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Preclinical Study (Prostate)

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<b>13. ABSTRACT (Maximum 200 Words)</b> We have previously reported that two polyamine antagonists currently undergoing clinical evaluation: the polyamine analog, N <sup>1</sup> , N <sup>11</sup> -diethylnorspermine (DENSPM) and the biosynthetic enzyme inhibitor, 4-amidinoindan-1-one 2'-amidinohydrazone (CGP-48664), appear to exert unusual regulatory effects in prostate carcinoma cell lines relative to other cell lines. We have proposed to develop strategies <i>in vitro</i> and <i>in vivo</i> for therapeutically exploiting this observation. In pursuit of this goal, we have discovered that CGP-48664 potently induces apoptosis via a p53 / caspase-3 dependent pathway that is unrelated to its intended mode of action as an inhibitor of polyamine biosynthesis. Such an effect has not been previously reported and may have relevance to ongoing clinical trials with the compound. Future studies will investigate the therapeutic significance of this finding <i>in vivo</i> . In another development, we have determined that a polyamine analog related to DENSPM exerts meaningful antitumor activity against DU145 human prostate carcinoma xenografts. This finding will be optimized with respect to drug effects on polyamine regulatory responses and on the antitumor activity of the compound. The overall goal of both research efforts is to identify and develop relevant prostate-directed therapies as rapidly as possible.				
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FOREWORD

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Carl W. Porter 9/21/99  
PI - Signature Date

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## **Introduction:**

Polyamines are organic cations found in all cells and known to be essential for growth. Intracellular levels are sensitively maintained by a series of regulatory responses. The relationship of polyamines to the prostate is unique among all tissues since in addition to synthesizing these molecules for cell growth, the gland produces massive quantities for export into semen. It might, therefore, be expected that prostatic tumors could exhibit unique polyamine-related regulatory responses. We have recently observed that in contrast to other normal and tumor cell types, two of three prostate carcinoma lines displayed an altered ability to regulate polyamine transport in responses to polyamine analog or inhibitors (1). More specifically, two lines could not down-regulate uptake in response to analogs and one could not up-regulate it in response to polyamine depletion caused by inhibitors. The findings have clear therapeutic implications for two polyamine antagonists currently undergoing clinical evaluation: the polyamine analog, N<sup>1</sup>, N<sup>11</sup>-diethylnorspermine (2, DENSPM; Parke-Davis) and the biosynthetic enzyme inhibitor, 4-amidinoindan-1-one 2'-amidinohydrazone (3-4, CGP-48664; Novartis). More specifically, it should be possible to develop drug treatment schedules capable of optimizing selective delivery of DENSPM (which utilizes the transporter) to prostate tumors and in the case of CGP-48664, to minimize antiproliferative escape via salvage of exogenous polyamines. Herein, we propose to further examine polyamine regulatory responses in prostate carcinoma cell lines treated with analogs and inhibitors; to examine the underlying molecular mechanism(s) responsible for alterations in those responses; and most importantly, to develop *in vitro* and *in vivo* strategies for therapeutically exploiting these fundamental observations. These latter studies will correlate variations in drug structure and schedule with prostate responses involving apoptosis and cell cycle arrest as they relate to unregulated versus regulated polyamine transport. We believe we are particularly well-poised in terms of long term involvement with both polyamine analogs and inhibitors. Given the current clinical status of both drugs, promising findings should be readily translatable into experimental therapies for prostate carcinoma patients as proposed in Phase II of this application

## **Body (Figures & Figure Legends located in Appendix 1)**

### **A. Proposed Tasks**

The project tasks as originally proposed are as follows:

- **Task 1.** To use available and newly acquired prostatic carcinoma cell lines and compounds to further determine the extent to which altered polyamine homeostatic regulatory responses are characteristic of this tumor type.
- **Task 2.** To develop effective *in vitro* strategies based on observations made in Task 1 which optimize the antiproliferative effects of DENSPM, CGP-48664 or related inhibitors and analogs.
- **Task 3.** To investigate mechanisms underlying aberrant regulatory responses as determined in Task 1.
- **Task 4.** To extend strategies developed in Task 2 to *in vivo* model systems involving appropriate human prostatic carcinoma lines growing in scid mice.

- Task 5. To further optimize the choice of analog and/or inhibitor using compounds available to us from various collaborating laboratories.

#### B. Task Modifications:

The ordered pursuit of the above proposed tasks has been altered by three significant developments: (i) our observation that CGP-48664 exerts a significant apoptotic effect in LNCaP prostate carcinoma cells and that this response seems to be unrelated to its intended activity as an inhibitor of polyamine biosynthesis; (ii) the progression of both CGP-48664 and DENSPM to Phase II clinical status and the need on our part, to explore the antitumor effectiveness in preclinical prostate carcinoma models in order to have the disease appropriately included in the ongoing clinical trials; (iii) clinical trials indicated that schedule modification of DENSPM (i.e. splitting a single daily dose to twice or three times daily, as we proposed in Tasks 1 and 2) is not well tolerated in patients. Thus, during the past year, greater emphasis has been given to Task 3 as it relates to the cell cycle effects of CGP-48664 and to Tasks 4 and 5 as they relate to the identification of antitumor activity of CGP-48664 and DENSPM and related analogs.

#### C. Mode of CGP-48664 Drug Action in LNCaP Cells:

As originally proposed in Task 1b, we began this project by attempting to confirm variant polyamine regulatory responses to CGP-48664 and DENSPM by examining the effects of other known polyamine inhibitors, and analogs. The goal was to determine that the responses could be generalized to compounds having comparable modes of action as CGP-48664 and DENSPM before moving on to examine the generality of altered polyamine responses in additional prostate carcinoma cell lines. An initial comparison of growth effects between CGP-48664 and MDL-46811, a well known mechanism-based specific inhibitor of S-adenosylmethionine decarboxylase (5, SAMDC), revealed that at 50  $\mu$ M, the former inhibitor induced an immediate and complete cessation of LNCaP cell growth while at 100  $\mu$ M, the latter had virtually no effect on the growth these cells (Figure 1). As expected for SAMDC inhibitors (3-5), both drugs similarly increased putrescine and decreased spermidine and spermine pools (data not shown). Our immediate suspicion was that CGP-48664 was exerting unexpected drug effects that were unrelated to inhibition of SAMDC. This possibility was further indicated by the observation that growth inhibition was not preventable with exogenous spermidine (Figure 1).

Flow cytometry cell cycle analysis of growth inhibition by 50  $\mu$ M CGP-48664 indicated a preG1 peak (data not shown) which suggested that a significant portion of the drug treated cells were undergoing apoptosis. The possibility was more rigorously examined by a qualitative gel DNA fragmentation assay which confirmed the presence of significant DNA fragmentation, a hallmark indicator of apoptosis (6). The effect was fully apparent at 50  $\mu$ M and barely detectable at 5  $\mu$ M in cells treated with CGP-48664 for 72 h (Figure 2). A time dependence study revealed that at 50  $\mu$ M CGP-48664, DNA fragmentation was first detectable at 24 h (Figure 3). The effect was sought in two other two human prostate carcinoma cell lines, DU145 and PC-3, but neither demonstrated detectable DNA fragmentation when treated with 50  $\mu$ M CGP-48664 or 100  $\mu$ M MDL-73811 for 72 h (Figure 4). Consistent with relative drug effects on apoptosis, treatment with 100  $\mu$ M MDL-43811 failed to induce DNA fragmentation in any of the cell lines including LNCaP. Taken together, the data indicate that CGP-48664 clearly induces apoptosis in LNCaP cells and this cellular response appears to be wholly unrelated to drug

effects on SAMDC. This raises two issues; namely, (i) that CGP-48664 drug action is non-specific in LNCaP cells and (ii) that drug action does not apply to all cell types.

The status of p53 is recognized as a determining factor in differential apoptotic responses among cell lines (7). In this regard, it is known from the literature (8), that LNCaP cells contain wild-type p53; DU145 cells contain p53 with two mutations and PC-3 cells are deficient in p53 due to a deletion mutation. Western blot analysis indicated that apoptosis was accompanied by upregulation of p53 (Figure 5). Induction of the protein was first noticeable at 3 h after which it increased steadily to 10-fold at 24 h. Thus, induction of apoptosis in LNCaP cells could be initiated by p53.

Determining the upstream events leading to the induction of p53 is beyond the scope of this proposal except to demonstrate that they appear to be unrelated to drug effects on polyamine biosynthesis. Since apoptosis and induction of p53 by CGP-48664 have not been previously described in other cell types, it could form the basis for selective drug action on well differentiated prostate carcinoma. Thus, it is relevant to determine the down-stream effectors of CGP-48664-induced apoptosis in LNCaP cells. In this regard, we observed that the anti-apoptotic protein Bcl-2 (9) was markedly down-regulated with CGP-48664 at 12 h (Figure 6), the time at which induction of p53 reaches a near maximum (Figure 5). A comparison of cytochrome c levels in mitochondrial versus the S-100 fractions of LNCaP treated cells revealed that CGP-48664 caused the mitochondrial protein to be released into the cytosolic fraction (Figure 7); a typical feature of p53/caspase-mediated apoptosis (10). Indeed, early activation of caspase-3 (Figure 8), and caspase-mediated cleavage of poly(ADP-ribose) polymerase (PARP; Figure 10) were seen in treated cells. Taken together, these findings suggest that in LNCaP cells, the apoptotic effect of CGP-48664 may be related to induction of p53 and more clearly, to activation of caspase-3 dependent apoptosis (11). The possible protein response interrelationships as they relate to caspase 3-induced apoptosis are shown in Figure 9. The apparent involvement of p53 and caspase 3 in apoptosis is based wholly on correlation and temporal sequence of events. The more defining studies involving various transfection strategies are beyond the goals and technical scope of this proposal.

As noted above, all of these events appear to be unrelated to perturbations in polyamine pools via inhibition of SAMDC. Although there exists some findings to indicate that CGP-48664 may have nonspecific side effects in certain cell lines, none of these implicates an apoptotic pathway. Clearly the lack of specificity seems to be cell-type specific since we have previously shown (12) that Chinese hamster ovary (CHO) cells made resistant to CGP-48664 overexpress SAMDC due to gene amplification and growth inhibition is fully preventable with exogenous spermidine, a down-stream product of SAMDC action.

#### D. Antitumor Studies with CGP-48664, DENSPM and Recently Available Polyamine Analogs:

##### 1. Establishing an *in vivo* LNCaP tumor model system.

In view of the above cell culture findings with CGP-48664 in human LNCaP tumors, it became obvious that the drug effect should be sought under *in vivo* conditions. Despite claims by others, we have been unable to grow LNCaP cells in a subcutaneous setting in mice—orthotopically implanted tumors are not readily amenable to antitumor drug studies. In our efforts, various inocula sizes were implanted in nude athymic mice or scid mice as free cells or

as cell embedded in matrigel or collagen. Androgen pellet were implanted into all mice prior to tumor implantation. In a further attempt, we purchased LNCaP cells expressing green fluorescent protein from Anticancer Inc (San Diego, CA) and first implanted them orthotopically with the intention of transferring tumor to a subcutaneous site once they grew out. Again, we failed to succeed. We continue to work on this problem and plan to contact Dr. Warren Heston (Sloan Kettering Institute, New York) and/or Dr. Tom Corbett (Wayne State Cancer Center, Detroit, MI) both of whom reportedly have been able to successfully grow LNCaP as a subcutaneous tumor and to conduct antitumor studies with it.

## 2. Antitumor studies with DU145 prostate carcinoma.

In view of the fact that we had previously shown (1) that DU-145 cells were the most sensitive of the three prostate carcinoma cell lines, antitumor studies were undertaken as proposed in Task 4 in an effort to establish base line antitumor activity for both DENSPM and CGP-48664. Tumors were grown subcutaneously in nude athymic mice and treatment was begun at either  $\sim 30 \text{ mm}^3$  or  $\sim 150 \text{ mm}^3$ . CGP-48664 at 50 mg/kg per day i.p. (commonly used dose for this drug) showed absolutely no antitumor activity (data not shown) while DENSPM at 80 mg/kg per day i.p. (near to the maximal tolerated dose) for 5 days yielded a modest antitumor activity which was apparent as a slowing in tumor growth followed by a full growth rate recover (data not shown). Experiments with DENSPM will be resumed after item 3 is completed.

## 3. New polyamine analogs.

Recently, we have gained access via S'LIL Pharmaceuticals (13, Madison, WI) to novel polyamine analogs which when tested via the company, showed *in vitro* activity against prostate carcinoma cells lines and which also showed *in vivo* activity in a benign prostate hyperplasia model (*PROPRIETARY INFORMATION from S'LIL*). The first (SL-11147) is an unsaturated analog of DENSPM with a double bond linking the two center carbons of the molecule. It produced antitumor activity nearly identical to DENSPM in the DU145 model. The second compound (SL-11144) is also an exaggerated analog of DENSPM—if the latter is equivalent to DE-333 where DE refers to diethyl and 333 to the intra-amine carbon spacings, then SL-11144 is DE-4x4db4-4 (where db = double bond). SL-11144 is an extremely competitor of polyamine transport which uniquely fails to down-regulate the transporter (*PROPRIETARY INFORMATION from S'LIL*). As shown in Figure 11, SL-11144 given as a single dose at the maximally tolerated daily dose of 10 mg/kg i.p. for 5 days, there is minimal antitumor activity. If after 10 days, a second 5 day round of SL-11144 dosing is administered, there is obviously significant antitumor activity (Figure 12). In another strategy, we followed the single round of SL-44 treatment with a 10 day dose of CGP-48664 with the idea that while the latter is not effective by itself, it may be capable of sustaining any initial antitumor activity produced by SL-11144. The result was nearly identical to SL-11144 alone (data not shown). Overall, we believe that the data with SL-11144 are encouraging and we are beginning to examine alternative schedules. In addition, we propose to study the biochemical polyamine parameter profile of tumors (focusing on drug accumulation) treated with these novel compounds in order to gain insight into their mode of *in vivo* drug action and how it compares to DENSPM (14, 15). Finally, we will attempt to optimize the biochemical and antitumor effects using analogs of SL-11144 found by investigators at S'LIL to have promising *in vitro* activity against prostate carcinoma cell lines.

### E. New Direction Under Consideration:

This proposal is intended to use clinically relevant polyamine analogs and inhibitors to therapeutically exploit aberrant polyamine regulatory responses in prostate carcinoma cells and tissues. While this primarily intended to focus on previous results indicating that regulation of transport is altered in certain prostate carcinoma lines, other regulatory responses were also proposed for consideration. In research efforts being undertaken in an NCI funded award and using transfected breast carcinoma cells, we have used conditional expression transfection strategies to show that the polyamine catabolic enzyme, spermidine/spermine N<sup>1</sup>-acetyltransferase (SSAT) is a critical determinant of DENSPM drug action and that the basal levels determine drug sensitivity. In our opinion, it would be highly worthwhile to use the same technology (conditional regulation of SSAT) to make similar determinations in a prostate carcinoma cell line and to use such findings as the basis for a possible gene therapy strategy to enhance the therapeutic potency and selectivity DENSPM towards prostate cancer.

#### ***Key Research Accomplishments:***

- Identification of a new site of action for CGP-48664 unrelated to inhibition polyamine biosynthesis in LNCaP cells and apparently responsible a caspase-3 mediated apoptosis which may be p53 dependent.
- Characterization of the antitumor activity of new and novel polyamine analogs against human DU145 prostate carcinoma xenografts.

#### ***Reportable Outcomes:***

- Mi, Z., Vredenburg, M.R., Kramer, D.L., Guo, B., Chen, Y. and Porter, C.W. CGP-48664 induces caspase-3 mediated apoptosis in LNCaP human prostate carcinoma cells via a polyamine independent mechanism. Manuscript under preparation.
- Mi, Z., Kramer, D.L., Miller, J.T., Bernacki, R.J., and Porter, C.W. Altered regulation of polyamine transport in human prostatic carcinoma cell lines in response to polyamine analogs and inhibitors. Proc. Am. Assoc. Cancer Res. 39:588, 1998.

#### ***Conclusions (& Future Directions):***

Conclusions of the studies to date are two-fold. Firstly, CGP-48664 is a potent inducer of apoptosis in LNCaP cells via a caspase-3 dependent pathway that appears to be unrelated to polyamine pool perturbations resulting from inhibition of SAMDC. Whether this action is therapeutically exploitable in early stage prostate carcinoma as represented by LNCaP cells, remains to be determined once a LNCaP *in vivo* tumor system is established. Secondly, newly identified and structurally novel polyamine analogs exhibit meaningful antitumor activity against DU145 prostate carcinoma xenografts. The basis for this antitumor activity as it most likely relates to polyamine regulatory responses remains to be characterized and then optimized with appropriate analogs.

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## APPENDIX 1

### FIGURE LEGENDS & FIGURES

**Figure 1.** Time-dependent effects on growth of LNCaP human prostate carcinoma cells treated with 50  $\mu$ M CGP-48664 (left panel) , 100  $\mu$ M MDL-73811 (right panel) or 50  $\mu$ M CGP-48664 in the presence of 10  $\mu$ M spermidine (center panel).

**Figure 2.** Qualitative agarose-gel analysis of DNA fragmentation in LNCaP cells treated for 72 h with 50  $\mu$ M CGP-48664 (664) for 72 h. DNA fragmentation is only faintly detectable at 5  $\mu$ M and fully apparent at 50  $\mu$ M. Lane Marker, a 1 kbp DNA marker. Ten  $\mu$ g of DNA were loaded onto each lane.

**Figure 3.** Time dependence of 50  $\mu$ M CGP-48664 induced DNA fragmentation in LNCaP cells. DNA fragmentation is first detectable at 24 h. Exogenous spermidine (SPD) seems to ameliorate the effect slightly. Lane marker (M), a 1 kbp DNA ladder. Ten  $\mu$ g of DNA were loaded onto each lane.

**Figure 4.** Differential induction of DNA fragmentation by 50  $\mu$ M CGP 48664 (664) in DU145, PC-3 and LNCaP human prostate carcinoma cells treated for 72 h. Only LNCaP cells show DNA fragmentation in response to CGP-48664 and none of the cell lines respond to MDL-73811 (MDL). Lane marker (M), a 1 kbp DNA ladder. Ten  $\mu$ g of DNA were loaded onto each lane.

**Figure 5.** Effect of CGP-48664 on p53 protein expression. LNCaP cells were harvested at 0-36 h during treatment with 50  $\mu$ M CGP-48664. Relative to actin, p53 protein increases to a maximum of ~10-fold. Twenty five  $\mu$ g of protein were loaded onto each lane.

**Figure 6.** Effect of 50  $\mu$ M CGP-48664 on Bcl-2 protein expression in LNCaP cells treated for 0 to 12 h. Twenty five  $\mu$ g of protein were loaded onto each lane.

**Figure 7.** Induction of cytochrome c release into the cytosol of LNCaP cells treated with 50  $\mu$ M CGP-48664 for 0 to 12 h. Note the absence of cytosolic cytochrome c at 0 h—other protein bands are included to show equality of loading in all lanes. Twenty five  $\mu$ g of protein were loaded onto each lane.

**Figure 8.** Increases in caspase-3 protein expression in LNCaP cells treated with 50  $\mu$ M CGP-48664 for 0 to 12 h. Note the increased expression of activated caspase. Twenty five  $\mu$ g of protein were loaded onto each lane.

**Figure 9.** Effect of CGP-48664 (at 50  $\mu$ M) on PARP protein level and degradation in LNCaP cells treated for 0 to 36 h. Twenty five  $\mu$ g of protein were loaded onto each lane. The

**Figure 10.** Diagram showing interactions of p53, Bcl-2, cytochrome c, caspase 3 and PARP as a possible pathway for apoptosis in LNCaP cells.

**Figure 11.** Antitumor effects of SL-11144 administered intraperitoneally at 10 mg/kg/d x 5 d in nude athymic mice bearing DU145 human prostate carcinoma subcutaneous xenografts. Bold lines indicate the median tumor volume for saline (squares) and SL-44 (crosses) treated tumors; broken lines indicate individual tumor volumes for animals treated with SL-44.

**Figure 12.** Antitumor effects of SL-11144 administered as two dosing rounds at 10 mg/kg/d x 5 d each separated by 10 days. Bold lines indicate the median tumor volume for saline (squares) and SL-11144 treated (triangles) tumors; broken lines indicate individual tumor volumes for animals treated with SL-11144. Note that tumor regression and arrest associated with the second treatment but not with the first.

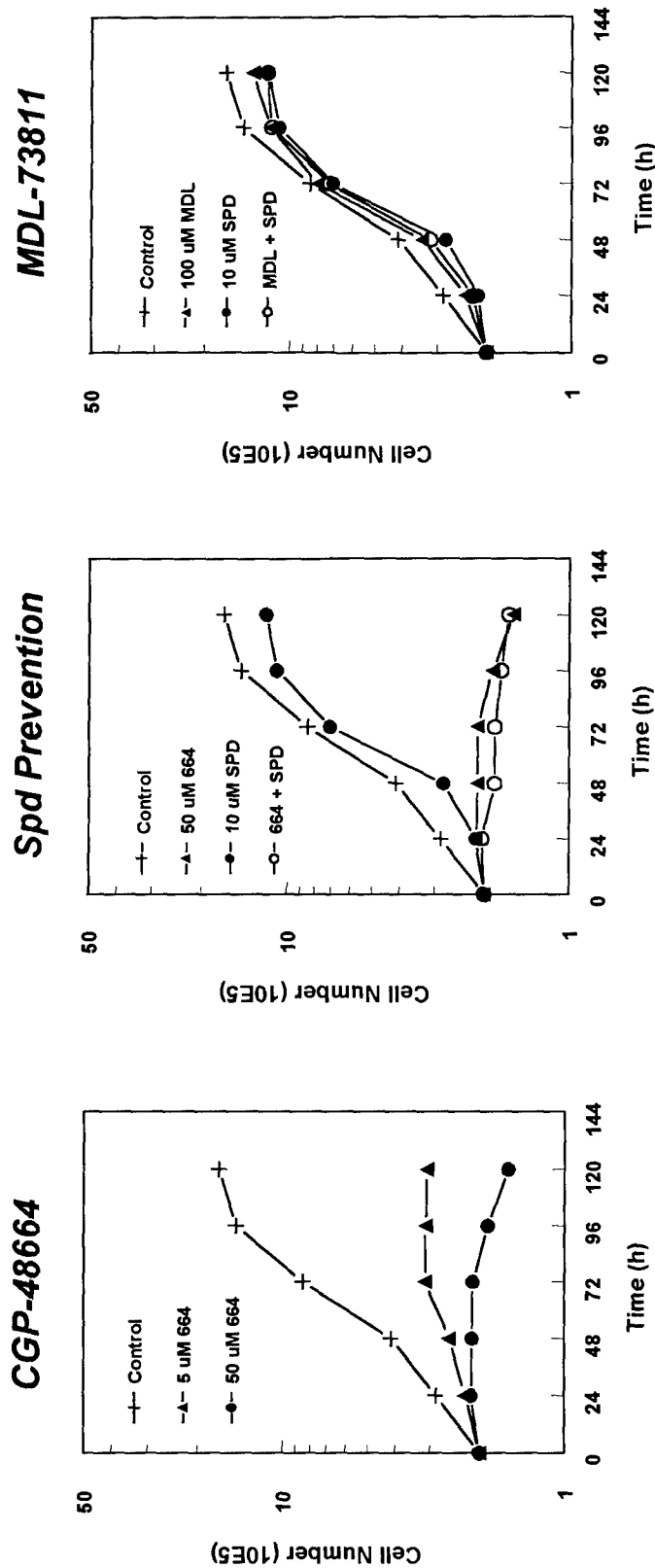
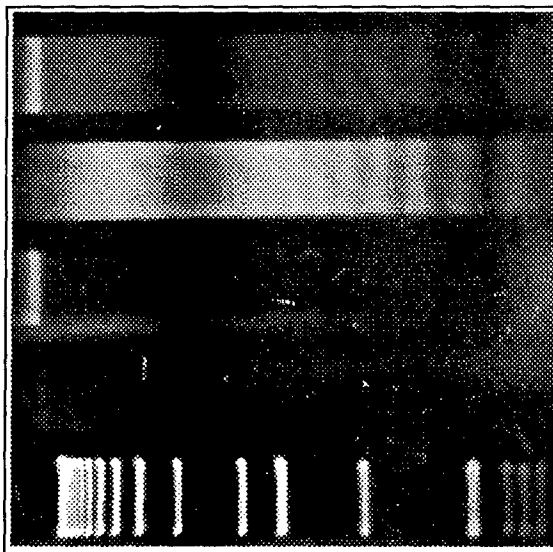


Figure 1. Comparison of CGP-48664 and MDL-73811 on the Growth of LNCaP Cells

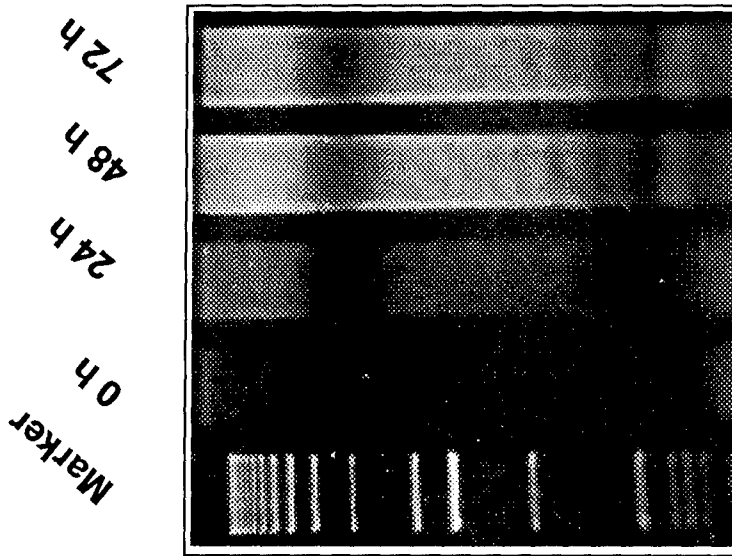
pro-apopf2

Marker  
0  $\mu$ M 664  
5  $\mu$ M 664  
50  $\mu$ M 664  
50  $\mu$ M 664 + 10  $\mu$ M SPD



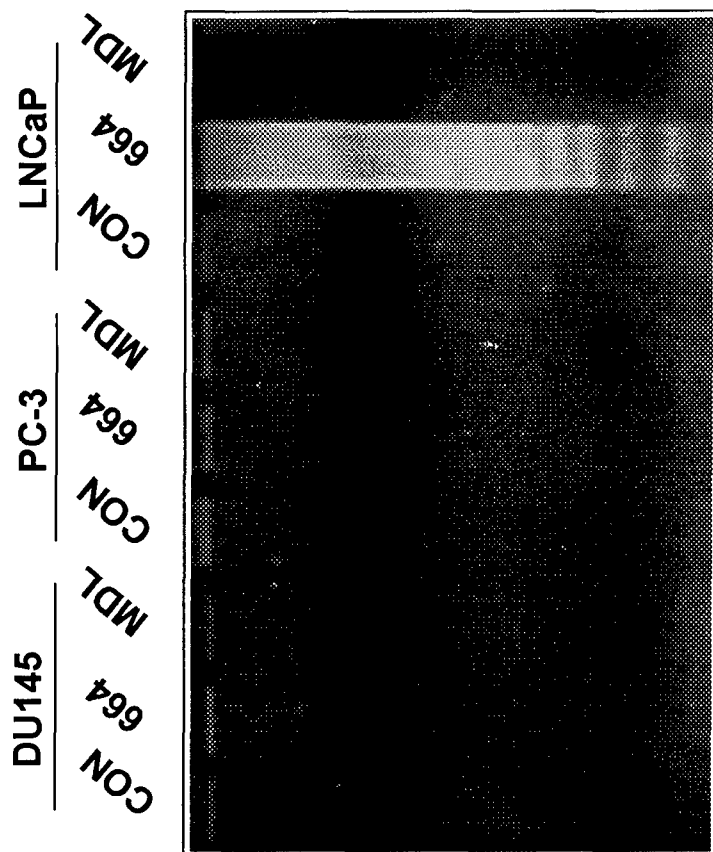
**Figure 2. CGP-48664-Induced DNA Fragmentation--Dose Dependence**

pro-apop3



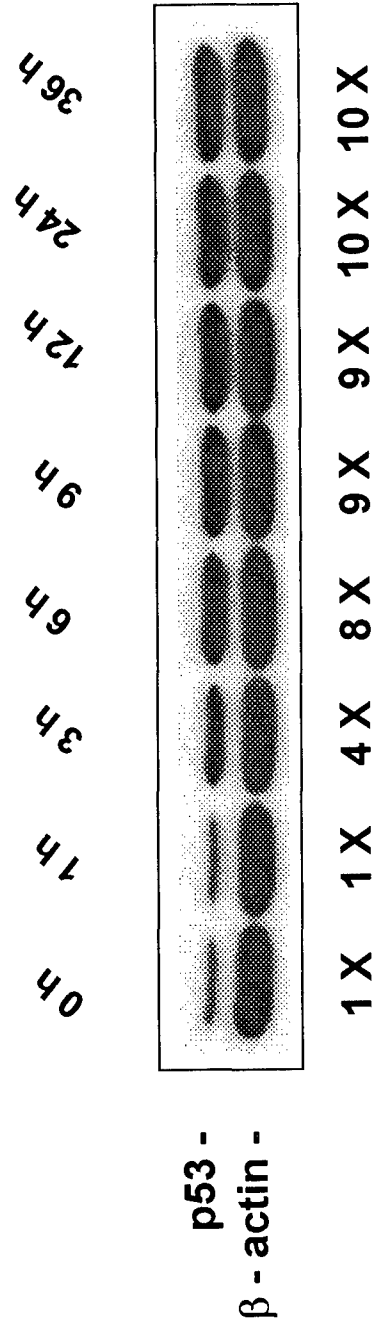
**Figure 3. CGP-48664-Induced DNA Fragmentation--Time Dependence**

pro-apopf4



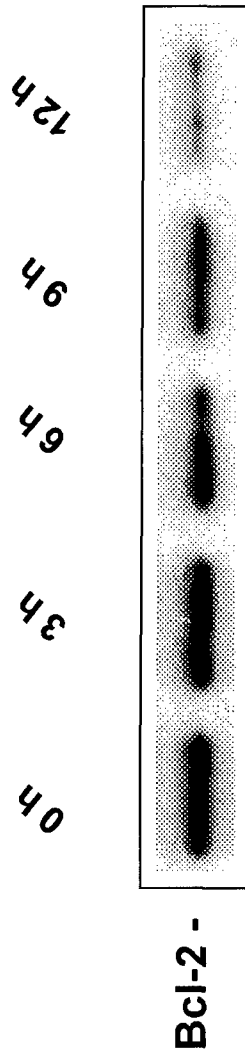
**Figure 4. CGP-48664-Induced DNA Fragmentation--Cell Line Comparisons**

pro-apopif5



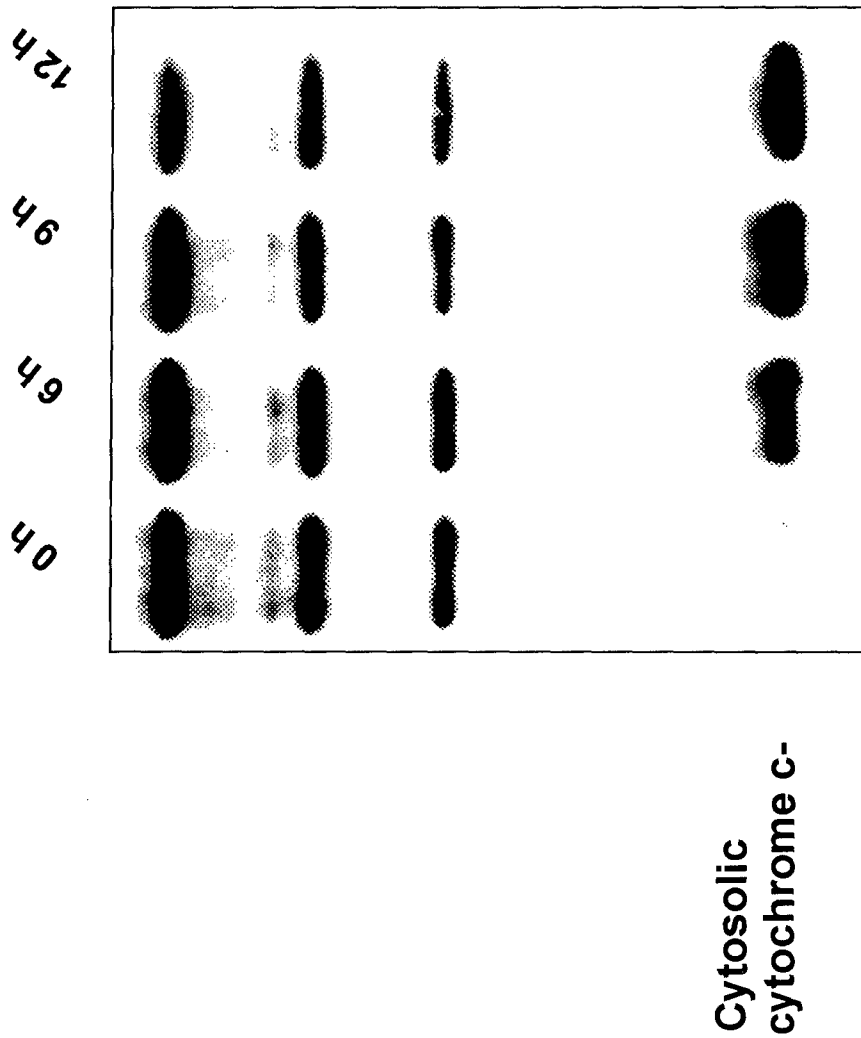
**Figure 5. Effect of CGP-48664 on p53 Protein Expression in LNCaP Cells**

pro-apopt6



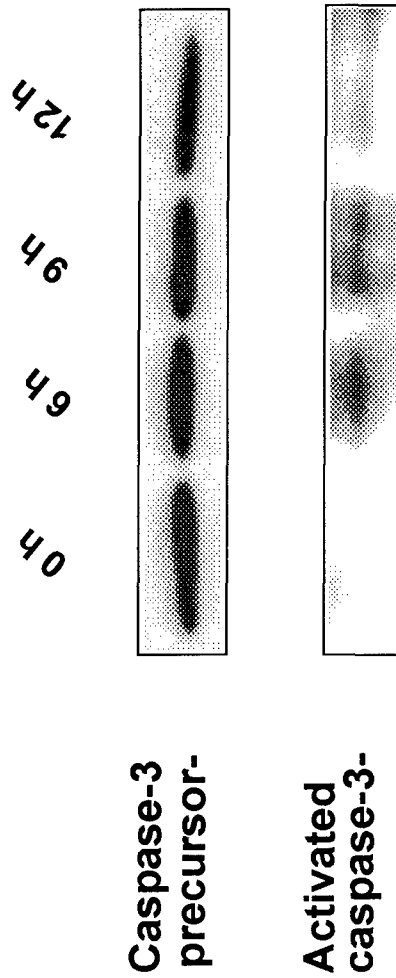
**Figure 6. Effect of CGP-48664 on Bcl-2 Protein Expression in LNCaP Cells**

pro-apopt7



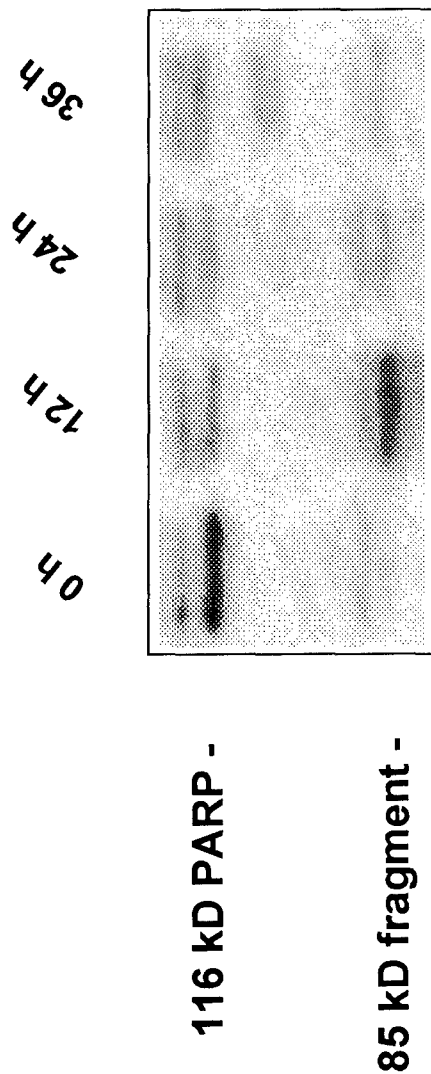
**Figure 7. CGP-48664 Induction of Cytochrome c Release in LNCaP Cells**

pro-apopt8



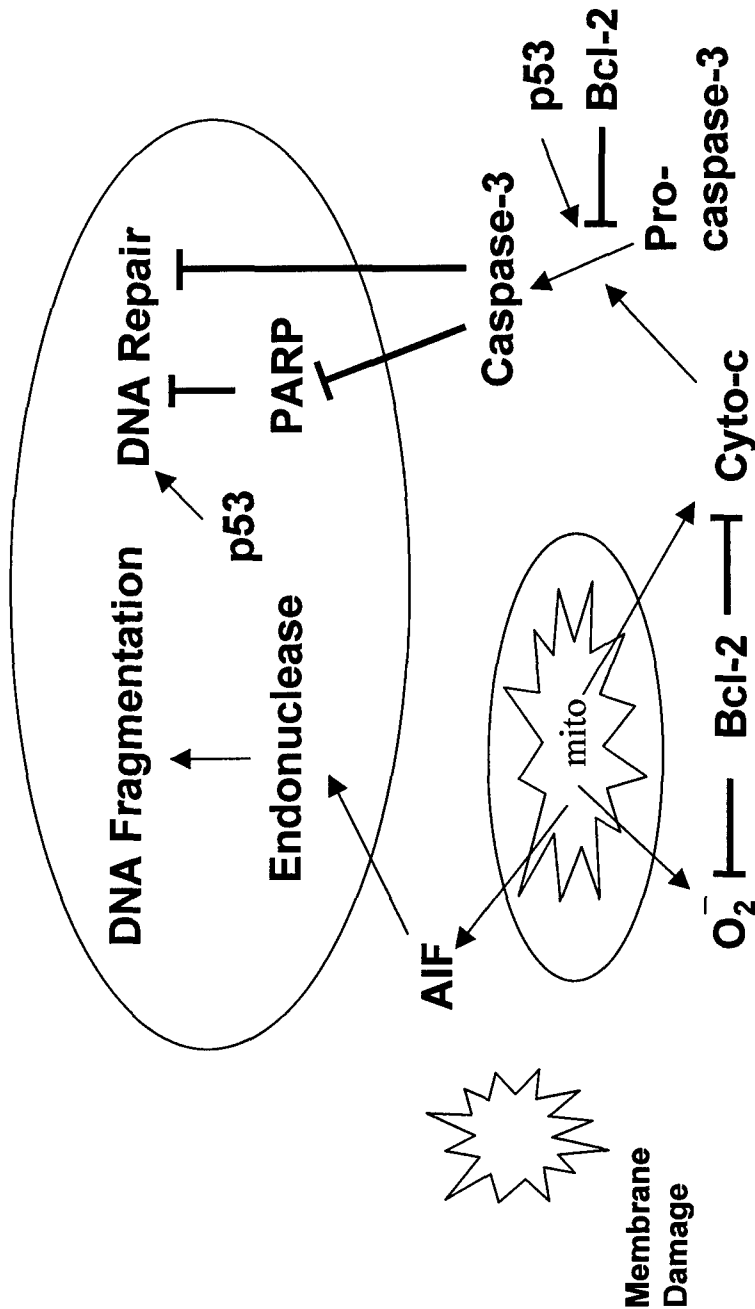
**Figure 8. Effect of CGP-48664 on Caspase-3 Protein Expression in LNCaP Cells**

pro-apopt9



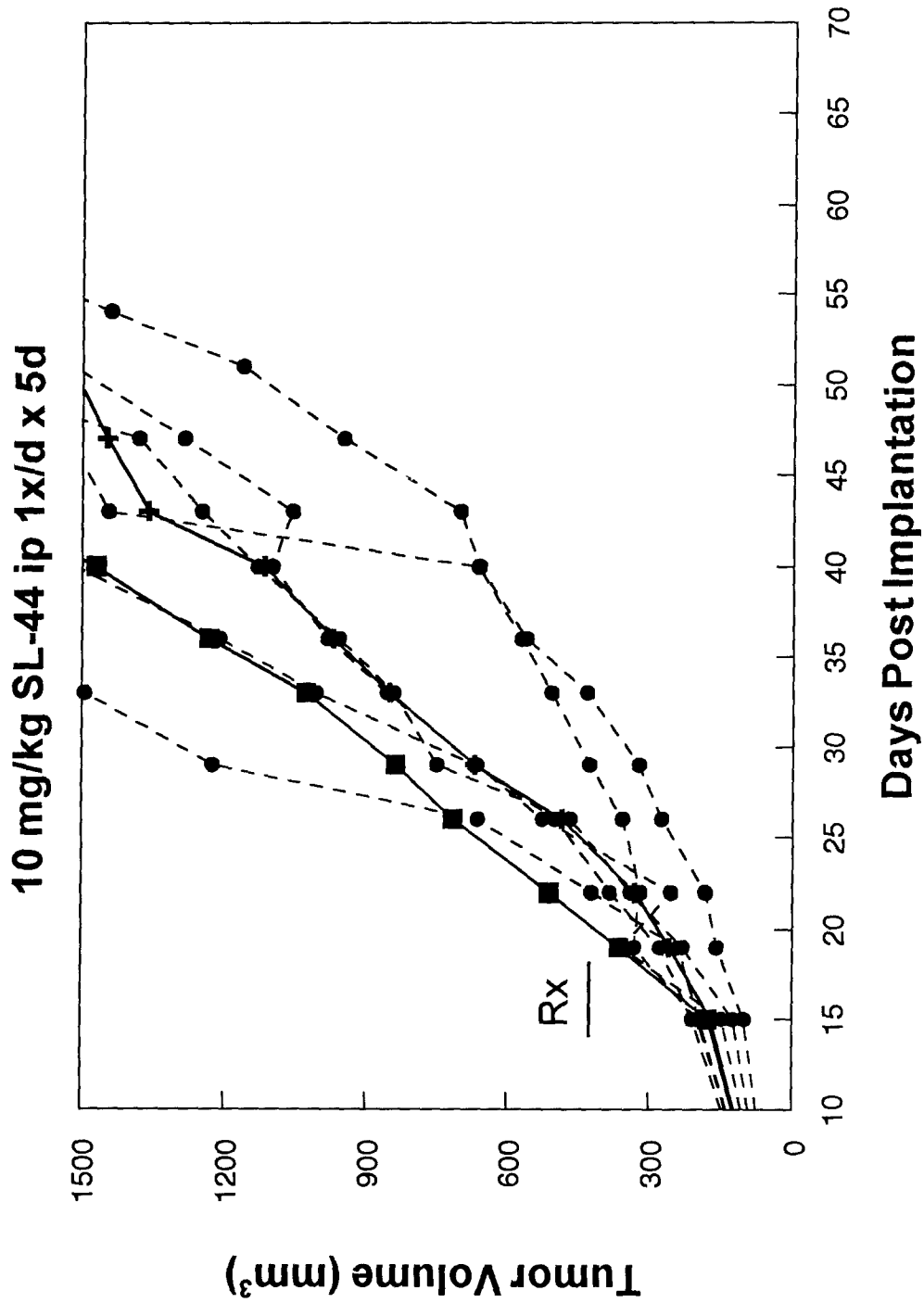
**Figure 9. Effect of CGP-48664 on PARP Protein Level and Degradation in LNCaP Cells**

pro-apopt0

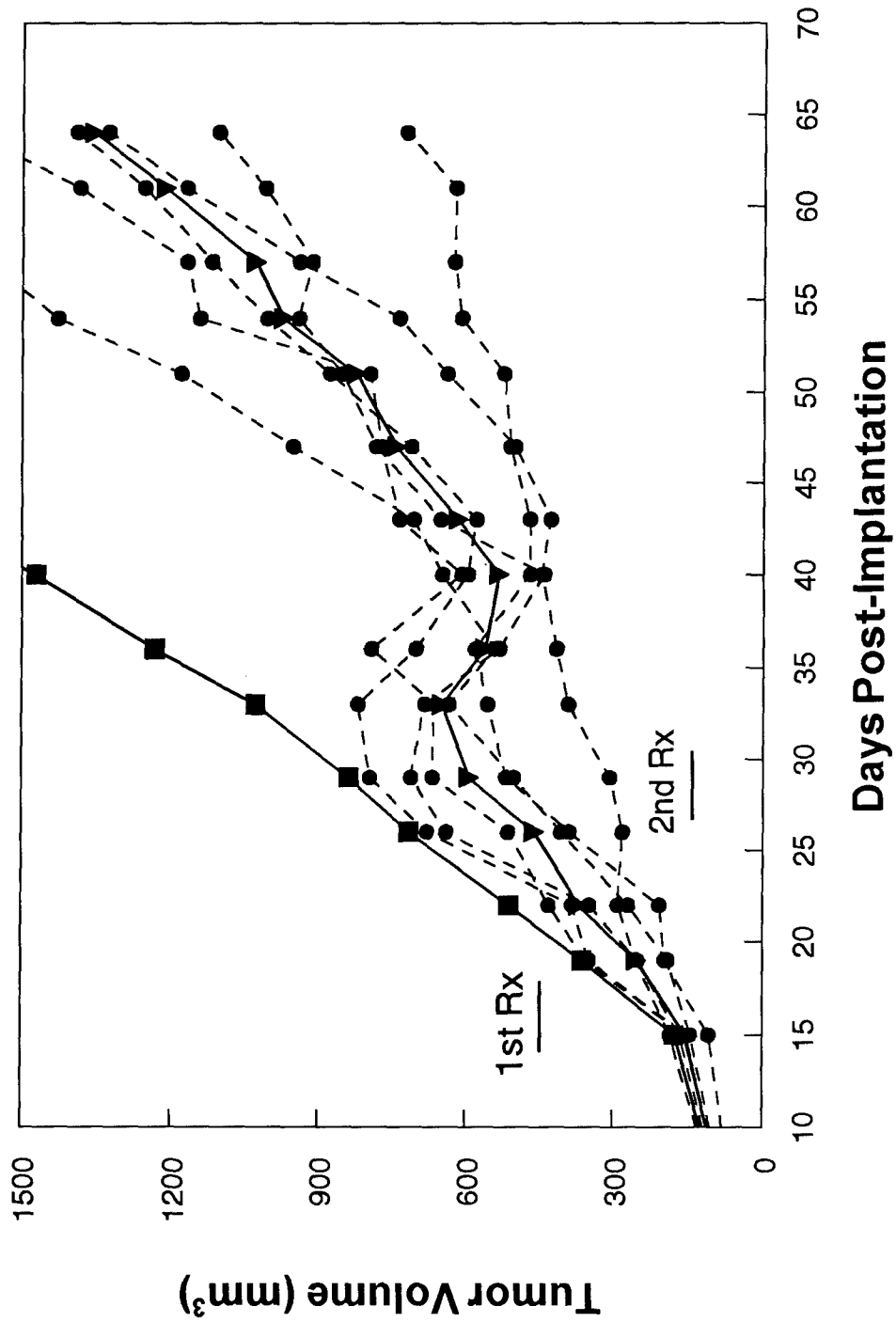


**Figure 10. Possible apoptotic pathways activated by CGP-48664 apoptosis in human prostate carcinoma LNCaP cells. (AIF =Apoptosis-Inducing Factor)**

**Figure 11. DU145 Prostate Carcinoma / Nude Mice**



**Figure 12. DU145 Prostate Carcinoma / Nude Mice**  
**10 mg/kg SL-44 ip 1x/d x 5; 10d off; Repeat Rx**





DEPARTMENT OF THE ARMY  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information  
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,  
VA 22060-6218

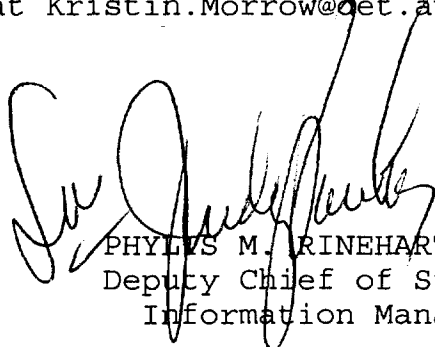
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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

  
PHYLLIS M. RINEHART  
Deputy Chief of Staff for  
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