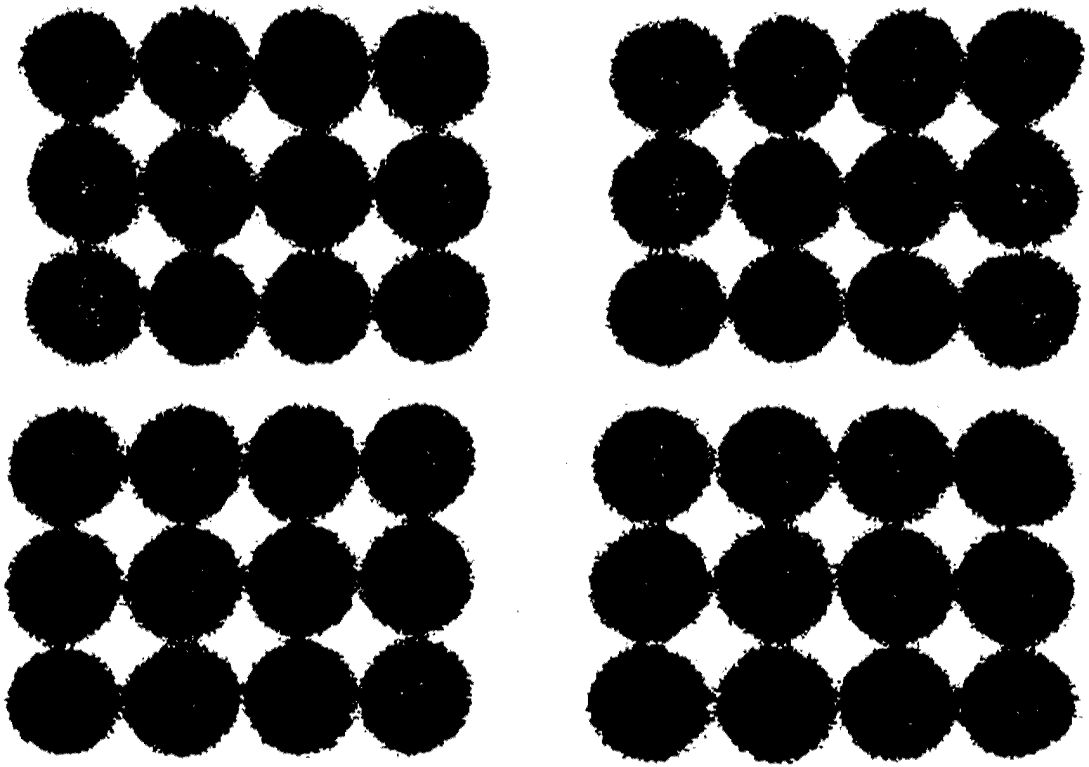


Figure 2

Plate gamma camera image of Ad infected cells with addition of ^{99m}Tc-P2045

Final Image (20 min)

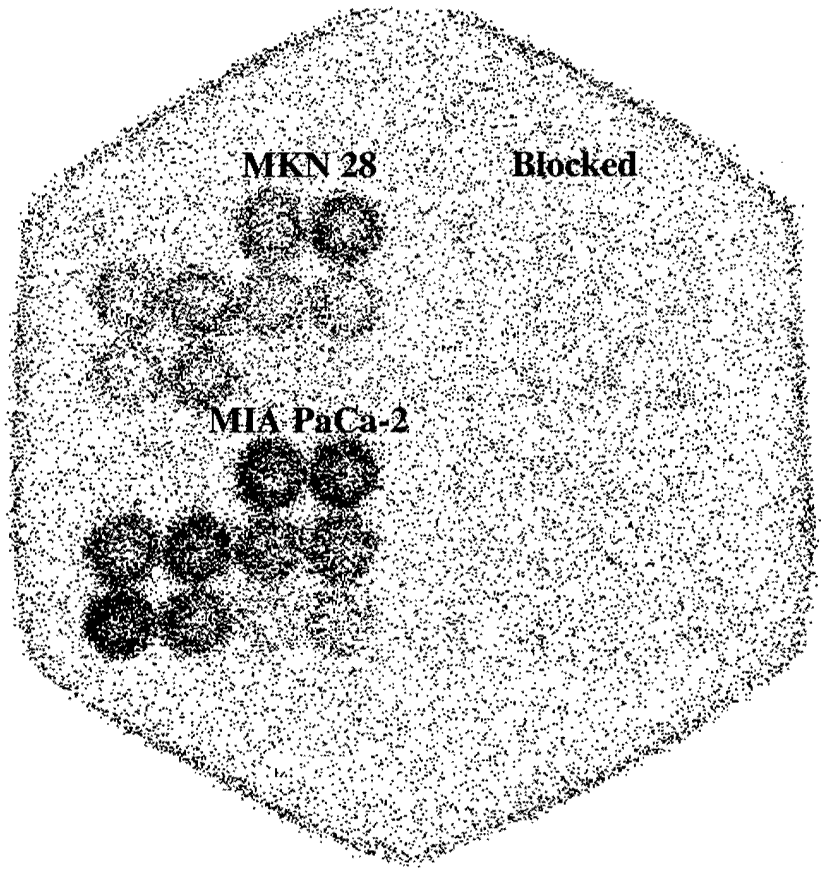


Figure 3

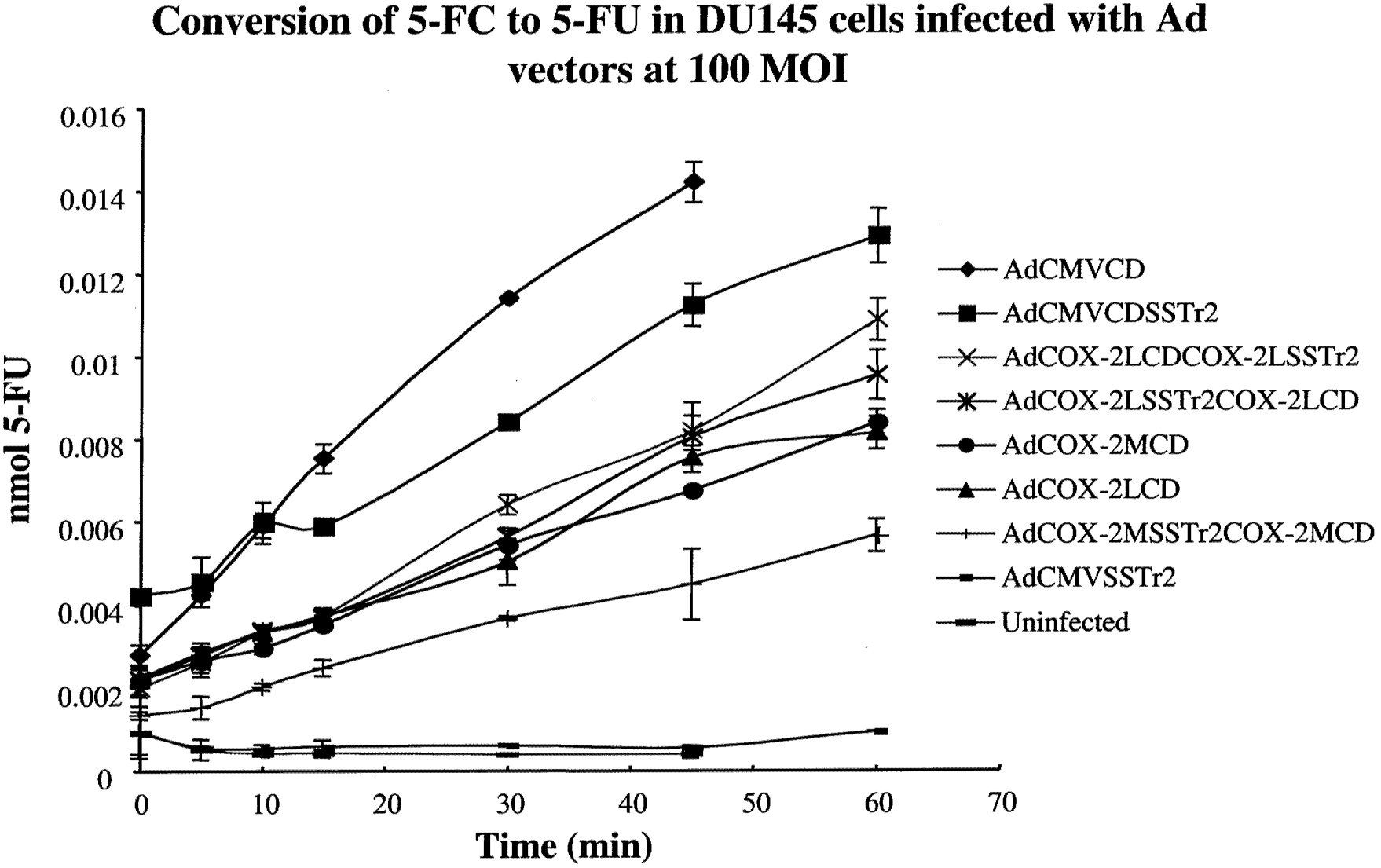

Conversion of 5-FC to 5-FU in DU145 cells infected with Ad vectors at 100 MOI
nmol 5-FU
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
10
20
30
40
50
60
70
Time (min)
AdCMVCD
AdCMVCDSSTr2
AdCOX-2LCDCOX-2LSSTr2
AdCOX-2LSSTr2COX-2LCD
AdCOX-2MCD
AdCOX-2LCD
AdCOX-2MSSTr2COX-2MCD
AdCMVSSTr2
Uninfected

Figure 4

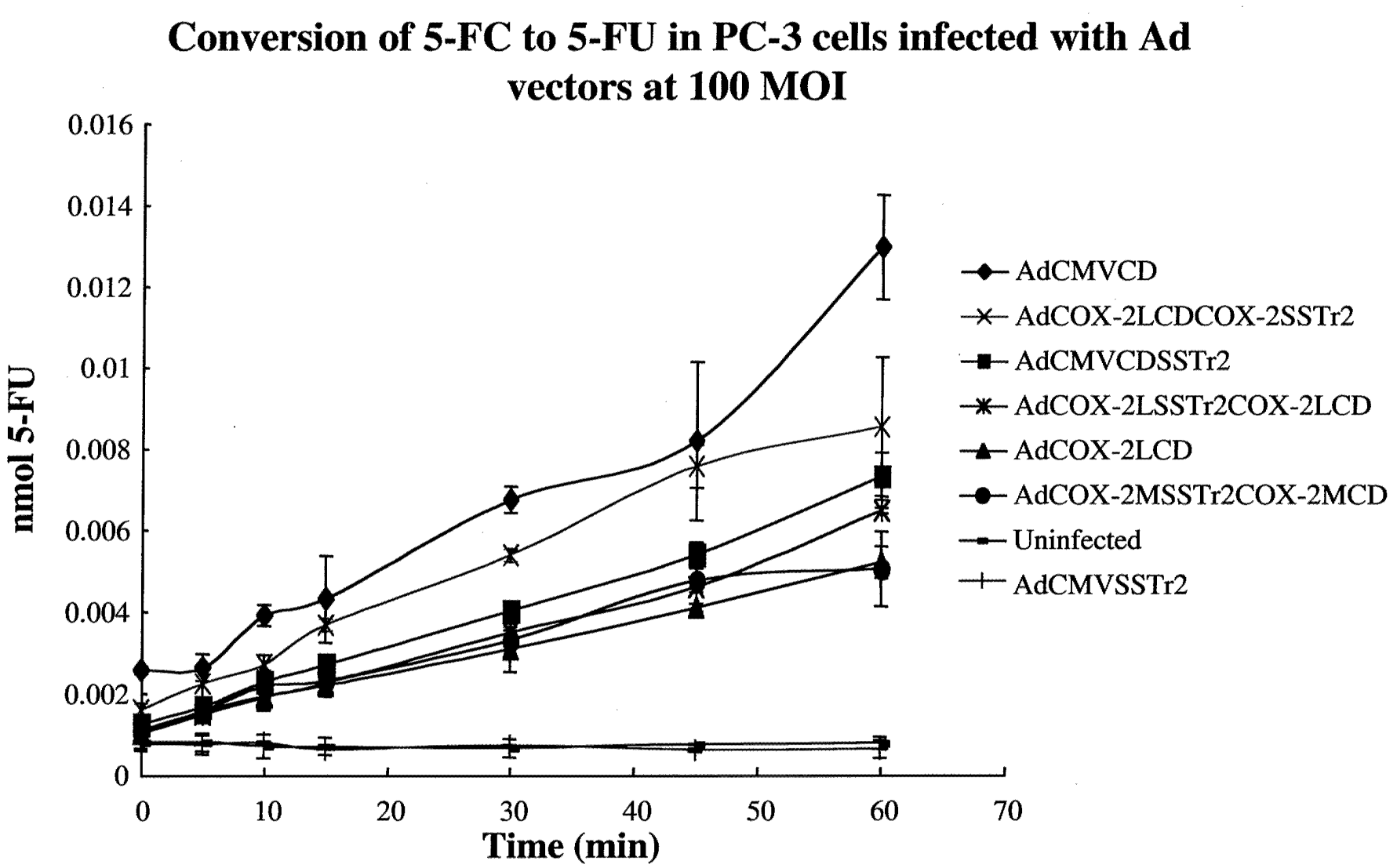

Conversion of 5-FC to 5-FU in PC-3 cells infected with Ad vectors at 100 MOI
nmol 5-FU
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
10
20
30
40
50
60
70
Time (min)
AdCMVCD
AdCOX-2LCDCOX-2SSTr2
AdCMVCDSSTr2
AdCOX-2LSSTr2COX-2LCD
AdCOX-2LCD
AdCOX-2MSSTr2COX-2MCD
Uninfected
AdCMVSSTr2

Figure 5

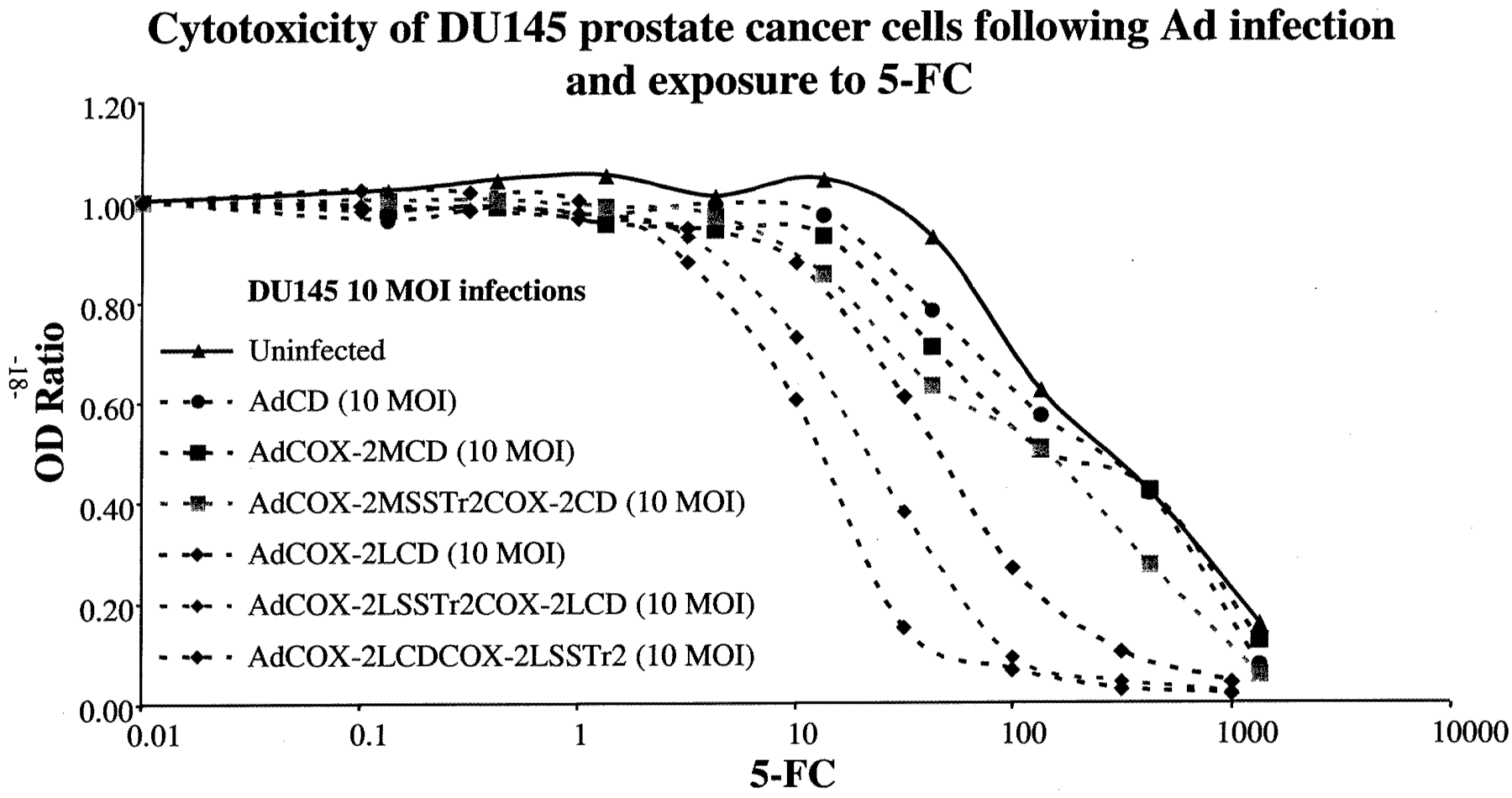

Cytotoxicity of DU145 prostate cancer cells following Ad infection and exposure to 5-FC
OD Ratio
1.20
1.00
0.80
0.60
0.40
0.20
0.00
DU145 10 MOI infections
Uninfected
AdCD (10 MOI)
AdCOX-2MCD (10 MOI)
AdCOX-2MSSTr2COX-2CD (10 MOI)
AdCOX-2LCD (10 MOI)
AdCOX-2LSSTr2COX-2LCD (10 MOI)
AdCOX-2LCDCOX-2LSSTr2 (10 MOI)
0.01
0.1
1
10
100
1000
10000
5-FC

Figure 6

Cytotoxicity of DU145 prostate cancer cells following Ad infection and exposure to 5-FC

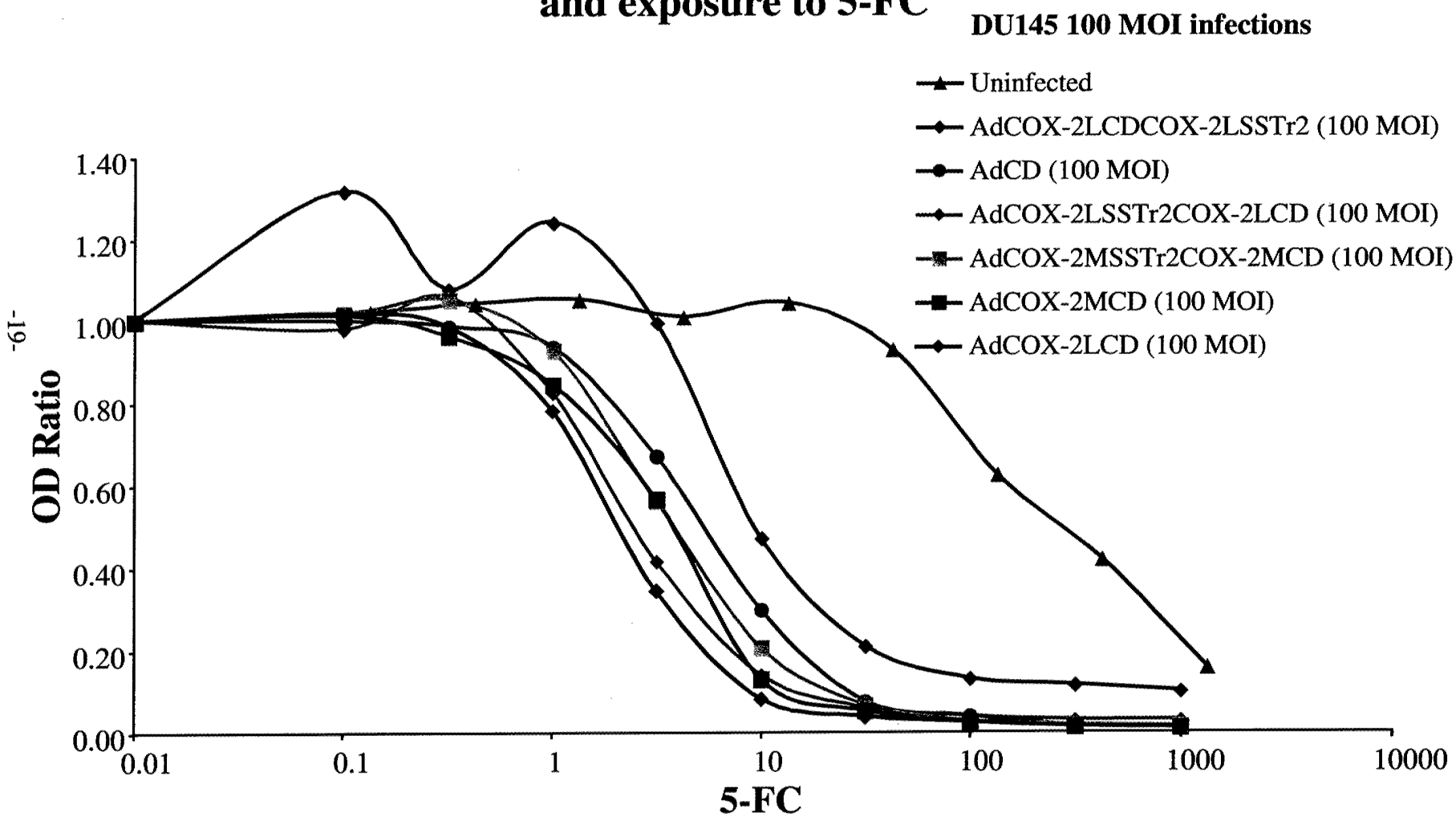


Figure 7

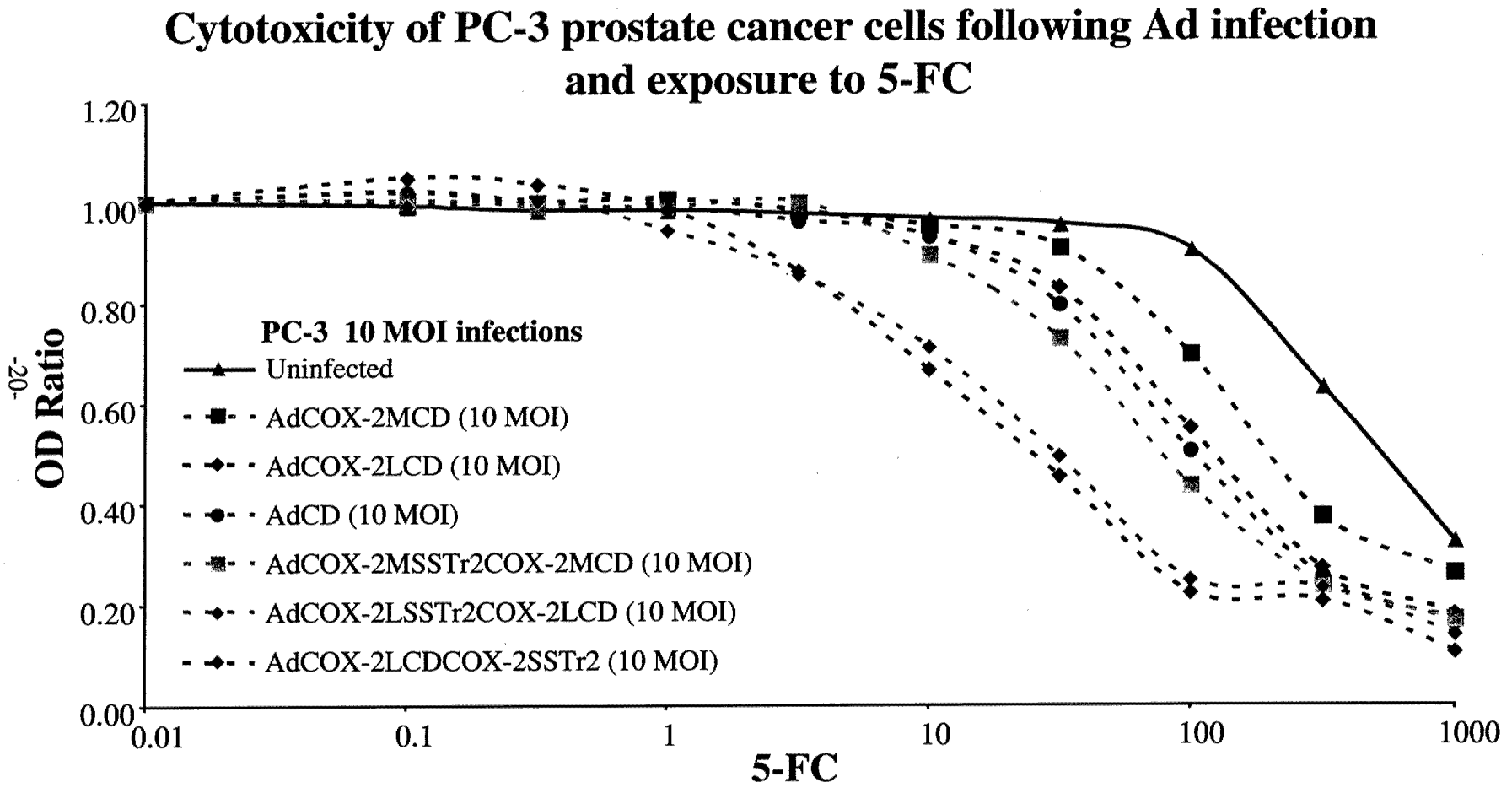
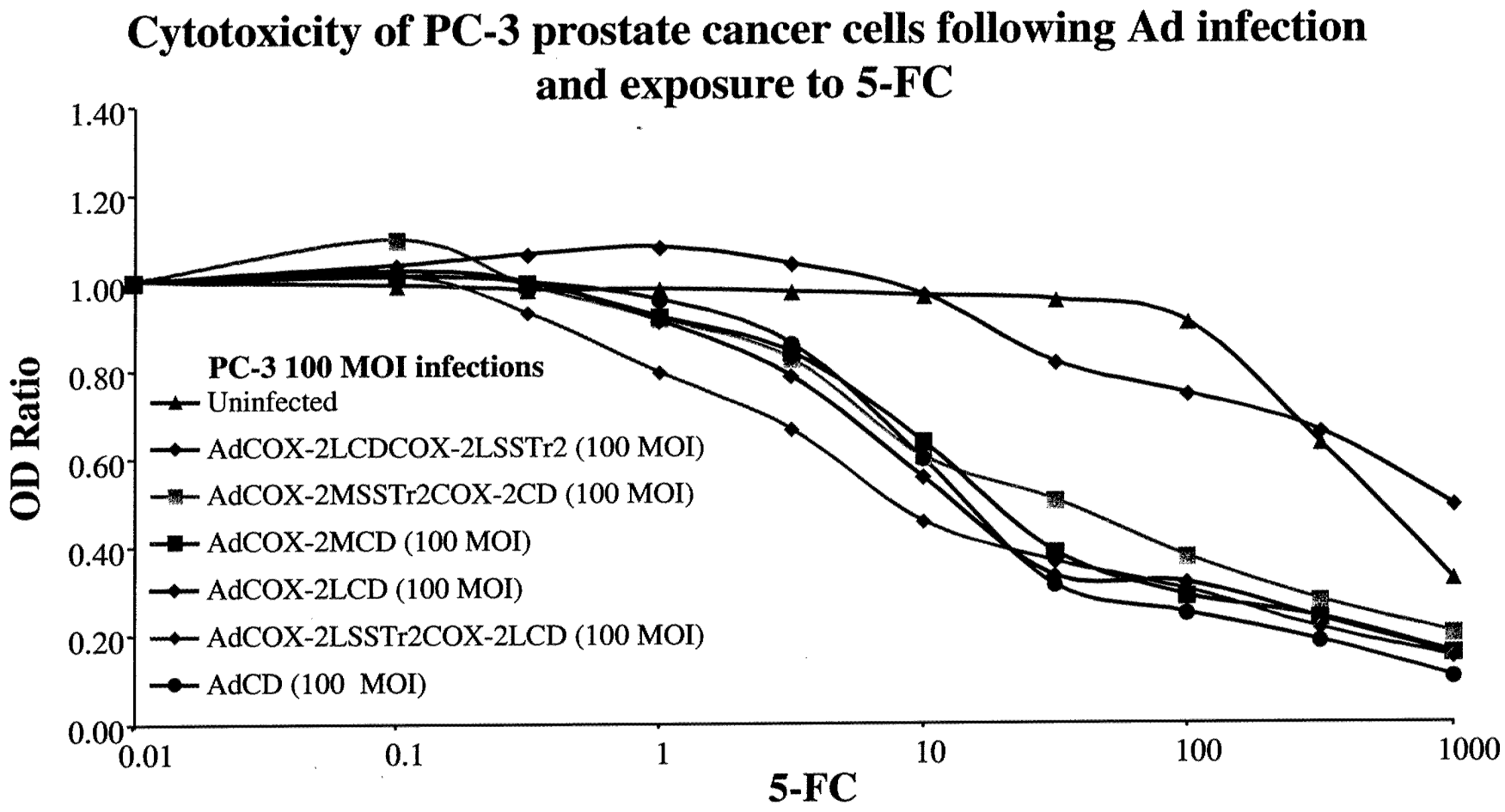

Cytotoxicity of PC-3 prostate cancer cells following Ad infection and exposure to 5-FC
OD Ratio
-20-
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.01
0.1
1
10
100
1000
5-FC
PC-3 10 MOI infections
Uninfected
AdCOX-2MCD (10 MOI)
AdCOX-2LCD (10 MOI)
AdCD (10 MOI)
AdCOX-2MSSTr2COX-2MCD (10 MOI)
AdCOX-2LSSTr2COX-2LCD (10 MOI)
AdCOX-2LCDCOX-2SSTr2 (10 MOI)

Figure 8


Cytotoxicity of PC-3 prostate cancer cells following Ad infection and exposure to 5-FC
OD Ratio
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.01
0.1
1
10
100
1000
5-FC
PC-3 100 MOI infections
Uninfected
AdCOX-2LCDCOX-2LSSTr2 (100 MOI)
AdCOX-2MSSTr2COX-2CD (100 MOI)
AdCOX-2MCD (100 MOI)
AdCOX-2LCD (100 MOI)
AdCOX-2LSSTr2COX-2LCD (100 MOI)
AdCD (100 MOI)