AD_____

Award Number: DAMD17-98-1-8588

TITLE: Intervention of Prostate Cancer by a Flavonoid Antioxidant
       Silymarin

PRINCIPAL INVESTIGATOR: Rajesh Agarwal, Ph.D.

CONTRACTING ORGANIZATION: AMC Cancer Research Center
                         Denver, Colorado   80214

REPORT DATE: October 1999

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.

1

20001019 051

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE October 1999	3. REPORT TYPE AND DATES COVERED Annual (1-Oct-98 - 30-Sep-99)

4. TITLE AND SUBTITLE
Intervention of Prostate Cancer by a Flavonoid Antioxidant Silymarin

5. FUNDING NUMBERS
DAMD17-98-1-8588

6. AUTHOR(S)
Rajesh Agarwal, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
AMC Cancer Research Center

Denver, Colorado 80214

E-MAIL:
agarwalr@amc.org

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

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Oncogenic potential of advanced and androgen-independent prostate cancer (PCA) is causally associated with a ligand/receptor autocrine growth loop, e.g. interaction of transforming growth factor α (TGFα) and epidermal growth factor receptor (erbB1). We rationalized that targeting this pathway would be useful for PCA intervention, and showed recently that a flavonoid antioxidant silymarin inhibits erbB1 activation followed by a G1 arrest and inhibition of PCA cell growth. Here we did more studies to define cause and effect relationship for the observed effect of silymarin at membrane receptor and cytoplasmic levels. Treatment of LNCaP and DU145 human prostate carcinoma cells with silibinin (the pure form of silymarin) resulted in highly significant inhibition of TGFα binding to erbB1 receptor and ligand internalization in both dose- and time-dependent manner. Conversely, silibinin does not result in the inhibition of intrinsic tyrosine kinase activity of erbB1 in both LNCaP and DU145 cells. The observed inhibitory effect of silymarin on ligand binding to erbB1 and ligand internalization also resulted in an inhibition of erbB1 activation followed by its dimerization that leads to activation of downstream mitogenic signaling. These inhibitory effects of silymarin on LNCaP and DU145 cells also corroborate with its inhibitory effect on both cellular and released expression of TGFα in these two cell lines. Together these effects of silymarin resulted in a strong inhibition of constitutive MAPK/ERK1/2 activation in both LNCaP and DU145 cells followed by a strong inhibition in their growth. These results suggest that more detailed mechanistic and tumor studies are needed to assess both preventive and interventive effects of silymarin (or silibinin) against human prostate cancer.

14. SUBJECT TERMS
Prostate Cancer, Silymarin, Antioxidants, Cell Signaling

15. NUMBER OF PAGES
70

16. PRICE CODE

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

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.
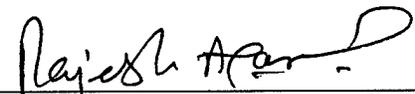
X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

_____ 10/28/99
PI - Signature   Date

3

4.   **TABLE OF CONTENTS**

5.   INTRODUCTION

Subject: Prostate cancer (PCA) is the most invasive and frequently diagnosed malignancy and the second leading cause (after lung) of cancer deaths in American males (1,2). Several studies have suggested that diet and androgen play a major role in the pathogenesis as well as in the promotion of PCA (2-8). Since PCA growth and development is initially androgen-dependent, androgen deprivation is used to control PCA (9). However, within a few years, tumor re-growth occurs which is largely due to progression/selection of initially androgen-dependent PCA cells to tumor cells that do not depend on androgen for their proliferation (10). At this stage, PCA growth is causally dependent on enhanced expression of growth factors and their receptors which lead to an autocrine loop for uncontrolled PCA growth and metastatic potential (11-20). In this regard, an enhanced expression of epidermal growth factor receptor (EGFR) and other members of this family and related ligands has been shown with high frequency in PCA (11-20). **Thus, new approaches are needed to control advanced androgen-dependent and -independent PCA as well as to prevent the disease from developing. One approach to reduce PCA incidence and associated mortality is chemoprevention/chemo-intervention targeted towards the impairment of mitogenic signaling mediated by growth factor receptors.** Several studies suggest that micro-chemicals present in fresh fruit, yellow-green vegetables and various herbs reduce the human cancer incidence and mortality due to stomach, colon, breast, lung, bladder, esophageal, prostate and other cancers (21-31). Among these, polyphenolic antioxidants are receiving increased attention in recent years as cancer preventive/interventive agents (32-34). **Silymarin** is also a polyphenolic flavonoid isolated from milk thistle (*Silybum marianum (L.) Gaertn*) and in its pure form composed mainly of stereoisomer **silibinin** (~90%, w/w) (35,36). For more than twenty-five years, silymarin and silibinin have been used clinically in Europe as an anti-hepatotoxic agent (37-40). In recent years, silymarin has also been used as a therapeutic agent in liver diseases in Asia and the United States, and marketed (in USA and Europe) as dietary supplement. Toxicity data on silymarin and silibinin as therapeutic agents show they are exceptionally well tolerated and largely free of adverse effects (41-44). **Together, we concluded that silymarin and silibinin are non-toxic and have been well studied as dietary supplements and as therapeutic plant flavonoids.**

Purpose: **The purpose of the studies in the current grant is the prevention/intervention of prostate cancer by silymarin targeted towards the impairment of EGFR-mediated mitogenic signaling involving the mechanistic approach on membrane receptor, cytoplasmic and nuclear signals and their biological significance in terms of PCA cell growth inhibition in nude mice tumor xenograft model.**

Scope: **An ideal cancer preventive agent should have a) little or no toxic effects; b) high efficacy; c) a known mechanism of action; d) low cost; and e) human acceptability (26). Therefore, we emphasize that silymarin (or silibinin) have promise and potential to be ideal cancer preventive/interventive agents against prostate cancer and based on our completed studies, as therapeutic agents for early recurrent disease.** The major scope of the studies in current grant is that their outcome will build a base for long-term phase II studies to a) further define the role of erbB family of RTKs and down stream events in human prostate cancer as molecular target(s) for intervention, and b) evaluate the therapeutic (and/or preventive) effects of silymarin (or silibinin) and other related agents against prostate cancer in investigative clinical trials with correlative laboratory studies.

6. BODY

Under this section "BODY", the research accomplishments associated with each Task outlined in the **approved** Statement of Work are described in sufficient detail in terms of experimental design, method employed, data obtained, interpretation of the results, and conclusion(s) drawn from the research findings. All the data are presented and discussed irrespective of their positive or negative outcome in a given experiment performed. We would also like to highlight here that each Task outlined in the **approved** Statement of Work, during first 12 months of funding, is described in detail in following pages in the same order as proposed in the original plan. In doing so, a separate sub-section is used for each Task.

Rationale for the studies proposed in the current grant: In addition to its exceptionally high anti-carcinogenic activity in different epithelial tumorigenesis protocols (45-47), in mechanistic studies we found that silymarin inhibits EGFR (erbB1) activation and induces anti-proliferative effects in epidermoid carcinoma cells A431 (48,49). As mentioned earlier, since erbB1 and other members of the erbB family play important roles in human prostate cancer (11-20), we reasoned that silymarin, by inhibiting erbB1 activation, may impair associated downstream events leading to growth inhibition of prostate cancer cells. In our preliminary studies (at the time of the submission of present grant) we showed that silymarin treatment of androgen-independent human prostate carcinoma DU145 cells results in a significant inhibition of transforming growth factor α (TGFα)-mediated activation of erbB1, but no change in its protein levels. Silymarin treatment also resulted in a significant decrease in tyrosine phosphorylation of an immediate down-stream target of erbB1, the adapter protein SHC, together with a decrease in its binding to erbB1. Blocking the activation of erbB1 by silymarin was associated with a significant induction of cyclin-dependent kinase inhibitors (CDKIs) Cip1/p21 and Kip1/p27, concomitant with a decrease in cyclin-dependent kinase (CDK) 4 expression. Cells treated with silymarin also showed an increased binding of CDKIs with CDKs together with a marked decrease in the kinase activity of CDKs and associated cyclins. Silymarin treatment also induced a G1 arrest, and resulted in a highly significant to complete inhibition of both anchorage-dependent and -independent growth of DU145 cells. The data from all these studies are published (prior to the current grant funding) in a journal article entitled "A flavonoid antioxidant silymarin inhibits activation of erbB1 signaling, and induces cyclin-dependent kinase inhibitors, G1 arrest and anti-carcinogenic effects in human prostate carcinoma DU145 cells" by Zi, X., Grasso, A.W., Kung, H.-J. and Agarwal, R.: Cancer Res., 58: 1920-1929, 1998.

Together, above summarized results suggested that silymarin may exert a strong anti-carcinogenic effect against prostate cancer, and that this effect is likely to involve impairment of erbB1-mediated signaling pathway, induction of CDKIs, and a resultant G1 arrest. This suggestion was the rationale for the proposed studies (in four aims/tasks) in the current funded grant.

6.1 Task (Aim) I: To study the effect of silymarin on membrane signaling, Months 1-9: All the studies proposed in this Task are completed, and described in detail below.

6.1.a Grow and maintain LNCaP and DU145 cells in culture: Both androgen-dependent LNCaP and androgen-independent DU145 human prostate carcinoma cell lines were obtained from American Type Culture Collection (Bethesda, MD). Both the cell lines were thawed quickly in a waterbath at 37oC, and seeded in T100 cell culture flask in PRMI 1640 culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics (all cell culture materials were from Gibco BRL, Gaithersburg, MD). Cultures from both the cell lines were maintained under standard culture conditions at 37oC, 95% air and 5% CO_2, and 90-95% humidity, in the above described culture medium. The cultures grown and maintained under these conditions were employed in all the studies detailed later in this section.

Negative finding(s): Not applicable.
Methodological problems: We have had no methodological or any other problems in growing and maintaining the two cell lines.

6.1.b & c Assess the effect of silymarin on ligand binding to erbB1 and the internalization of the ligand in both cell lines: For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' (obtained from Sigma Chemical Co, Milwaukee, WI) was used in the studies.

Experiment 1: Experimental design and Method: **First**, we did a dose-dependent study to assess the effect of silibinin on ligand binding to erbB1 and ligand internalization in both cell lines. These studies were performed as described by Baulida et al (50) with desired modifications. LNCaP and DU145 cells were seeded at 0.12 million cells/well in 12-well dishes under standard culture conditions, and after 24 hrs, the cells were subjected to serum starvation. Briefly, the attached cells were quickly washed two times with phosphate buffered saline (PBS) and replaced with fresh medium without serum. This serum starvation was necessary to shutdown the constitutive activation of erbB1 and to make the receptor available for the ligand binding. After 34 hrs under these serum starvation conditions, the cultures were treated with dimethyl sulfoxide (DMSO) vehicle alone or varying concentrations (50, 75, 100 and 150 µg/ml) of silibinin in DMSO. The final concentration of DMSO in each treatment including control was 0.5% (v/v) of the medium. Two hrs after these treatments, cultures were incubated with ^{125}I-epidermal growth factor (EGF) [2 ng (0.28 µCi)/ml, specific activity 900 Ci/m mol obtained from Amersham Pharmacia Biotech] at 37°C for 6 min. At the end, medium was aspirated, and cultures were rapidly washed with ice-cold medium. The surface bound ^{125}I-EGF was removed by a rapid wash with 0.2 ml of glacial acetic acid (pH 2.8), added to 5 ml scintillation fluid and quantitated as a measure of surface bound ligand. The cells were then solubilized in 0.2 ml of 1 M NaOH, added to 5 ml scintillation fluid and counted to determine internalized ^{125}I-EGF (ligand). The nonspecificity of binding and internalization of ligand was determined by adding 400 ng unlabeled EGF 5 min prior to hot ligand.

Results: As shown by data in **Figure 1**, silibinin treatment of LNCaP (panel A) and DU145 (panel B) cells resulted in a highly significant inhibition (in a dose-dependent manner) of both ligand binding to erbB1 as well as internalization of the ligand. When the results were analyzed for LNCaP cells (**Figure 1A**), silibinin treatment at 50 µg/ml dose showed very little inhibitory effect towards ligand binding to erbB1, however higher doses of 75, 100 and 150 µg/ml silibinin resulted in a 30, 50 and 75% inhibition ($P < 0.001$, Student's t test), respectively. In terms of ligand internalization in LNCaP cells, these four doses of silibinin resulted in much stronger effect accounting for 55, 30, 83 and 95% inhibition ($P < 0.001$, Student's t test) at 50, 75, 100 and 150 µg/ml doses, respectively. In case of DU145 cells (**Figure 1B**), a reverse trend was observed towards the inhibitory effect of silibinin on ligand binding to erbB1 and its internalization. In this case, silibinin treatment at 50, 75, 100 and 150 µg/ml doses resulted in 20, 35, 50 and 64% inhibition ($P < 0.05$ to 0.001, Student's t test)in ligand binding to erbB1, but 2, 12, 22 and 27% inhibition ($P < 0.1$ to 0.001, Student's t test) in ligand internalization, respectively. An analysis of the results for nonspecific binding study using 200 fold excess of cold ligand showed that it was with 5% of specific binding for hot ligand (data not shown).
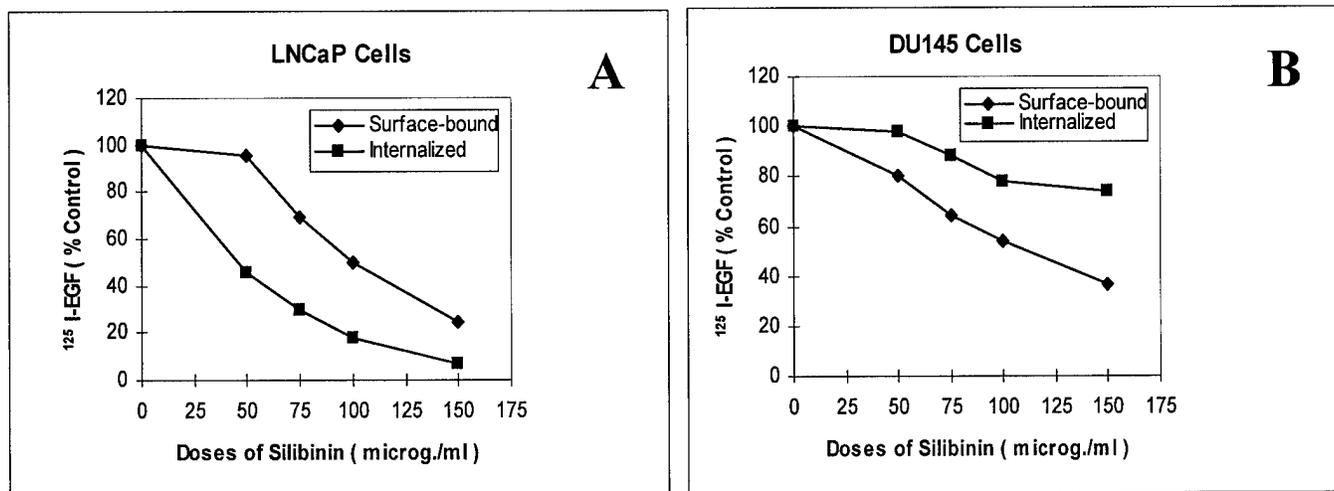

Figure 1: Dose-dependent inhibitory effect of silibinin on ligand binding to erbB1 and the internalization of the ligand in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In each case, the data shown are mean (with less than 5% error) of two independent experiment, each done in duplicate wells.

Experiment 2: Experimental design and Method: Based on the results from dose-dependent study detailed above, **second,** we did a time-response study to assess the effect of time of silibinin treatment on ligand binding to erbB1 and ligand internalization in both cell lines. The highest effective dose from **experiment 1,** 150 µg/ml silibinin was used in this set of experiment. LNCaP and DU145 cells were seeded at 0.12 million cells/well in 12-well dishes under standard culture conditions, and after 24 hrs, the cells were subjected to serum starvation as detailed above. After 34 hrs under these serum starvation conditions, the cultures were treated with DMSO vehicle alone or 150 µg/ml of silibinin in DMSO for 10 min (0.16 hr), 0.5, 8 or 16 hrs. After these treatments, cultures were incubated with ^{125}I-EGF (2 ng/ml) at 37°C for 6 min. At the end, medium was aspirated, and cultures were rapidly washed with ice-cold medium. The levels of surface bound ^{125}I-EGF and internalized ^{125}I-EGF (ligand) were then determined as described above.

Results: As shown by data in **Figure 2**, silibinin treatment, at 150 µg/ml dose, of LNCaP (panel A) and DU145 (panel B) cells resulted in a highly significant inhibition (in a time-dependent manner) of both ligand binding to erbB1 as well as internalization of the ligand. When the results were analyzed for LNCaP cells **(Figure 2A)**, silibinin treatment for 10 min showed little inhibitory effect (15%) towards ligand binding to erbB1, however higher treatment times resulted in a 39, 64 and 74% inhibition ($P < 0.001$, Student's t test), respectively. In terms of ligand internalization in LNCaP cells, all the time points examined showed stronger effect accounting for 32, 44, 77 and 88% inhibition ($P < 0.001$, Student's t test) after 10 min, 0.5, 8 and 16 hrs of 150 µg/ml dose of silibinin treatment, respectively. In case of DU145 cells **(Figure 2B)**, similar time-dependent inhibitory effect of silibinin on ligand binding to erbB1 and its internalization was observed. In this case, silibinin treatment at 150 µg/ml doses for 10 min, 0.5, 8 and 16 hrs resulted in 11, 50, 69 and 78% inhibition ($P < 0.05$ to 0.001, Student's t test) in ligand binding to erbB1, and 17, 34, 65 and 87% inhibition ($P < 0.05$ to 0.001, Student's t test) in ligand internalization, respectively.
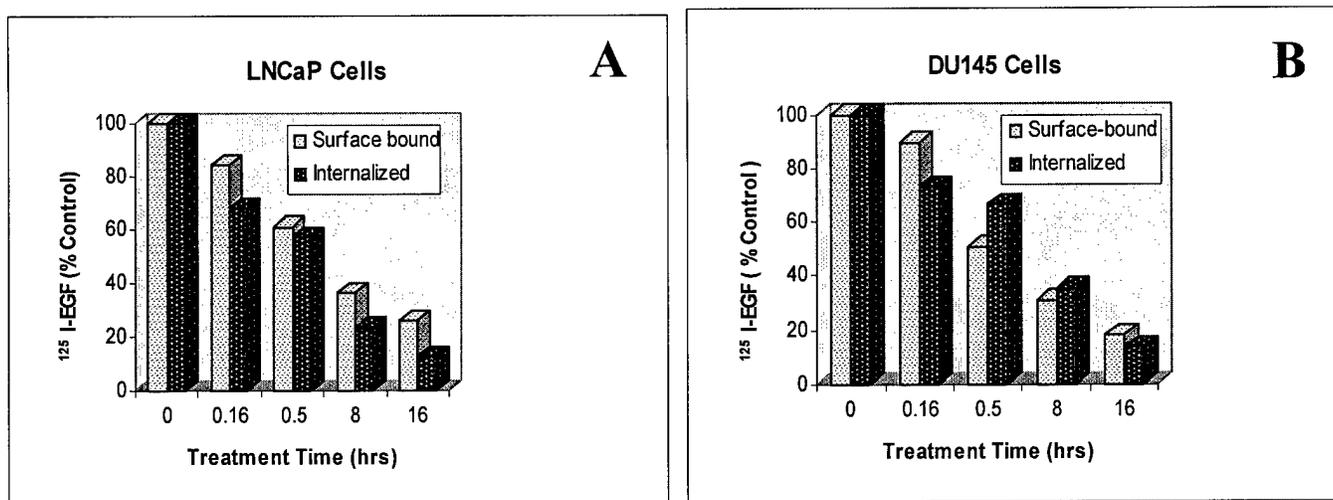

Figure 2: Time-dependent inhibitory effect of 150 µg/ml dose of silibinin on ligand binding to erbB1 and the internalization of the ligand in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In each case, the data shown are mean (with less than 5% error) of two independent experiment, each done in duplicate wells.

Taken together, the results shown in **Figures 1 and 2**, convincingly suggest that silibinin inhibits the binding of the ligand EGF to erbB1 receptor in both LNCaP and DU145 human prostate carcinoma cells that possibly also results in an inhibition of internalization of the ligand in these two prostate carcinoma cells. These findings also establish a cause for the observed inhibitory effect of silymarin (silibinin) on ligand (TGFa)-caused activation of erbB1 in DU145 cells reported recently by our group (51). It could be suggested that this effect of silymarin (silibinin) is directly due to its inhibitory effect on ligand binding to erbB1 and ligand internalization that leads to an inhibition of erbB1 activation.

<u>Negative finding(s):</u> In a dose-response study, the treatment of 34 hrs serum starved LNCaP and DU145 cells with 0.1, 1, 5, 10 and 25 µg/ml doses for 2 hrs followed by hot ligand treatment for 6 min, did not result in an inhibition of either ligand binding to erbB1 or ligand internalization (all these data are not shown). This experiment was done following same experimental strategy and methods as described above for **Experiment 1**.

<u>Methodological problems:</u> We have had no methodological or any other problems in performing the studies in these Tasks.

<u>6.1.d</u>   <u>*In vivo* and *in vitro* erbB1 intrinsic kinase activity assays evaluating the effect of silymarin in both cell lines:</u> For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies.

<u>Experimental design and Method:</u> We did a dose-dependent study to assess the *in vivo* effect of silibinin on erbB1 intrinsic kinase activity in both cell lines. These studies were performed as described recently by us (48) with desired modifications. LNCaP and DU145 cells were grown to 80% confluency in 100 mm dishes under the standard culture conditions detailed above, and treated with varying doses (10, 25, 50, 75, 100 and 150 µg/ml) of silibinin in DMSO or DMSO vehicle alone. Sixteen hrs after these treatments, medium was removed, cultures were washed with ice cold PBS, and cell lysates were prepared under native lysis conditions. Briefly, 0.5 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40 and 0.2 U/ml aprotonin) was added per plate. After 15 min in lysis buffer at 4°C, the cell lysate was scraped from the plate, collected in microcentrifuge tubes and left on ice for additional 15 min followed by centrifugation, and clear supernatant was collected as soluble cell lysate for the desired studies. Equal amount of protein (200 µg/sample lysate) was subjected to immunoprecipitation with anti-EGFR antibody (clone 528 from Neomarkers, Union City, CA) and protein A beads as detailed by us recently (48,51). The immunoprecipitated erbB1 embedded in protein A beads was suspended in 25 µl of kinase assay buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 10 mM $MnCl_2$ and 1 mM $MgCl_2$) containing 10 µCi of [^{32}P]-ATP (from Amersham). The reaction mixture was incubated at 4°C for 15 min, terminated by adding 5 µl of 6 x sample buffer followed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gel. The gel was dried followed by autoradiography.

<u>Results:</u> The cytoplasmic domain of erbB1 contains tyrosine kinase domain that is phosphorylated causing an activation of the receptor for further events (52). Recent studies from our laboratory have shown that treatment of A431 cells with silymarin results in a dose-dependent inhibition of intrinsic kinase activity of tyrosine in cytoplasmic domain of erbB1 suggesting this pathway as one of mechanisms of silymarin's effect on the inhibition of erbB1 activation (48). Interestingly, treatment of LNCaP and DU145 cells with different doses of silibinin for 16 hrs did not result in any inhibition of erbB1 intrinsic kinase activity in both the cell lines examined (**Figure 3**). In case of LNCaP cells (**Figure 3A**), compared to vehicle treated control, the lowest dose assessed (10 µg/ml silibinin) showed no change in receptor kinase activity. The higher doses examined (25-150 µg/ml), however, showed an increase in intrinsic kinase activity (**Figure 3A**). Unlike the results with LNCaP cells, in case of DU145 cells (**Figure 3B**), there was no considerable change in kinase activity following silibinin treatment at different doses. Together, these findings suggest that unlike A431 cells, in case of prostate carcinoma LNCaP and DU145 cells, the inhibitory effect of silibinin of erbB1 activation does not involve its effect on receptor tyrosine kinase activity inhibition.

<u>Negative findings:</u> As discussed above in the result section and shown in **Figure 3**, the data obtained were in contrast to our anticipation, and showed that silibinin does not inhibit erbB1 intrinsic kinase activity in either of LNCaP and DU145 cells examined, and in fact causes some increase in the activity at higher doses in LNCaP cells. A cause for such an increase in kinase activity by silibinin in LNCaP cells remains to be studied.

<u>Methodological problems:</u> We have had no methodological or any other problems in performing the studies in this Task. In fact the positive A431 cell lysate sample and negative sample without any cell lysate were useful in confirming the validity of the assay in these studies (**Figure 3**).
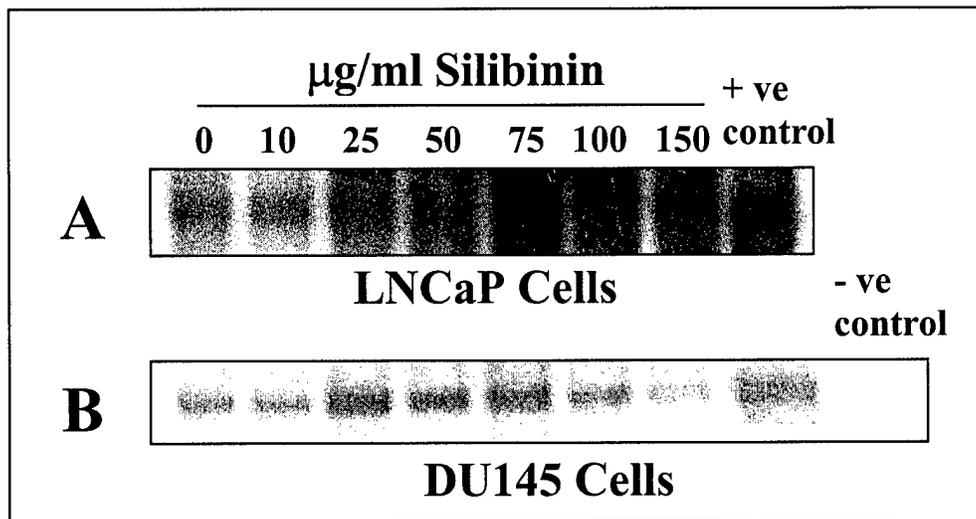
Figure 3: A lack of effect of silibinin on *in vivo* erbB1 intrinsic kinase activity in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In each case, the data shown are representative of three independent experiments with similar results. + ve control, cell lysate from A431 human epidermoid carcinoma cells that contain high erbB1 levels (48) was used in place of sample extract in the assay; - ve control, no cell lysate was used in the assay.

<u>6.1.e</u>   <u>erbB1 dimerization studies in intact cells and membrane preparation to assess the effect of silymarin:</u>
For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies.

<u>Experimental design and Method:</u> We did a dose-dependent study to assess the inhibitory effect of silibinin on erbB1 dimerization in both cell lines. These studies were performed as described by Cochet et al (53) with desired modifications. LNCaP and DU145 cells were grown in 35 mm dishes to 80% confluency and then serum starved for 36 hrs. Cells were then treated with DMSO vehicle alone or varying concentrations of silibinin (50, 75, 100 and 150 µg/ml) for 2 hrs followed by ligand stimulation (50 ng/ml of TGFα,obtained from Gibco BRL) for 10 min at 37°C. The cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC, obtained from Sigma Chem. Co.), was then added at 15 mM concentration for 15 min, and then cell lysates were prepared under non-denaturing conditions as described above. Equal protein (100 µg) from each cell lysate was subjected to SDS-PAGE on 4% gel followed by Western blotting. Membranes were probed with anti-EGFR and anti-phosphotyrosine antibodies followed by desired secondary antibodies and detection by ECL system as described recently by us (51).

<u>Results:</u> As shown by data in **Figure 4**, compared to vehicle treated controls, treatment of serum starved LNCaP and DU145 cells with different doses of silibinin followed by ligand stimulation resulted in a dose-dependent inhibition of ligand-caused activation of erbB1 in both the cell lines examined. As reported earlier that ligand-caused activation of erbB1 results in its dimerization leading to a new band at 340 kDa (53), we also observed the formation of this band in positive control samples from both the cell lines where serum starved cultures were treated with ligand for 10 min followed by EDAC for 15 min (**Figure 4, lane 3 in each panel**). Whether assessed in terms of erbB1 expression or tyrosine-phosphorylated erbB1, the formation of 340 kDa band following ligand stimulation was at the expense of a decrease in 170 kDa erbB1 which further confirms that 340 kDa band was dimerized erbB1 (**Figure 4, lane 3 versus lane 2 in each panel**). Consistent with its effect on inhibition of ligand-caused activation of erbB1, silibinin also showed a concentration-dependent inhibition of ligand-caused erbB1 dimerization at all the doses examined in both LNCaP and DU145 cells (**Figure 4**).

<u>Negative finding(s):</u> We did not get any negative results in this experiment. In fact, the data obtained were anticipated based on the findings from Tasks 'b' and 'c' showing that silibinin inhibits ligand binding to

erbB1 and ligand internalization. These effects of silibinin were anticipated to result in an inhibition of erbB1 activation followed by inhibition in erbB1 dimerization, which is a receptor activation-dependent phenomenon.

Methodological problems: We have had two major methodological problems in performing the studies in this Task. Firstly, treatment of both the cell lines during the last step with EDAC for erbB1 dimerization for 15 min turn out to be toxic for the cells. This problem is being handled by lowering the concentration of EDAC to 5 or 10 mM and reducing its treatment time to 5 or 10 min. In these scenarios, however, there was much reduced dimer formation. The second major problem encountered in this Task was separating a clear band at 340 kDa. As is evident from the data shown in **Figure 4**, it is almost impossible to get a clean crisp 340 kDa band from the samples where EDAC was used for receptor dimerization. A strong background (like protein trailing) was always evident in these samples. Further standardization of the method and technique is in progress to overcome these two problems.
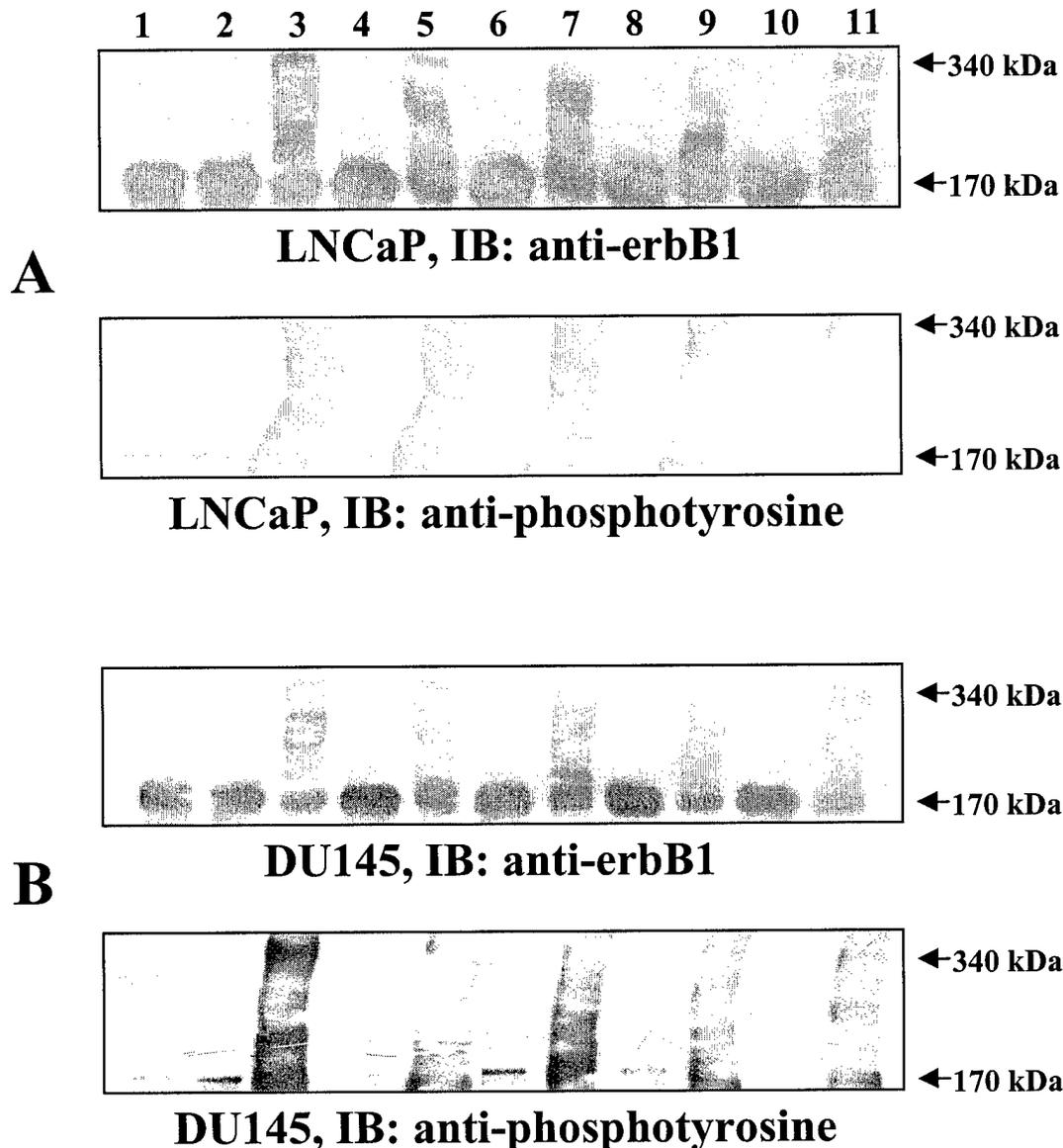

Figure 4: Inhibitory effect of silibinin on ligand-caused erbB1 dimerization in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In each case, the data shown are representative of three independent experiments with similar results. Lanes **1**, serum starved cells; **2**, serum starved cells treated with TGFα; **3**, same as lane 2 but with EDAC; **4, 6, 8 & 10**, same as lane 2 but treated with 50, 75, 100 & 150 µg/ml silibinin for two hrs prior to ligand stimulation; **5, 7, 9 & 11**, same as lanes 4, 6, 8 & 10 but with EDAC. IB, immunoblotting.

<u>6.1.f & g</u>   Evaluate the effect of silymarin on TGFα release and TGFα expression in both LNCaP and DU145 cells: For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies.

Experimental design and Method: We did both dose- and time-dependent study to assess the inhibitory effect of silibinin on TGFα release (the secreted form in the medium) and TGFα expression (cellular levels in the cells) in both cell lines. LNCaP and DU145 cells were grown to 60% confluency in 60 mm dishes under standard culture conditions detailed above. At this point, cultures were treated with DMSO vehicle alone or varying concentrations of silibinin (25, 50, 75 and 100 μg/ml) for 0, 6, 12, 24, 48 and 72 hrs. After these treatments, medium was collected from each dish and stored at –80oC till further assay. At the same time, cells were also collected, washed in ice cold PBS, and cellular extracts were prepared following step-by-step protocol provided by the manufacturer for TGFα ELISA assay kit (cat #QIA 61 from Oncogene Research Products, Cambridge, MA). Employing medium and cellular samples collected and prepared above, released (secreted) and cellular TGFα levels were determined using the ELISA kit and following the protocol provided with the kit.

Results: Several experiments are done in this Task, and a number of important findings were obtained from them. As shown by data in **Figure 5A**, in case of LNCaP cells, the cell growth alone in the absence of any silibinin treatment showed time-dependent increase in TGFα release in medium. Compared to a zero time point data, 6 hrs cell culture resulted in a 12-fold increase in ligand release which doubled by 24 hrs of culture; maximum TGFα release was observed following 48 hrs of LNCaP culture (**Figure 5A**). This observation was consistent no matter the data were analyzed in terms of amount of ligand/ml medium or per 10^6 cell. In the studies assessing the effect of silibinin on TGFα release in LNCaP cells, as shown by data in **Figure 5A**, it showed a highly significant inhibition in both dose- and time-dependent manner. Maximum effect of silibinin was evident following 48 hrs of its treatment and at the dose of 100 μg/ml (when the data are analyzed in terms of amount of ligand/10^6 cells) (**Figure 5A**). In terms of its effect towards ligand release/ml medium, silibinin showed much stronger inhibition even at lower doses and at other time points (**Figure 5A**).

Similar results were also observed in case of DU145 cells where culture growth alone over the period of time showed strong increase in TGFα release in the medium with optimum levels at 48 hrs followed by a small decline at 72 hrs (**Figure 5B**). When the effect of silibinin was assessed on ligand release in DU145 cells, compared to that observed in LNCaP cells, much stronger inhibitory effect was evident that was also dependent on silibinin dose and time of its treatment (**Figure 5B**). Once again, the maximum inhibition was evident at 100 μg/ml silibinin dose but was at 24 hrs of treatment (when the data are analyzed in terms of amount of ligand/10^6 cells) (**Figure 5B**). In terms of ligand release/ml of medium, even lower doses of silibinin showed strong inhibition (**Figure 5B**). <u>Taken together, the findings from both LNCaP and DU145 cells convincingly suggest that silibinin inhibits the release of TGFα, and that this inhibition is not due to a decrease in total number of cells in silibinin treated samples.</u>

Conversely to ligand release studies discussed above and shown in **Figure 5**, in case of cellular expression of TGFα, 6 to 72 hrs following initial culture, no significant change in its level was evident in either of the two prostate carcinoma cells examined in terms of both TGFα levels/μg cellular protein or /10^6 cells (**Figure 6**). A very strong inhibitory effect of silibinin, however, was observed on the cellular levels of TGFα in both LNCaP and DU145 cells (**Figure 6**). In case of LNCaP cells (**Figure 6A**), the inhibitory effect of silibinin was as high as 80% at both 75 and 100 μg/ml doses following 72 hrs of treatment; almost comparable inhibition was also evident at these doses after 48 hrs of treatment (**Figure 6A**). Even lower doses of silibinin showed strong inhibition following 24 hrs of treatment (**Figure 6A**). Similarly, silibinin also showed strong inhibitory effect on cellular TGFα expression in DU145 cells (**Figure 6B**). In this case, as high as 73% inhibition was observed at 50, 75 and 100 μg/ml silibinin doses after 72 hrs of treatment and 55-65% inhibition after 48 hrs of treatment at same doses (**Figure 6B**). Significant inhibitory effect of silibinin was also observed 24 hrs following its treatment at these doses and accounted for 37-41% inhibition (**Figure 6B**). <u>Taken together, these findings from both LNCaP and DU145 cells corroborate those obtained for secreted TGFα, and suggest that silibinin inhibits the cellular levels of TGFα that results in a decrease in TGFα release.</u>

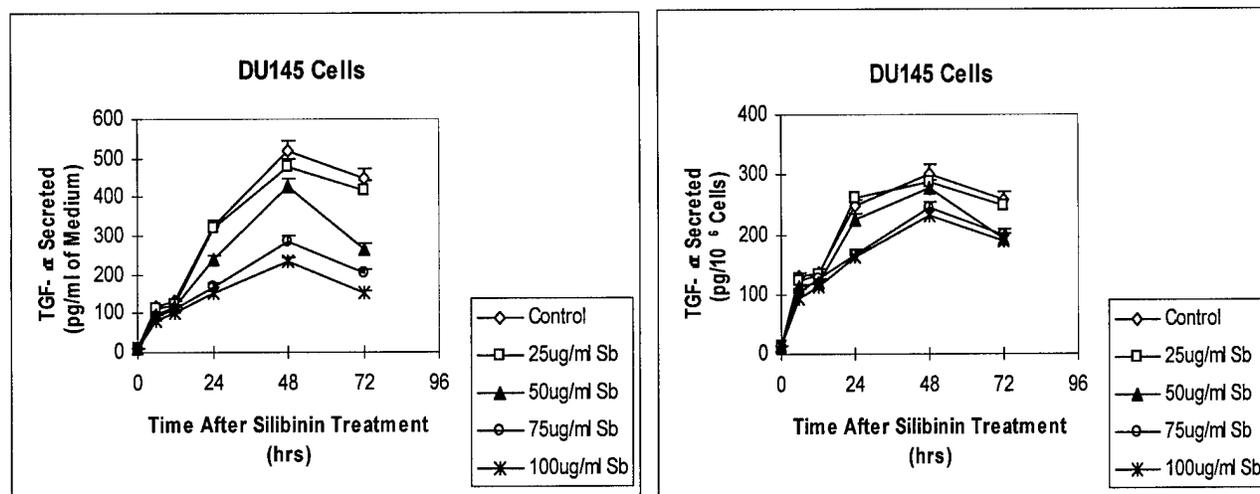Negative finding(s): We did not get any negative results in these studies.

A
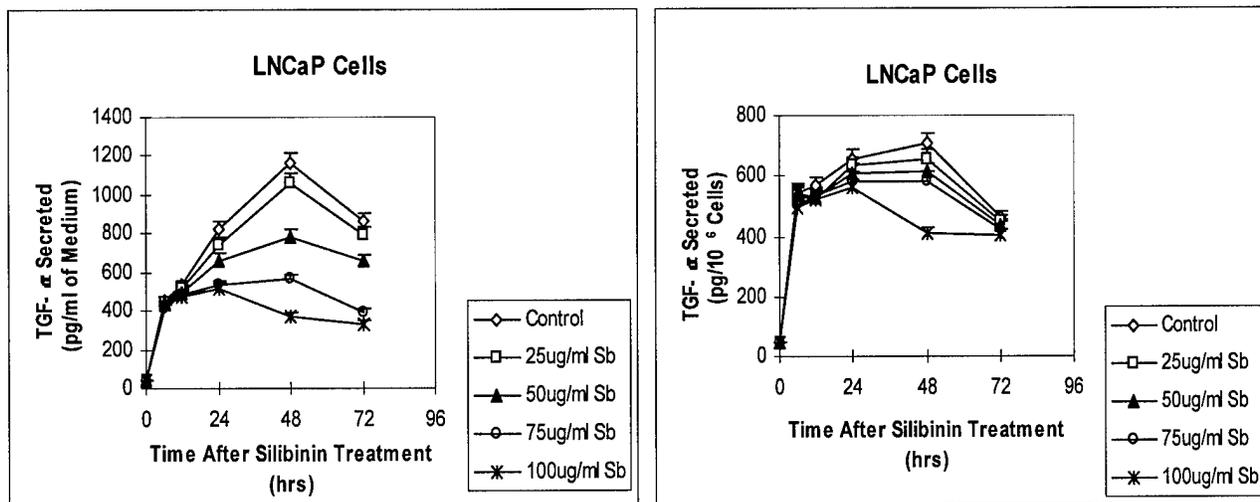
B

Figure 5: Dose- and time-dependent inhibitory effect of silibinin on TGFα release (in medium) in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In order to show the significance of the observed inhibitory effect of silibinin on TGFα release in both the cell lines, the data are calculated as the amount of TGFα (in pg) secreted in 1 ml of medium, and the amount of TGFα secreted by 10^6 cells following vehicle (control) treatment or different doses of silibinin treatment. The later calculation was important because it can be argued that since silibinin treatment inhibits the growth of cells, a decrease in the observed TGFα release is due to a decrease in total number of cells in silibinin treated cases. However, as can be seen from the data presented, after the correction for cell numbers, that is not the case. Each data point shown is the mean (± SE) of two independent experiment each done in triplicate.
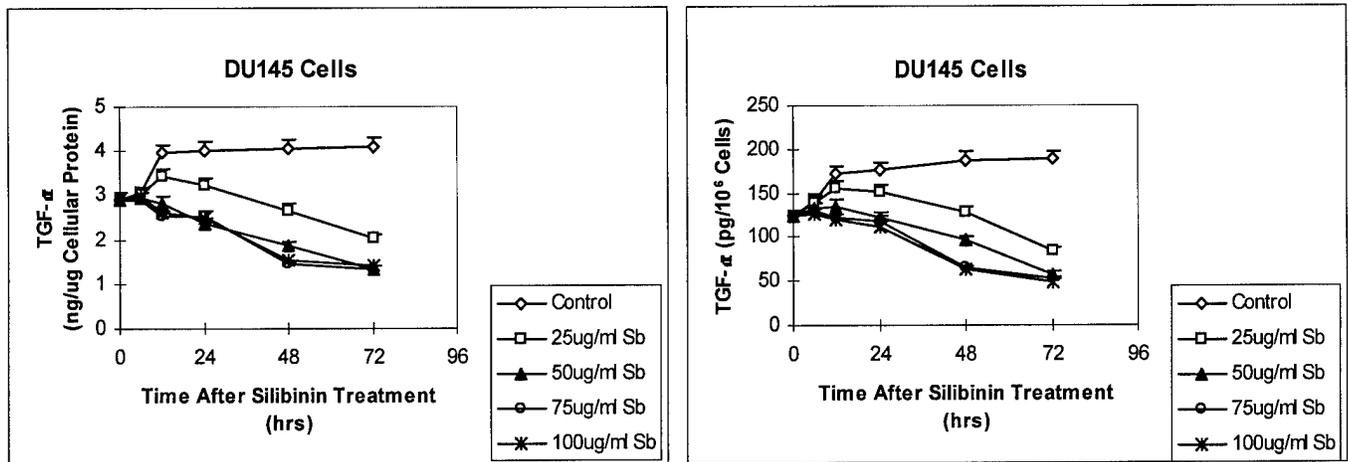
A
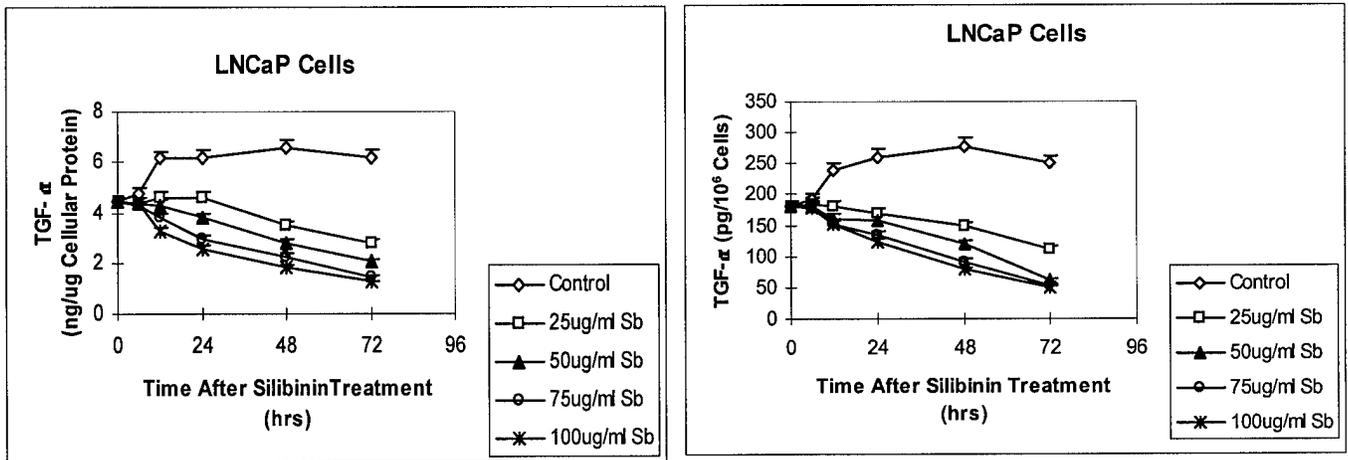
B

Figure 6: Dose- and time-dependent inhibitory effect of silibinin on cellular TGFα expression in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In order to show the significance of the observed inhibitory effect of silibinin on cellular TGFα expression in both the cell lines, the data are calculated as the amount of TGFα (in ng) per 1 μg of cellular protein, and the amount of TGFα per 10^6 cells following vehicle (control) treatment or different doses of silibinin treatment. Both the calculations lead to comparable pattern, and suggest that the observed inhibitory effect of silibinin on TGFα release be due to a highly significant inhibition of cellular TGFα expression following silibinin treatment. Each data point shown is the mean (± SE) of two independent experiment each done in triplicate.

6.2 **Task (Aim) II: To study the effect of silymarin on cytoplasmic signaling, Months 9-18:** Part of the studies proposed in this Task are completed, and described in detail below.

6.2.a & c **Assess the effect of silymarin on activation of erbB1 in LNCaP and DU145 cells:** For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies.

Experimental design and Method: Based on the findings shown in **Figure 1** that 150 μg/ml dose of silibinin results in a highly significant inhibition of ligand binding to erB1 as well as ligand internalization, in the present experiment, we used same dose of silibinin to assess its time-dependent inhibitory effect on erbB1 activation in both LNCaP and DU145 cells. LNCaP and DU145 cells were grown to 60% confluency in 100 mm dishes under standard culture conditions detailed above. At this point, cultures were treated with DMSO vehicle alone or 150 μg/ml dose of silibinin in DMSO. After 6, 12, 24, 48 and 72 hrs., medium was removed, cultures were washed two times with ice cold PBS, and cell lysates were prepared under non-denaturing conditions as described above in detail. Equal amount of protein (200 μg) from each cell lysate was diluted to 1 ml with lysis buffer and added with 2 μg of anti-EGFR (erbB1) antibody (from UPSTATE Biotechnology, Lake Placid, NY) followed by rotating this mixture at 4°C for 4 hrs. Thereafter, 25 μl of protein A agarose beads were added, and this mixture was incubated overnight at 4°C. The next day, beads were collected by centrifugation, washed four times with lysis buffer and the immunoprecipitated erbB1 was denatured with 30 μl of 1x SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE on 8% gel, and separated proteins were transferred on to nitrocellulose membrane by Western blotting. The membranes were probed with anti-phosphotyrosine and anti-EGFR antibodies (from UPSTATE Biotechnology, Lake Placid, NY) followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system.

Results: As expected, treatment of both LNCaP and DU145 cells with silibinin resulted in a highly significant inhibition (in a time-dependent manner) of constitutive erbB1 activation (**Figure 7**). In case of LNCaP cells, as shown in **Figure 7A**, compared to DMSO treated control, silibinin treatment at 150 μg/ml dose resulted in the inhibition of erbB1 activation as early as by 6 hrs. By 48 hrs most of activated erbB1 expression was inhibited, and by 72 hrs it was not detectable. The observed inhibitory effect of silibinin of erbB1 activation (tyrosine phosphorylation) was not due to a decrease in total erbB1 protein expression as evident by a no change in its content following silibinin treatment for the time points studied (**Figure 7A, lower panel**). In case of DU145 cells, much stronger inhibitory effect of silibinin at this dose was evident on erbB1 activation (**Figure 7B**). Whereas 6 hrs of silibinin treatment was not effective, by 12 and 24 hrs, most of activated erbB1 expression was inhibited, and by 48 hrs it was not detectable (**Figure 7B**). Once again, this effect of silibinin in DU145 cells was not due to a change in total erbB1 protein levels (**Figure 7B, lower panel**). Together, these results convincingly suggest that silibinin inhibits erbB1 activation in both LNCaP and DU145 human prostate carcinoma cells, and that this effect is due to: **a)** inhibition of ligand binding to erbB1 followed by an inhibition in ligand internalization, and **b)** inhibition in cellular expression of TGFα followed by a decrease in its release.

Negative finding(s): We did not get any negative results in these studies.

Methodological problems: We have had no problems in performing the studies in these Tasks.

Studies in progress in these Tasks:
- Dose-dependent effect of silibinin (after 24 hrs of treatment) on constitutive erbB1 activation in both LNCaP and DU145 cells.

- Both dose- and time-dependent effect of silibinin on ligand-caused activation of erbB1 in both LNCaP and DU145 cells.
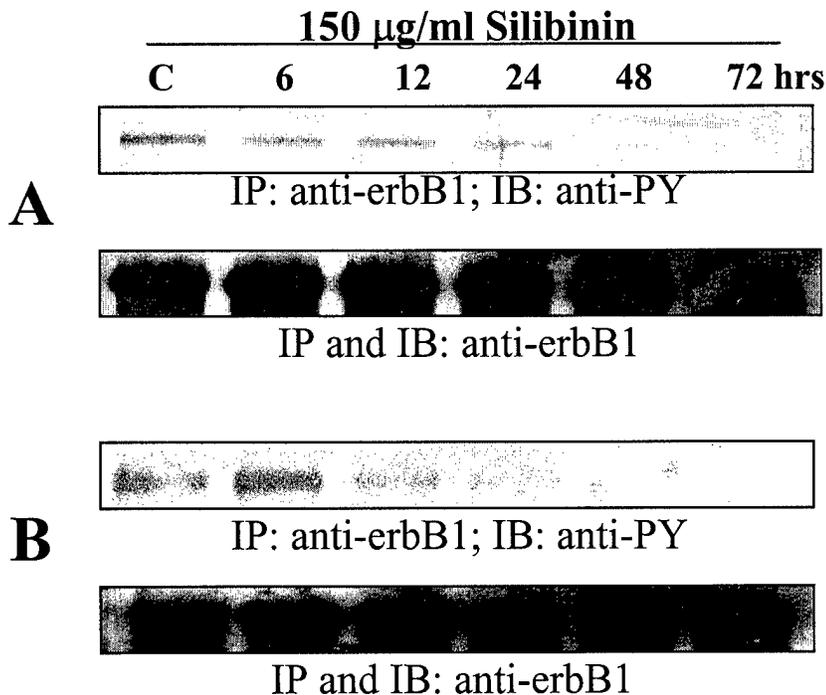
150 µg/ml Silibinin

| C | 6 | 12 | 24 | 48 | 72 hrs |

A

IP: anti-erbB1; IB: anti-PY

IP and IB: anti-erbB1

B

IP: anti-erbB1; IB: anti-PY

IP and IB: anti-erbB1

Figure 7: Time-dependent inhibitory effect of silibinin on constitutive erbB1 activation in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above, and the treatments are labeled as such in the figure shown; C, vehicle treated control at 36 hrs. In each case, the data shown are representative of three independent experiments with similar results. IP, immunoprecipitation; IB, immunoblotting.

6.2.b & d   Assess the effect of silymarin on MAPK activation in LNCaP and DU145 cells: For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies. The MAPK activation studied was ERK1/2.

Experimental design and Method: Based on the findings shown in **Figure 1** that 150 µg/ml dose of silibinin results in a highly significant inhibition of ligand binding to erB1 as well as ligand internalization, and **Figure 7** showing that this dose of silibinin significantly inhibits constitutive erbB1 activation in a time-dependent manner, in the present experiment, we used same dose of silibinin to assess its time-dependent inhibitory effect on MAP/ERK1/2 activation in both LNCaP and DU145 cells. LNCaP and DU145 cells were grown to 60% confluency in 100 mm dishes under standard culture conditions detailed above. At this point, cultures were treated with DMSO vehicle alone or 150 µg/ml dose of silibinin in DMSO. After 12, 24, 48 and 72 hrs., medium was removed, cultures were washed two times with ice cold PBS, and cell lysates were prepared under non-denaturing conditions as described above in detail. Equal amount of protein (80 µg) from each cell lysate was denatured with SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE on 12% gel, and separated proteins were transferred on to nitrocellulose membrane by Western blotting. The membranes were probed with anti-phospho MAPK/ERK1/2 and anti-MAPK/ERK1/2 antibodies (from New England Biolab Inc, Beverly, MA) followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system.

Results: Similar to our anticipation, treatment of both LNCaP and DU145 cells with silibinin resulted in a highly significant inhibition (in a time-dependent manner) of constitutive MAPK/ERK1/2 activation (**Figure 8**). In case of LNCaP cells, as shown in **Figure 8A**, compared to DMSO treated control, silibinin treatment at

150 µg/ml dose resulted in a strong inhibition of MAPK activation after 48 hrs and by 72 hrs it was not detectable. The observed inhibitory effect of silibinin on MAPK/ERK1/2 activation (tyrosine phosphorylation) was not due to a decrease in total MAPK/ERK1/2 protein expression as evident by a no change in its content following silibinin treatment for the time points studied (**Figure 8A, lower panel**). In case of DU145 cells, much stronger inhibitory effect of silibinin at this dose was evident on MAPK activation (**Figure 8B**). Whereas 12 and 24 hrs of silibinin treatment showed small inhibitory effect, by 48 hrs, most of activated MAPK/ERK1/2 expression was inhibited, and by 72 hrs it was almost not detectable (**Figure 8B**). This effect of silibinin in DU145 cells was not due to a change in total MAPK/ERK1/2 protein levels (**Figure 8B, lower panel**). Together, these results convincingly suggest that silibinin inhibits erbB1 activation in both LNCaP and DU145 human prostate carcinoma cells, and that this effect leads to a significant inhibition of mitogenic signaling mediated downstream by MAPK/ERK1/2. The biological significance of these findings is discussed later in **section 9 – conclusion under so what section**.

Negative finding(s): We did not get any negative results in these studies.

Methodological problems: We have had no problems in performing the studies in these Tasks.

Studies in progress in these Tasks: At this point in our annual progress report, we would like to highlight that the other studies proposed in these tasks are in progress, and will be completed by 18 months of initial funding as proposed in the approved Statement of Work. At that point, we will start the studies proposed to be completed in 15 months to 24 months of the funding. We would also like to mention here that in addition to the studies proposed during this funding period, we have already done part of the work from Task (Aim) III (to be done during Months 15-24) that has already resulted in an outstanding publication (54). Since, this work was not part of the first 12 months of Task, it will be discussed in detail and reported in next annual report.

Figure 8: Time-dependent inhibitory effect of silibinin on constitutive MAPK/ERK1/2 activation in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above, and the treatments are labeled as such in the figure shown; C, vehicle treated control at 36 hrs. In each case, the data shown are representative of three independent experiments with similar results. IP, immunoprecipitation; IB, immunoblotting.

7.   KEY RESEARCH ACCOMPLISHMENTS

There were several key research accomplishments during the current (annual) progress of this grant, which include both academic and professional achievements. The **academic accomplishments** are summarized below.

- Treatment of LNCaP and DU145 human prostate carcinoma cells with silymarin results in a highly significant inhibition of TGFα binding to erbB1 receptor in both dose- and time-dependent manner.
- Consistent with above finding, silymarin also showed a strong inhibition of ligand (TGFα) internalization in these two cell lines.
- Conversely, silymarin does not result in the inhibition of intrinsic tyrosine kinase activity of erbB1 in both LNCaP and DU145 cells.
- The observed inhibitory effect of silymarin on ligand binding to erbB1 and ligand internalization also resulted in an inhibition of erbB1 activation followed by its dimerization that leads to activation of downstream mitogenic signaling.
- These inhibitory effects of silymarin on LNCaP and DU145 cells also corroborate with its inhibitory effect on both cellular and released expression of TGFα in these two cell lines.
- Together these effects of silymarin resulted in a strong inhibition of constitutive MAPK/ERK1/2 activation in both LNCaP and DU145 cells.

In terms of **professional accomplishments**, based on the results from these studies published in two respected journals (51,54), the P.I. has been invited to present these findings at three separate meetings, two international, and one national. The P.I. was also able to develop his academic career in prostate cancer research that is another major accomplishment. Based on the knowledge gained by him in the area of receptor mitogenic signaling in prostate cancer, he has received a fundable priority score for a RO1 grant funding from NCI, NIH starting December 1, 1999 in the area of receptor mitogenic and anti-apoptotic signaling in prostate cancer and their impairment by phytochemicals. These accomplishments are further highlighted (in a bullet format) in the next section.

8.   REPORTABLE OUTCOMES

Manuscript:

1.   Zi, X. and **Agarwal, R.**: Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: Implications for prostate cancer intervention. Proc. Natl. Acad. Sci. USA, 96:7490-7495, 1999. (**Appendix 1**).

2.   Zi, X. and **Agarwal, R.**: Impairment of erbB1 receptor-mediated and fluid-phase endocytosis, and associated mitogenic signaling by inositol hexaphosphate in human prostate carcinoma DU145 cells: a novel approach for the intervention of prostate cancer. Submitted. (**Appendix 2**).

3.   Bhatia, N. and **Agarwal, R.**: Detrimental effect of polyphenolic antioxidants on membrane receptor, cytoplasmic and nuclear signaling in human prostate carcinoma DU145 cells: a comparison of silymarin and genistein. In Preparation.

4.   Sharma, Y, and **Agarwal, R.**: A flavonoid antioxidant silibinin inhibits constitutive activation of erbB1 and MAPK by a strong inhibitory effect on ligand binding to erbB1 and both cellular and released expression of ligand in human prostate carcinoma cells. In Preparation.

Abstracts and Presentations

5.   **Agarwal, R.**: Prostate Cancer Prevention by an antioxidant silymarin. **Invited Talk** at International Conference on Diet and Prevention of Cancer, Tampere, Finland, June, 1999. (**Appendix 3**).

6.   **Agarwal, R.**: Mitogenic and anti-apoptotic signaling as molecular targets for prostate cancer prevention by an antioxidant silymarin. **Invited Talk** to be delivered at Endocrinology Seminar at University of Colorado Cancer Center, December, 1999.

7.   **Agarwal, R.**: Cell signaling, regulators of cell cycle and apoptosis as molecular targets for prostate cancer intervention by dietary agents. **Invited Talk** to be delivered at Third Molecular and Cellular Biology Meeting, Luxembourg, January 26-29, 2000.

8.   Sharma, Y, and **Agarwal, R.**: A flavonoid antioxidant silibinin inhibits TGFa expression and it's binding to erbB1 resulting in impairment of ligand/receptor autocrine growth loop in human prostate carcinoma cells. Abstract submitted for presentation in Annual Meeting of the AACR, 2000. (**Appendix 4**).

9.   CONCLUSIONS

9.1 Summary of results from completed studies including their importance and/or implications: The findings obtained thus far from the studies detailed above in section 6, clearly and convincingly suggest that silymarin (or silibinin) has exceptionally strong inhibitory effect on erbB1-mediated mitogenic signaling in case of both androgen-dependent and androgen-independent human prostate carcinoma cells. These results alone are major accomplishments towards the notion that more detailed mechanistic and tumor studies are needed to assess both preventive and interventive effects of silymarin (or silibinin) against human prostate cancer.

The **major implication** of these findings is that this effect of silymarin could be exploited to inhibit the growth of those prostate carcinomas where an interaction of autocrine/paracrine growth factors and their receptors plays a causal role in malignant cell growth and metastasis. As elaborated earlier that advanced and androgen-independent prostate cancer growth causally depends on such interactions where an autocrine loop exists between ligand and receptor, our findings showing an inhibitory effect of silymarin on these molecular events are extremely important in developing interventive strategies against prostate cancer by silymarin.

9.2 "So what section": From the findings detailed earlier and summarized above, it can be argued that silymarin exerts its inhibitory effect on prostate carcinoma cells by different mechanisms that are possibly linked to each other by a cause and effect relationship. For example, it can be argued that as an initial step, silymarin inhibits the binding of the ligand to erbB1 that results in an inhibition of ligand internalization. Since these steps are essential for erbB1 activation, their inhibition results in an inhibition of erbB1 activation followed by a lack of its dimerization that ultimately causes a decrease in the activation of MAPK/ERK1/2. As activation of MAPK/ERK leads to activation of transcription factor for cell growth and proliferation, its inhibition would be anticipated to lead in an inhibition of trascriptional followed by translational effects including a decrease in TGFα expression followed by its release, as observed by us in silymarin treated cells. An important question from these arguments is **so what** if silymarin inhibits these molecular events in prostate carcinoma cells, and **so what** if these studies establish a cause and effect relationship. What is the biological significance of these findings in terms of prostate carcinoma growth? **This issue was identified to be extremely important to establish the implications of the findings observed thus far**. A two-tier approach was made to answer this issue where we planned to conduct prostate carcinoma cell growth (and death) studies employing both *in vitro* and *in vivo* (nude mice tumor xenograft) systems. Whereas nude mice tumor xenograft studies will be performed in year 02 of the funding (as proposed in the approved Statement of Work), the cell growth studies (in culture) were performed during current funding period (though not part of the approved Statement of Work).

As shown by data in **Figure 9**, treatment of LNCaP and DU145 human prostate carcinoma cells with silymarin (in its pure form, silibinin) resulted in a highly significant inhibition of cell growth in both dose- and time-dependent manner. Whereas low doses of silibinin (10 and 25 μg/ml) were not effective at all the time points studied, the doses higher than these (50, 75 and 100 μg/ml silibinin) showed strong inhibitory effect on cell growth in both the cell lines (**Figure 9**). The treatment of these cells with higher doses of silibinin for longer treatment period also resulted in a moderate cell death (**Figure 9**).

The observed dose- and time-dependent inhibitory effect of silibinin on cell growth strongly corroborated with its dose- and time-dependent inhibitory effect on the molecular events detailed above suggesting that the two are causally related in inhibiting prostate cancer growth by silymarin.
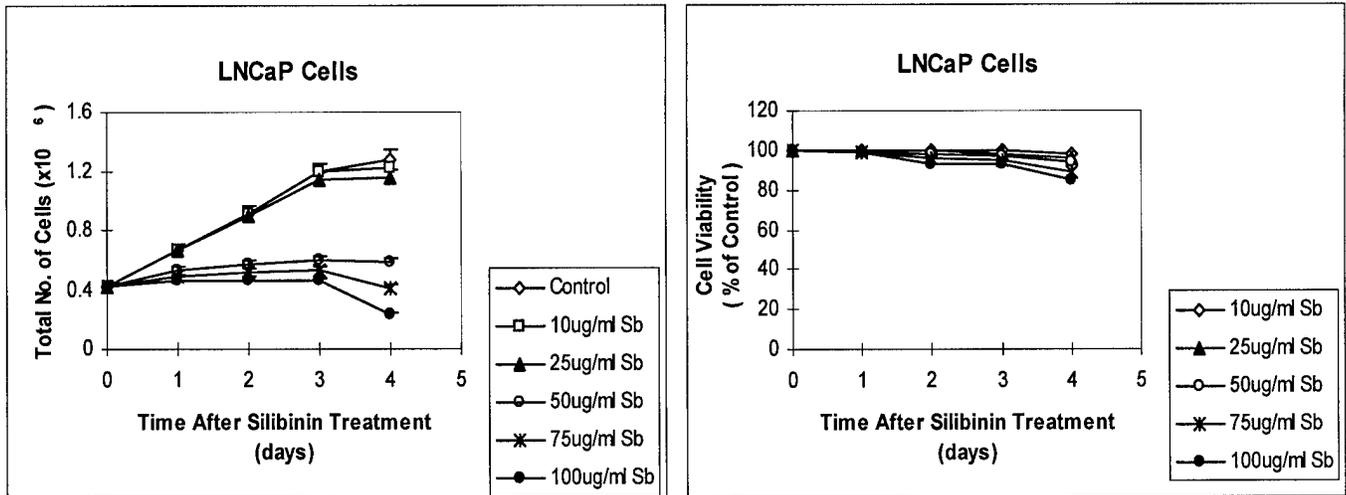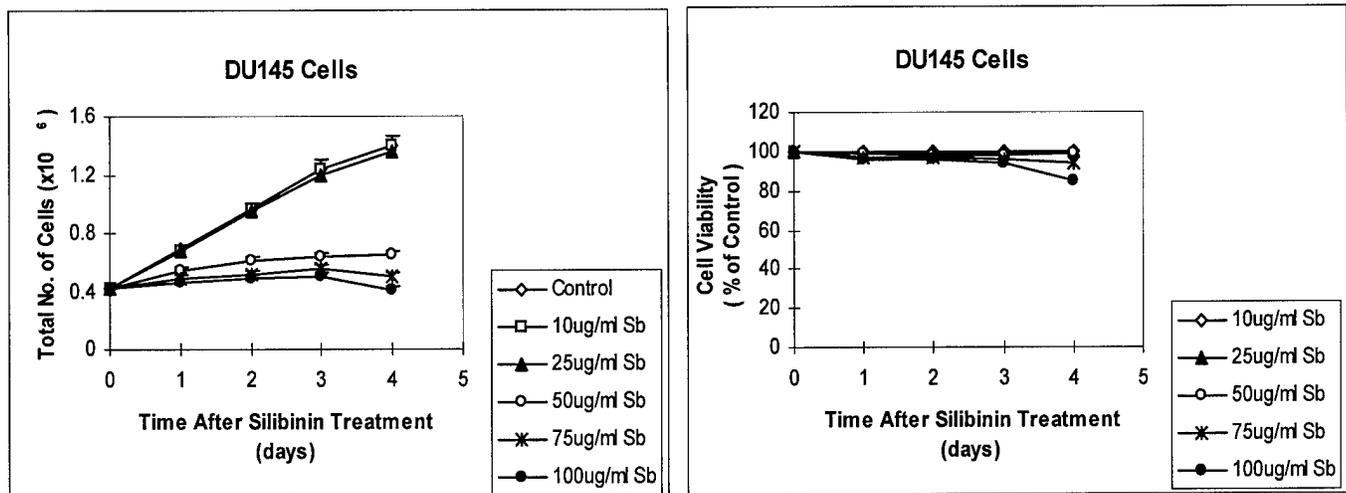
A

B

Figure 9: Inhibitory effect of silibinin on the growth of LNCaP and DU145 human prostate carcinoma cells in culture. LNCaP and DU145 cells were seeded at 4 x 105 cells/35 mm dish under standard culture conditions in RPMI 1640 medium with 10% serum and 1% P-S. After 24 hrs, the medium was removed and cultures were treated with either DMSO alone (control) or varying doses of silibinin in DMSO. The medium was changed alternate days with desired amount of fresh silibinin upto the end of the study. At 1, 2, 3 and 4 days after these treatments, cells were trypsinized and both live and dead (by trypan blue staining) cells were counted. Each data point shown is a mean ± SE of triplicate plates, the experiment was done two times.

10. REFERENCES

1. Parker SL, Tong T, Bolden S, Wingo PA: Cancer Statistics. CA Cancer J Clin 47:5-27, 1997.
2. Godley PA, Campbell MK, Gallagher P, Martinson FEA, Mohler JL, Sandler RS: Biomarkers of essential fatty acid consumption and risk of prostate carcinoma. Cancer Epidemiol Biomarkers Prevention 5:889-895, 1996.
3. Hsing AW: Essential fatty acids and prostate cancer: an emerging hypothesis? Cancer Epidemiol Biomarkers Prevention 5:859-860, 1996.
4. Ross RK, Henderson BE: Do diet and androgens alter prostate cancer risk via a common etiologic pathway? J Natl Cancer Inst 86:252-254, 1994.
5. Giovannucci E, Rimm EB, Colditz GA, Stampfer MJ, Ascherio A, Chute CC, Willett WC: A prospective study of dietary fat and risk of prostate cancer. J Natl Cancer Inst 85:1571-1579, 1993.
6. Gann PH, Hennekens CH, Sacks FM, Grodstein F, Giovannucci EL, Stampfer MJ: Prospective study of plasma fatty acids and risk of prostate cancer. J Natl Cancer Inst 86:281-286, 1994.
7. Wynder EL, Rose DP, Cohen LA: Nutrition and prostate cancer: a proposal for dietary intervention. Nutrition and Cancer 22:1-10, 1994.
8. Fournier DB, Erdman Jr JW, Gordon GB: Soy, its components, and cancer prevention: a review of the in vitro, animal, and human data. Cancer Epidemiol Biomarkers Prevention 7:1055-1065, 1998.
9. Aquilina JW, Lipsky JJ, Bostwick DG: Androgen deprivation as a strategy for prostate cancer chemoprevention. J Natl Cancer Inst 89:689-696, 1997.
10. Umekita Y, Hiipakka RA, Kokontis JM, Liao S: Human prostate tumor growth in athymic mice: inhibition by androgens and stimulation by finasteride. Proc Natl Acad Sic USA 93:11802-11807, 1996.
11. Karp JE, Chiarodo A, Brawley O, Kelloff GJ: Prostate cancer prevention: Investigational approaches and opportunities. Cancer Res 56:5547-5556, 1996.
12. Bostwick DG: c-erbB-2 oncogene expression in prostatic intraepithelial neoplasia: mounting evidence for a precursor role. J Natl Cancer Inst 86:1108-1110, 1994.
13. Myers RB, Srivastava S, Oelschlager DK, Grizzle WE: Expression of p160erbB3 and p185erbB2 in prostatic intraepithelial neoplasia and prostatic adenocarcinoma. J Natl Cancer Inst 86:1140-1145, 1994.
14. Pretlow TG, Pelley RJ, Pretlow TP: Biochemistry of prostatic carcinoma. In Biochemical and Molecular Aspects of Selected Cancers. Pretlow TG, Pretlow TP (eds). Acad Press, San Diego pp 169-237, 1994.
15. Bostwick DG, Aquilina JW: Prostatic intraepithelial neoplasia (PIN) and other prostatic lesions as risk factors and surrogate endpoints for cancer chemoprevtion trials. J Cell Biochem, 25S:156-164, 1996.
16. Tilley WD, Wilson CM, Marcelli M, McPhaul MJ: Androgen receptor gene expression in human prostate carcinoma cell lines. Cancer Res 50: 5382-5386, 1990.
17. Hofer DR, Sherwood ER, Bromberg WD, Mendelsohn J, Lee C, Kozlowski JM: Antonomous growth of androgen-independent human prostatic carcinoma cells: role of transforming growth factor alpha. Cancer Res 51:2780-2785, 1991.
18. Fong CJ, Sherwood ER, Mendelsohn J, Lee C, Kozlowski JM: Epidermal growth factor receptor monoclonal antibody inhibits constitutive receptor phosphorylation, reduces autonomous growth, and sensitizes androgen-independent prostatic carcinoma cells to tumor necrosis factor alpha. Cancer Res 52:5887-5892, 1992.
19. Peng D, Fan Z, Lu Y, DeBlasio T, Scher H, Mendelsohn J: Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates p27KIP1 and induces G1 arrest in prostatic carcinoma cell line DU145. Carcen Res 56:3666-3669, 1996.
20. Scher H, Sarkis A, Reuter V, Cohen D, Netto G, Petrylak D, Lianes P, Fuks Z, Mendelsohn J, Cordon-Cardo C: Changing pattern of expression of the epidermal growth factor receptor and transforming growth factor alpha in the progression of prostatic neoplasms. Clin Cancer Res 1:545-550, 1995.
21. Singh DK, Lippman SM: Cancer chemoprevention Part 1: retinoids and carotenoids and other classic antioxidants. Oncology, 12:1643-1659, 1998.
22. Singh, D.K. and Lippman, S.M. Cancer chemoprevention Part 2: hormones, nonclassic antioxidant natural agents, NSAIDs, and other agents. Oncology, 12:1787-1800, 1998.
23. Kelloff, G. J., Boone, C. W., Crowell, J. A., Steele, V. E., Lubet, R., and Sigman C. C. Chemopreventive drug development: prospectives and progress. Cancer Epidemiol. Biomarkers Preven., 3: 85-98, 1994.

24. Kelloff, G. J., Boone, C. W., Crowell, J. A., Nayfield, S. G., Hawk, E., Malone, W. F., Steele, V. E., Lubet, R. A., and Sigman, C. C. Risk biomarkers and current strategies for cancer chemoprevention. J. Cellular Biochem., 25S: 1-14, 1996.
25. Birt, D. F., Pelling, J. C., Nair, S., and Lepley, D. Diet intervention for modifying cancer risk. Prog. Clin. Biol. Res., 395: 223-234, 1996.
26. Morse, M. A., and Stoner, G.D. Cancer chemoprevention: principles and prospects. Carcinogenesis, 14: 1737-1746, 1993.
27. Lipkin, M. New rodent models for studies of chemopreventive agents. J. Cell. Biochem. Suppl., 28-29: 144-147, 1997.
28. Conney, A. H., Lou, Y. R., Xie, J. G., Osawa, T., Newmark, H. L., Liu, Y., Chang, R. L., and Huang, M. T. Some perspectives on dietary inhibition of carcinogenesis: studies with curcumin and tea. Proc. Soc. Exp. Biol. Med., 216: 234-245, 1997.
29. Goodman, G. E. The clinical evaluation of cancer prevention agents. Proc. Soc. Exp. Biol. Med., 216: 253-259, 1997.
30. Boone, C. W., Bacus, J. W., Bacus, J. V., Steele, V. E., and Kelloff, G. J. Properties of intraepithelial neoplasia relevant to the development of cancer chemopreventive agents. J. Cell. Biochem. Suppl., 28-29: 1-20, 1997.
31. Hong, W. K., and Sporn, M. B. Recent advances in chemoprevention of cancer. Science, 278: 1073-1077, 1997.
32. Perchellet, J.-P., and Perchellet, E.M. Antioxidants and multistage carcinogenesis in mouse skin. Free Radical Biol. Med., 7: 377-408, 1989.
33. Dragsted, L. O. Natural antioxidants in chemoprevention. Arch. Toxicol. Suppl., 20: 209-226, 1998.
34. Mukhtar, H., and Agarwal, R. Skin cancer chemoprevention. J. Invest. Dermatol. Sym. Proc., 1: 209-214, 1996.
35. Wagner, H., Seligmann, O., Horhammer, L. and Munster, R. The chemistry of silymarin (silybin), the active principle of the fruits of Silybum Marianum (L) Gaertn. (Carduus marianus) (L). Arzneimittelforsch 18:688-696, 1968.
36. Wagner, V.H., Diesel, P. and Seitz, M. Chemistry and analysis of silymarin from Silybum marianum Gaertn. Arzneimittelforsch 24:466-471, 1974.
37. Mereish, K. A., Bunner, D. L., Ragland, D. R., and Creasia, D. A. Protection against microcystin-LR-induced hepatotoxicity by silymarin: biochemistry, histopathology, and lethality. Pharm. Res., 8: 273-277, 1991.
38. Letteron, P., Labbe, G., Degott, C., Berson, A., Fromenty, B., Delaforge, M., Larrey, D., and Pessayre, D. Mechanism for the protective effects of silymarin against carbon tetrachloride-induced lipid peroxidation and hepatotoxicity in mice. Biochem. Pharmacol., 39: 2027-2034, 1990.
39. Ferenci, P., Dragosics, B., Dittrich, H., Frank, H., Benda, L., Lochs, H., Meryn, S., Base, W., and Schneider, B. Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver. J. Hepatol., 9: 105-113, 1989.
40. Luper, S. A review of plants used in the treatment of liver disease: part 1. Altern. Med. Rev. 3: 410-421, 1998.
41. Mourelle, M., Muriel, P., Favari, L., and Franco, T. Prevention of CCl_4-induced liver cirrhosis by silymarin. Fundam. Clin. Pharmacol., 3: 183-191, 1989.
42. Vogel, G., Trost, W., and Braatz, R. Studies on the pharmacodynamics, including site and mode of action, of silymarin: The antihepatotoxic principle from Silybum mar. (L) Gaertn. Arzneimittelforsch, 25: 82-89, 1975.
43. Hahn, V. G., Lehmann, H. D., Kurten, M., Uebel, H., and Vogel, G. Pharmacology and toxicology of silymarin, the anti-hepatotoxic agent of Silybum marianum (L.) Gaertn. Arzneimittelforsch, 18: 698-704, 1968
44. Ely, H. Dermatologic therapies you've probably never heard of. Derm. Clinics, 7: 19-35, 1989.
45. Katiyar, S. K., Korman, N. J., Mukhtar, H., and Agarwal, R. Protective effects of silymarin against photocarcinogenesis in mouse skin model. J. Natl. Cancer Inst., 89: 556-566, 1997.
46. Zi, X., Mukhtar, H., and Agarwal, R. Novel cancer chemopreventive effects of a flavonoid antioxidant silymarin: Inhibition of mRNA expression of an endogenous tumor promoter TNFa. Biochem. Biophys. Res. Commun., 239: 334-339, 1997.

47. Lahiri-Chatterjee, M., Katiyar, S.K., Mohan, R.R. and Agarwal, R.: A flavonoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in SENCAR mouse skin tumorigenesis model. Cancer Res., 59:622-632, 1999.
48. Ahmad, N., Gali, H., Javed, S. and Agarwal, R.: Skin cancer chemopreventive effects of a flavonoid antioxidant silymarin are mediated via impairment of receptor tyrosine kinase signaling and perturbation in cell cycle progression. Biochem. Biophys. Res. Commun., 247: 294-301, 1998.
49. Zi, X. and Agarwal, R.: Modulation of mitogen-activated protein kinase activation and cell cycle regulators by the potent skin cancer preventive agent silymarin. Biochem. Biophys. Res. Commun., 263: 528-536, 1999.
50. Baulida J, Kraus MH, Alimandi M, Di Fiore PP, Carpenter G: All erbB receptors other than epidermal growth factor receptor are endocytosis impaired. J Biol Chem 271:5251-5257, 1996.
51. Zi, X., Grasso, A.W., Kung, H.-J. and Agarwal, R.: A flavonoid antioxidant silymarin inhibits activation of erbB1 signaling, and induces cyclin-dependent kinase inhibitors, G1 arrest and anti-carcinogenic effects in human prostate carcinoma DU145 cells. Cancer Res., 58: 1920-1929, 1998.
52. Goldman R, Levy RB, Peles E, Yarden Y: Heterodimerization of the erbB1 and erbB2 receptors in human breast carcinoma cells: A mechanism for receptor transregulation. Biochemistry 29: 11024-11028, 1990.
53. Cochet C, Kashles O, Chambaz EM, Borrello I, King CR, Schlessinger J: Demonstration of epidermal growth factor-induced receptor dimerization in living cells using a chemical covalent cross-linking agent. J Biol Chem 263: 3290-3295, 1988.
54. Zi, X. and Agarwal, R.: Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: Implications for prostate cancer intervention. Proc. Natl. Acad. Sci. USA, 96:7490-7495, 1999.

11. APPENDICES

1. Zi, X. and **Agarwal, R.**: Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: Implications for prostate cancer intervention. Proc. Natl. Acad. Sci. USA, 96:7490-7495, 1999. (**Appendix 1**).

2. Zi, X. and **Agarwal, R.**: Impairment of erbB1 receptor-mediated and fluid-phase endocytosis, and associated mitogenic signaling by inositol hexaphosphate in human prostate carcinoma DU145 cells: a novel approach for the intervention of prostate cancer. Submitted. (**Appendix 2**).

3. **Agarwal, R.**: Prostate Cancer Prevention by an antioxidant silymarin. **Invited Talk** at International Conference on Diet and Prevention of Cancer, Tampere, Finland, June, 1999. (**Appendix 3**).

4. Sharma, Y, and **Agarwal, R.**: A flavonoid antioxidant silibinin inhibits TGFa expression and it's binding to erbB1 resulting in impairment of ligand/receptor autocrine growth loop in human prostate carcinoma cells. Abstract submitted for presentation in Annual Meeting of the AACR, 2000. (**Appendix 4**).

Appendix 1

Silibinin decreases prostate-specific antigen with cell growth inhibition via G_1 arrest, leading to differentiation of prostate carcinoma cells: Implications for prostate cancer intervention

XIAOLIN ZI* AND RAJESH AGARWAL*†‡

*Center for Cancer Causation and Prevention, AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214; and †University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, CO 80262

ABSTRACT   Reduction in serum prostate-specific antigen (PSA) levels has been proposed as an endpoint biomarker for hormone-refractory human prostate cancer intervention. We examined whether a flavonoid antioxidant silibinin (an active constituent of milk thistle) decreases PSA levels in hormone-refractory human prostate carcinoma LNCaP cells and whether this effect has biological relevance. Silibinin treatment of cells grown in serum resulted in a significant decrease in both intracellular and secreted forms of PSA concomitant with a highly significant to complete inhibition of cell growth via a G_1 arrest in cell cycle progression. Treatment of cells grown in charcoal-stripped serum and 5α-dihydrotestosterone showed that the observed effects of silibinin are those involving androgen-stimulated PSA expression and cell growth. Silibinin-induced G_1 arrest was associated with a marked decrease in the kinase activity of cyclin-dependent kinases (CDKs) and associated cyclins because of a highly significant decrease in cyclin D1, CDK4, and CDK6 levels and an induction of Cip1/p21 and Kip1/p27 followed by their increased binding with CDK2. Silibinin treatment of cells did not result in apoptosis and changes in p53 and bcl2, suggesting that the observed increase in Cip1/p21 is a p53-independent effect that does not lead to an apoptotic cell death pathway. Conversely, silibinin treatment resulted in a significant neuroendocrine differentiation of LNCaP cells as an alternative pathway after Cip1/p21 induction and G_1 arrest. Together, these results suggest that silibinin could be a useful agent for the intervention of hormone-refractory human prostate cancer.

Prostate cancer (PCA) is the most common invasive malignancy and second leading cause of cancer deaths in United States males (1). Clinical PCA incidence is low in Asians and highest in African-Americans and Scandinavians (2, 3). However, once moved to the United States, incidence and mortality because of PCA increase in Asians, approximating those of Americans (3). Epidemiological studies suggest that dietary and environmental factors are major causes for an increase in PCA (2, 3). Low-fat and high-fiber diets significantly affect sex hormone metabolism in men (4). In Japan and other Asian countries, despite the same incidence of latent small or noninfiltrating PCA, mortality rate is low (3). This could be explained, at least partly, by a diet-related lowering of biologically active androgen (4). The importance of androgen in PCA also is suggested by the observations that PCA rarely occurs in eunuchs or men with deficiency in 5α-reductase, the enzyme that converts testosterone to its active metabolite 5α-dihydrotestosterone (DHT) (5). In addition, at least 75% of PCAs with metastatic potential are androgen-dependent at initial diagnosis (6).

Androgen receptors (ARs) are required for development of both normal prostate and PCA (7). A high proportion of mutations are shown in the ligand-binding domain of AR in hormone-refractory and metastatic PCA (7), and mutant ARs could be activated by estrogen and progesterone (7). Changes in specificity of AR may provide a selective advantage in metastatic androgen-independent PCA because they remain active after androgen ablation (7). A notable gene regulated by androgen in normal prostate and PCA cells is prostate-specific antigen (PSA) (8). PSA is demonstrated to be a sensitive and specific tumor marker for PCA screening and assessment (9) and is used as an indicator of disease and response to PCA therapy (10). Several trials also have shown a direct relationship between decline in PSA and shrinkage of PCA (11). Whereas stimulation of mutant AR in human PCA LNCaP cells by androgen does not differ from stimulation of wild-type AR, estrogenic substance and some antiandrogens bind to AR in LNCaP cells with higher affinity, efficiently stimulate its transactivation function, and increase PSA (7).

Traditional Asian diets are low in animal proteins and fat, high in starch and fiber, and rich in "weak plant estrogens," which are released in large amounts in urine and serum (12, 13). Some of these phytoestrogens possess weak estrogenic, antiestrogenic, and antioxidant activity, and, therefore, possess the potential for exerting an influence on hormone-dependent cancers including PCA (12, 13). Two groups of phytoestrogens, polyphenolic flavonoid antioxidants and lignans, are receiving attention for the prevention and intervention of human cancers including PCA (12–14). Silymarin, a polyphenolic flavonoid isolated from the seeds of milk thistle (Silybum marianum), is composed mainly of silibinin (or silybin; Fig. 1A), with small amounts of other stereoisomers isosilybin, dihydrosilybin, silydianin, and silychristin (15). Silymarin and silibinin have human acceptance, being used clinically in Europe and Asia for the treatment of liver diseases (reviewed in refs. 16–19). Human populations in Europe have been using silymarin or silibinin in a whole range of liver conditions (16, 17). As therapeutic agents, both silymarin and silibinin are well tolerated and largely free of adverse effects (15–19). Silymarin is sold in the United States and Europe as a dietary supplement, and silibinin is used clinically as silipide, a lipophilic silibinin–phosphatidylcholine complex (16).

Recently, we showed that silymarin affords high to complete protection against tumorigenesis in mouse skin models (18, 19). Likewise, in a mammary gland culture initiation–promotion protocol, silymarin inhibits tumor promotion (19). More recent studies by us found that both silibinin and silymarin possess comparable inhibitory effects on human carcinoma cell growth

Medical Sciences: Zi and Agarwal

Proc. Natl. Acad. Sci. USA 96 (1999) 7491

and DNA synthesis and are equally strong antioxidants (R.A. and colleagues, unpublished observations). Based on (*i*) structural similarity of silibinin with phytoestrogens for a polyphenolic flavonoid skeleton, (*ii*) strong antioxidant and anticarcinogenic effects of silibinin, (*iii*) the fact that silibinin is used clinically and marketed as dietary supplement, and (*iv*) the bioavailability of silibinin in prostate after its oral administration to mice (R.A. and colleagues, unpublished observations), we reasoned that silibinin also could be a useful agent for the intervention of human PCA. Here, we show that silibinin decreases intracellular and secreted levels of PSA in human PCA LNCaP cells under both serum- and androgen-stimulated conditions concomitant with inhibition of cell growth via a G_1 arrest in cell cycle progression. The G_1 arrest by silibinin does not lead to apoptosis but causes neuroendocrine differentiation of the cells.

MATERIALS AND METHODS

Cells and Cultures. Human prostate carcinoma LNCaP cells and NIH 3T3 cells were obtained from American Type Culture Collection. Normal human epithelial prostate cells were from Clonetics (San Diego). LNCaP and NIH 3T3 cells were cultured in RPMI 1640 medium and DMEM, respectively, with 10% FBS and 1% penicillin-streptomycin (P-S). LNCaP cells also were cultured in 10% charcoal-stripped FBS (cFBS) and 1% P-S with or without 1 nM DHT. Normal prostate cells were cultured in defined medium as suggested by the vendor.

Silibinin and Its Purity. Silibinin (Fig. 1*A*), International Union of Pure and Applied Chemistry name: 3,5,7-trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxan-6-yl]-4-chromanon, was from U. Mengs (MADAUS AG, Cologne, Germany) and Sigma. Purity of silibinin from both sources was checked by HPLC equipped with UV followed by electrochemical detectors (EC). The HPLC system consisted of two ESA 580 pumps, an ESA RP-C18 column (3 mm, 4.6 × 250 mm), a UV detector (at λ 270 nm), an EC detector (at 500 mV potential), and an ESA 5600 control and analysis software. HPLC mobile phase contained solvent A [7.5% methanol in 100 mM of acetate buffer with 50 mM of triethylamine (TEA)/1 mM of 1-octanesulfonic acid (OSA), pH 4.8] and solvent B (80% methanol in 100 mM of acetate buffer with 50 mM TEA/1 mM OSA, pH 4.8). The linear gradient, at 0.6 ml/min, was 0–5 min, 75% A and 25% B; 5–15 min, 50% of both A and B; 15–20 min, 30% A and 70% B; 20–25 min, isocratic 30% A and 70% B; and 25 min, stop of run. Column eluate was monitored at 270 nm followed by EC detection. As shown in Fig. 1*B*, using these HPLC conditions, silibinin showed a single peak in both 270 nm UV and EC detections, with a retention time of 13.5 min. These HPLC profiles also show the purity of silibinin to be 100%.

Silibinin Treatments. Silibinin was dissolved in ethanol. Final volume of ethanol in culture during silibinin treatment and controls did not exceed 0.5%. LNCaP cells were grown in 10% FBS to 80% confluency and treated with ethanol or varying doses

FIG. 1. Chemical structure of silibinin (*A*) and HPLC profiles of silibinin by UV and EC detection (*B*).

of silibinin for 20 hr or 75 μg/ml of silibinin for varying times. Cells also were treated with paclitaxel (1 μM final concentration) for 20 hr. Cells then were lysed in 0.5 ml lysis buffer as detailed recently (20). In another study, cells grown in 10% FBS were treated with ethanol or 25 and 75 μg/ml of silibinin for 24, 48, and 72 hr, and medium was collected. Cells also were grown in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 24 hr, were treated with ethanol or 50 μg/ml of silibinin. Cell lysates then were prepared (20).

Western Blotting and Kinase Assays. Levels of PSA, cell cycle and apoptosis regulatory molecules, cytokeratins 8 and 18 (K8 & K18), and chromogranin A were determined by Western blotting. Equal amounts of protein (10–80 μg) from cell lysate or 20 μl of medium sample was denatured in sample buffer and subjected to SDS/PAGE on a 12% gel, and proteins were transferred onto membrane. The blots were probed with specific primary followed by secondary antibody and visualized by enhanced chemiluminescence. The binding of cyclin-dependent kinase inhibitors (CDKIs) with CDKs, CDK2- and cyclin E-H1 histone kinase activity, and CDK4-, CDK6-, and cyclin D1-retinoblastoma (RB) kinase activity were determined as detailed recently (20).

Cell Growth Assay. LNCaP cells were plated at 1×10^4 cells per 60-mm plate in RPMI 1640 medium containing 10% FBS. To assess the effect of silibinin on normal cell growth, NIH 3T3 cells were plated at the same density, and normal human prostate cells were plated at 2,500 cells/cm². On day 2, cells were fed with fresh medium and treated with ethanol or varying doses of silibinin (5, 25, 50, and 75 μg/ml). The cultures were fed with fresh medium with the same treatments on alternate days. After 1–6 days of treatments, cells were trypsinized and counted (20). In other studies, LNCaP cells were cultured in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 24 hr, were treated with ethanol or 50 μg/ml of silibinin. Cells then were collected and counted (20). To assess cytotoxicity of silibinin, cell viability was determined by Trypan blue assay.

FACS Analysis. LNCaP cells were cultured in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 24 hr, were treated with ethanol or 50 μg/ml of silibinin. Cells then were trypsinized, and cell cycle distribution was analyzed as detailed recently (20).

DNA Ladder Assay. LNCaP cells at 70–80% confluency were treated with different doses of silibinin for 24 and 48 hr, and, thereafter, trypsinized cells (together with any floating cells) were collected. The DNA ladder analysis then was done as detailed recently (21).

Morphological Analysis. LNCaP cells were cultured in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 48 hr, were treated with ethanol or 50 μg/ml of silibinin. Pictures then were taken by using a phase-contrast microscope at ×200 magnification.

RESULTS

Silibinin Decreases Serum- and DHT-Stimulated PSA Expression in LNCaP Cells. PSA has its acceptance and approval from FDA as a screening tool for human PCA. Therefore, to evaluate the usefulness of silibinin for PCA intervention, we assessed its effect on PSA levels in LNCaP cells. Consistent with an earlier study (8), LNCaP cells showed high levels of intracellular PSA as evidenced by a 33- to 34-kDa band (Fig. 2*A*). However, treatment of cells grown in 10% FBS with silibinin resulted in a highly significant decrease in intracellular PSA levels in a dose- and time-dependent manner (Fig. 2*A*). In a quantitative analysis, 50, 75, and 100 μg/ml of silibinin showed 54, 66, and 79% reduction in intracellular PSA levels, respectively. Similarly, cells grown in 10% FBS with 25 and 75 μg/ml of silibinin for 24 and 48 hr also showed a significant decrease in secreted PSA (Fig. 2*B*). Silibinin treatment for 24 hr at 25- and 75-μg/ml doses led to a 45 and 59% reduction in PSA secretion in medium, respectively. Because promoter of PSA gene contains functional androgen-responsive element (8) and DHT increases PSA production in LNCaP cells
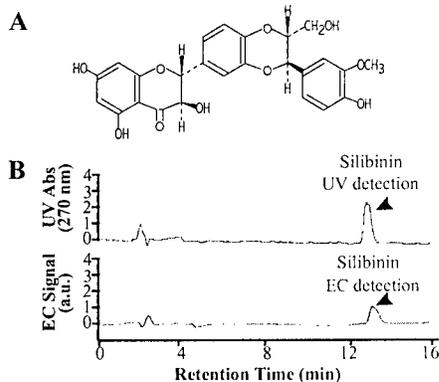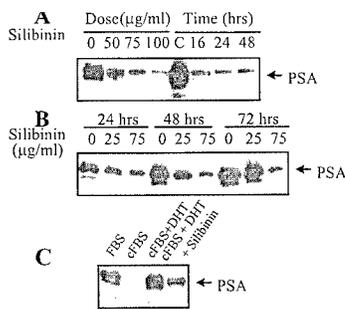
FIG. 2. Silibinin decreases serum- and DHT-stimulated PSA expression in LNCaP cells. (*A*) Effect of silibinin on intracellular PSA in cells grown in 10% FBS. Cells were treated with silibinin for 20 hr or for indicated times at 75 μg/ml; C, control cells treated with ethanol for 48 hr. (*B*) Effect of silibinin on secreted (medium) PSA in cells grown in 10% FBS. Cells were treated with silibinin for the indicated doses and time, and medium was collected. (*C*) Effect of silibinin on intracellular PSA in cells grown in 10% cFBS + 1 nM DHT. Cells were grown in: 1, 10% serum; 2, 10% cFBS; 3, 10% cFBS + 1 nM DHT; or 4, 10% cFBS supplemented with 1 nM DHT + 50 μg/ml of silibinin, and cell lysates were prepared. The data in *C* are at 5 days of cultures; silibinin was added at day 4. PSA protein levels were determined in cell lysates and medium as detailed in *Materials and Methods*. The Western blot data shown are representative of three independent experiments with similar findings.

(7), we next examined whether inhibitory effects of silibinin on PSA levels are mediated via AR. Compared with cells grown in 10% FBS showing strong PSA levels, cells grown in 10% cFBS showed no reactivity for PSA protein (Fig. 2*C*). However, cells grown in 10% cFBS + 1 nM DHT showed levels of PSA comparable to that for 10% FBS (Fig. 2*C*). Treatment of cells grown in 10% cFBS and 1 nM DHT with 50 μg/ml of silibinin resulted in a 56% reduction in DHT-stimulated intracellular PSA levels (Fig. 2*C*).

Silibinin Inhibits Serum- and DHT-Stimulated Growth of LNCaP Cells with No Effects on Normal Cells. To assess whether an observed decrease in PSA by silibinin is a biological response, we examined its effect on LNCaP cell growth. Treatment of cells grown in 10% FBS with silibinin resulted in a highly significant to complete inhibition of their growth in both a dose- and time-dependent manner (Fig. 3*A*). An inhibitory effect of silibinin was evident at 2 days, but a more profound effect was observed during 4–6 days of treatment. The 5- and 25-μg/ml doses of silibinin showed 42 and 61% inhibition in cell growth, respectively (Fig. 3*A*). Cells treated with 50 and 75 μg/ml of silibinin showed 93% and complete growth inhibition, respectively (Fig. 3*A*). At these doses of silibinin, cells stopped growing as early as 1 and 2 days, with a small reduction in initial cell number at 75 μg/ml (Fig. 3*A*). In studies assessing the effect of silibinin on androgen-stimulated growth of LNCaP cells, compared with cells grown in 10% FBS, cells grown in 10% cFBS showed a 68% reduction in growth (Fig. 3*B*). This was an expected finding because cFBS is devoid of hormones and other growth agents. Cells grown in 10% cFBS + 1 nM DHT showed much higher growth, but it was only 77% of that observed in 10% FBS (Fig. 3*B*). Silibinin treatment, however, showed 38% inhibition of DHT-stimulated cell growth (Fig. 3*B*). Together, the inhibitory effects of silibinin on FBS- and DHT-stimulated LNCaP cell growth were consistent with a decrease in PSA levels. Silibinin, however, did not show a considerable inhibition of NIH 3T3 and normal human prostate cell growth (data not shown). In cell viability, silibinin did not show cytotoxicity at present doses (data not shown).

Silibinin Induces G₁ Arrest and Decreases CDK and Cyclin Kinase Activity in LNCaP Cells. We next assessed whether cell growth-inhibitory effects of silibinin are via perturbation in cell cycle progression. Fluorescence-activated cell sorter (FACS) analysis of control and silibinin-treated cells grown in 10% FBS clearly indicated a G₁ arrest by silibinin (Fig. 4). The increase in
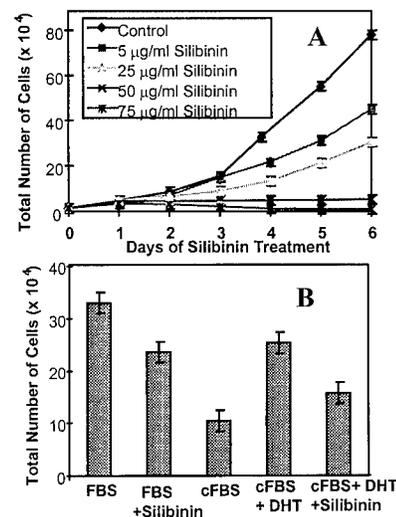

FIG. 3. Silibinin inhibits serum- and DHT-stimulated growth of LNCaP cells. (*A*) Dose- and time-dependent inhibitory effect of silibinin on serum-stimulated cell growth. Cells were treated with ethanol (control) or indicated doses of silibinin. (*B*) Inhibitory effect of silibinin on DHT-stimulated cell growth. Cells were grown in FBS, 10% serum; FBS + silibinin, 10% serum + 50 μg/ml of silibinin; cFBS, 10% cFBS; cFBS + DHT, 10% cFBS + 1 nM DHT; or cFBS + DHT + silibinin, 10% cFBS + 1 nM DHT + 50 μg/ml of silibinin. The data in *B* are at 5 days of cultures; silibinin was added at day 4. After desired treatments, cells were trypsinized and counted as described in *Materials and Methods*. Each data point represents mean ± SE of four independent plates; each sample was counted in duplicate.

G₁ population by silibinin (82.8 vs. 63% in control) was accompanied by a large decrease of cells in both S and G₂/M phases (Fig. 4*B* vs.*A*). G₁ arrest by silibinin also was found at other time points (data not shown). Similar to silibinin, when cells were
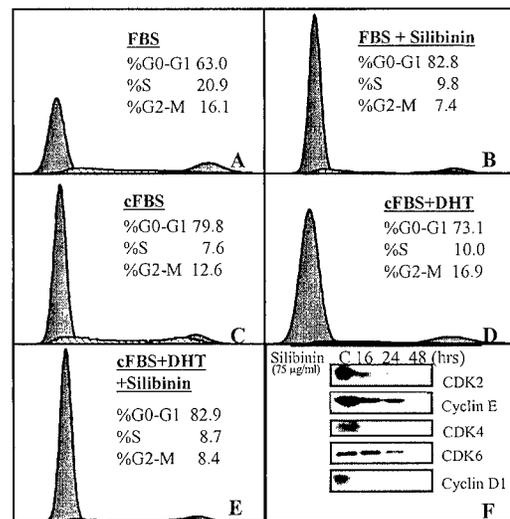

FIG. 4. Silibinin induces G₁ arrest and decreases CDK and cyclin-associated kinase activity in LNCaP cells. Cell cycle phase distribution of LNCaP cells grown in 10% serum (*A*); 10% serum + 50 μg/ml of silibinin (*B*); 10% cFBS (*C*); 10% cFBS + 1 nM DHT (*D*); and 10% cFBS + 1 nM DHT + 50 μg/ml of silibinin (*E*). The data are at 5 days of cultures; silibinin was added at day 4. After desired treatments, cells were trypsinized and FACS analysis was done as described in *Materials and Methods*. (*F*) Inhibitory effect of silibinin on CDK and cyclin kinase activity. Cells were treated with 75 μg/ml of silibinin for the indicated time, and CDK and cyclin kinase activity was determined as described in *Materials and Methods*; C, control cells treated with ethanol for 48 hr. The cell cycle phase distribution and kinase activity data shown are representative of three independent experiments with similar findings.
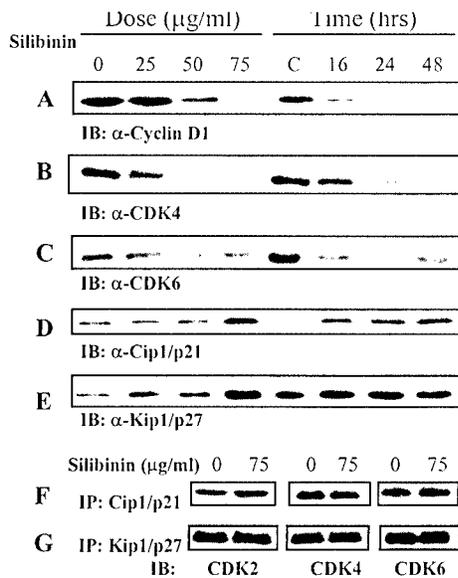
FIG. 5. Silibinin modulates protein levels of cyclin D1, CDKs, and CDKIs and increases binding of CDKIs to CDK2 in LNCaP cells. Dose- and time-dependent effect of silibinin on levels of cyclin D1 (*A*); CDK4 (*B*); CDK6 (*C*); Cip1/p21 (*D*); and Kip1/p27 (*E*). Cells were treated with silibinin for 20 hr or for the indicated time at 75 μg/ml; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared and subjected to SDS/PAGE, Western blotting, and enhanced chemiluminescence detection as described in *Materials and Methods*. Shown also is the effect of silibinin on binding of CDKs with Cip1/p21 (*F*) and Kip1/p27 (*G*). Cells were treated with vehicle or 75 μg/ml of silibinin for 16 hr, and cell lysates were prepared. CDKIs binding with CDKs was determined as described in *Materials and Methods*. The data shown are representative of three independent experiments with similar findings.

grown in 10% cFBS, a G_1 arrest also was observed (Fig. 4 *C* vs. *A*). This finding suggests a possibility that observed G_1 arrest by silibinin may be due to its inhibitory effect on growth-stimulating factors that are not present in cFBS. Additional studies also were performed to answer two questions: first, whether absence of androgen in cFBS was a major factor for observed G_1 arrest in 10% cFBS grown cells and, second, whether silibinin inhibits DHT-stimulated cell cycle progression. Compared with 10% cFBS, cells grown in 10% cFBS + 1 nM DHT showed a release from G_1 arrest (Fig. 4 *D* vs. *C*). However, when FACS data for 10% cFBS + 1 nM DHT were compared with 10% FBS, DHT-stimulated release from G_1 arrest in 10% cFBS cells was not complete (Fig. 4 *D* vs. *A*). DHT-stimulated release of cells from G_1 arrest, however, was blocked completely by silibinin (Fig. 4 *D* vs. *E*). Together, these data suggest that, in addition to androgen, there are other growth factors in serum responsible for growth and cell cycle progression of LNCaP cells and that silibinin results in a G_1 arrest in cell cycle progression of cells that are stimulated for growth by serum or only androgen.

Cell cycle progression is regulated via irreversible transitions propelled by CDKs and cyclins (22, 23). Whereas CDK4 (or CDK6)/cyclin D1 are involved in early G_1 phase, transition from G_1 to S is regulated by CDK2/cyclin E (23). Therefore, we reasoned that observed G_1 arrest by silibinin could be due to a decrease in kinase activity of CDKs and cyclins. Indeed, 75 μg/ml of silibinin showed a time-dependent decrease in CDK2 and cyclin E kinase activity (Fig. 4*F*); at 48 hr, kinase activity was not detectable in both cases. Similarly, silibinin also resulted in a highly significant decrease in CDK4, CDK6, and cyclin D1 kinase activity (Fig. 4*F*). Together, these data suggest that G_1 arrest induced by silibinin is due to a significant decrease in kinase activity of both CDKs and cyclins associated with early G_1 phase and late G_1- to S-phase transition.
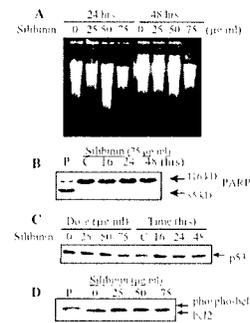

FIG. 6. Silibinin does not induce apoptosis and modulation of p53 and Bcl2 in LNCaP cells. (*A*) Agarose gel electrophoresis of cellular DNA showing a lack of DNA ladder by silibinin treatment. Cells, at 80% confluency, were treated with silibinin for the indicated doses and time. Cells were collected and cellular DNA was isolated, followed by agarose gel electrophoresis as described in *Materials and Methods*. (*B*) A lack of silibinin's effect on PARP cleavage. Cells were treated with paclitaxel (P) for 20 hr at 1 μM or for the indicated time at 75 μg/ml of silibinin; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared, and PARP protein level and cleavage were detected as described in *Materials and Methods*. (*C*) Dose- and time-dependent effect of silibinin on p53 expression. Cells were treated with silibinin for 20 hr or for the indicated time at 75 μg/ml of silibinin; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared, and p53 levels were detected as described in *Materials and Methods*. (*D*) Dose-dependent effect of silibinin on bcl2 expression. Cells were treated with paclitaxel (P) for 20 hr at 1 μM or silibinin for 20 hr, cell lysates were prepared, and bcl2 levels were detected as described in *Materials and Methods*. The data shown are representative of three independent experiments with similar findings.

Silibinin-Induced Decrease in Kinase Activity of CDKs and Cyclins Is Mediated via a Decrease in Cyclin D1, CDK4, and CDK6 Levels and an Induction of Cip1/p21 and Kip1/p27 and Their Increased Binding with CDK2 in LNCaP Cells. CDK activity is regulated positively by cyclins and negatively by CDKIs (22, 23). Based on silibinin's effect on kinase activity, we assessed its effect on (*i*) CDK and cyclin levels and (*ii*) CDKI Cip1/p21 and Kip1/p27 levels and their binding with CDKs. Silibinin resulted in a significant to complete reduction in cyclin D1 protein (Fig. 5*A*) and showed a strong decrease in CDK4 and CDK6 (Fig. 5 *B* and *C*). No effect of silibinin, however, was evident on CDK2 and cyclin E (data not shown). In other studies, silibinin resulted in both dose- and time-dependent induction of CDKIs Cip1/p21 (Fig. 5*D*) and dose-dependent induction of Kip1/p27 (Fig. 5*E*); maximum increase was evident at 24 and 16 hr, respectively. Because an induction in CDKI normally leads to an increase in its binding to and subsequent inactivation of CDK-cyclin complex (22, 23), we also investigated whether an observed decrease in CDK and cyclin kinase activity also is due to an increased CDK binding with up-regulated Cip1/p21 and Kip1/p27 by silibinin. As shown in Fig. 5 *F* and *G*, silibinin resulted in an increase only in CDK2 binding to Cip1/p21 and Kip1/p27; quantification of bands showed 1.4- and 2.6-fold increases, respectively. No effect of silibinin, however, was observed on CDK4 and CDK6 binding to either Cip1/p21 or Kip1/p27 (Fig. 5 *F* and *G*). Together, these results clearly indicate that whereas the resultant effect of silibinin was a G_1 arrest, its causes were different in terms of molecular mechanisms at early G_1 and late G_1- to S-phase transition.

Silibinin Does Not Induce Apoptosis and Modulation of p53 and bcl2 Protein Levels in LNCaP Cells. Based on observed effects of silibinin, we next assessed whether silibinin causes apoptotic death of LNCaP cells. The 25-, 50-, and 75-μg/ml doses of silibinin for 24 and 48 hr did not result in apoptosis as evidenced by a lack of DNA fragmentation (Fig. 6*A*) and a lack of poly (ADP ribose) polymerase (PARP) cleavage that otherwise was clearly evident in a paclitaxel-treated sample used as a positive control (Fig. 6*B*). Because p53 and bcl2 are considered to be crucial in apoptosis (24), we also assessed their levels after

silibinin treatment. As shown in Fig. 6 *C* and *D*, silibinin also did not result in any change in p53 and bcl2 expression; however, paclitaxel (a positive control) showed a clear phosphorylation of bcl2 (Fig. 6*D*), a process associated with inactivation of bcl2 that causes apoptosis in LNCaP cells (25). Paclitaxel also showed clear morphological changes suggestive of apoptosis (data not shown), but no such effect was evident with silibinin, and, in fact, cells started showing differentiation (Fig. 7). These results suggest that silibinin-induced G_1 arrest in LNCaP cells does not lead to an apoptotic cell death.

Silibinin Induces Neuroendocrine Differentiation and Expression of K8 & K18 and Chromogranin A in LNCaP Cells. LNCaP cells treated with silibinin manifested unique morphologic changes. Compared with cells growing in 10% FBS as piled up layers attached loosely to the surface, cells treated with silibinin primarily were monolayer and attached firmly to the surface with better anchoring (Fig. 7*A* vs. *B*). Significant changes in morphology also were observed with silibinin as cells became elongated with prominent dendrite-like cytoplasmic extensions where some of the dendrite-like extensions were connected to each other among neighboring cells (Fig. 7*B*). These morphological changes were similar to that of neuroendocrine morphology, suggesting that silibinin induces neuroendocrine differentiation of LNCaP cells (Fig. 7*B*). LNCaP cells grown in 10% cFBS also showed similar morphological changes (Fig. 7*C*), which were reversed to normal growth morphology by 1 nM DHT (Fig. 7*D*); the addition of silibinin reversed DHT-stimulated growth effect and induced similar neuroendocrine morphology in LNCaP cells (Fig. 7*E*). Silibinin treatment of cells grown in 10% FBS (or cells grown in 10% cFBS + 1 nM DHT; data not shown) also resulted in a significant induction of K8 & K18 and chromogranin A expression under identical conditions that showed neuroendocrine differentiation (Fig. 7*F*). The observed increases in K8 & K18 and chromogranin A by silibinin were optimum at both 24 and 48 hr
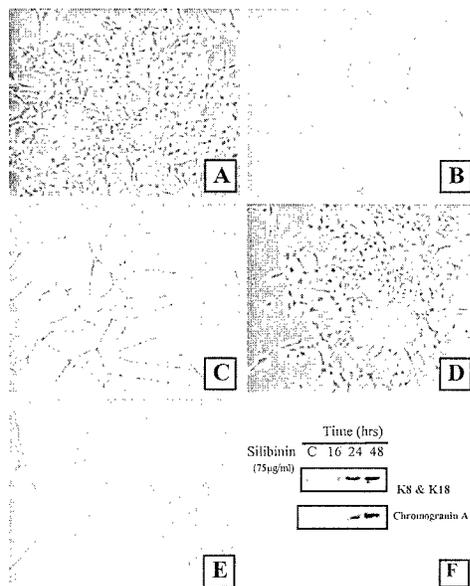

FIG. 7.   Silibinin induces neuroendocrine differentiation and expression of K8 & K18 and chromogranin A in LNCaP cells. Morphology of LNCaP cells grown in 10% serum (*A*); 10% serum + 50 μg/ml silibinin (*B*); 10% cFBS (*C*); 10% cFBS + 1 nM DHT (*D*); and 10% cFBS + 1 nM DHT + 50 μg/ml of silibinin (*E*). The data are at 5 days of cultures; silibinin was added at day 3. The phase-contrast photography was done at ×200 magnification as described in *Materials and Methods*. (*F*) Stimulatory effect of silibinin on K8 & K18 and chromogranin A levels. Cells were treated with 75 μg/ml of silibinin for the indicated time; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared, and levels of K8 & K18 (*Upper*) and chromogranin A (*Lower*) were determined as described in *Materials and Methods*. The data shown are representative of three independent experiments with similar findings.

(Fig. 7*F*). K8 & K18 have been shown to be markers of prostate tissue differentiation, and both K8 & K18 and chromogranin A are induced during differentiation of LNCaP cells with similar neuroendocrine-morphological changes (26, 27). These data suggest that silibinin induces neuroendocrine differentiation of LNCaP cells after G_1 arrest in cell cycle progression coupled with inhibition of growth-stimulatory pathways mediated by both serum as well as androgen.

DISCUSSION

LNCaP cells are one of the best *in vitro* models for human PCA studies because they possess an aneuploid male karyotype, produce PSA, and express a high-affinity mutant AR (28). These cells are responsive to androgenic stimulation and form tumors in nude mice (29). Because reduction in serum PSA levels has been proposed as an endpoint biomarker for hormone-refractory human PCA intervention (9–11), our results showing that silibinin significantly decreases both intracellular and secreted levels of PSA in androgen-dependent human PCA LNCaP cells have useful implications for human PCA intervention.

PSA is an abundant serine protease produced by prostate epithelial cells (30) and can cleave predominant seminal vesicle protein (31). PSA secretion by tumor cells into prostate stroma might augment cleavage of IGFBP3-IGF-1 and the activation of transforming growth factor β or other growth factors in extracellular matrix and then endow cancerous cells with a growth advantage leading to tumor progression (8). This hypothesis explains why PCA cells tend to diffusely infiltrate prostatic stroma rather than forming a localized tumor (8). Therefore, inhibition of PSA secretion may be an important strategy to prevent PCA progression. Here, we showed that a percentage decrease by silibinin in secreted PSA levels was comparable to intracellular PSA, suggesting that a decrease in PSA secretion by silibinin may be due to its inhibitory effect on PSA protein expression in LNCaP cells. Because silibinin also inhibited DHT-induced PSA and cell growth, we suggest that silibinin may have a direct effect on AR-mediated PSA expression.

Mammalian cell growth and proliferation are mediated via cell cycle progression (22, 23). However, defects in cell cycle are one of the most common features of cancer cells, because they divide under conditions in which their normal counterparts do not (22, 23). Androgen is shown to regulate genes controlling cell cycle, and that abnormally activated AR activity (e.g., gain-of-function by mutations in AR) may malignantly stimulate cell growth (32). Therefore, agents that inhibit cell cycle progression of cancer cells could lead to a cell growth arrest. We provide convincing evidence that silibinin inhibits both serum- and androgen-stimulated LNCaP cell growth by inducing G_1 arrest. The results from molecular mechanism studies showed that G_1 arrest by silibinin involves a significant decrease in cyclin D1, CDK4, and CDK6, resulting in a marked decrease in their kinase activity, and a significant increase in Cip1/p21 and Kip1/p27 that leads to their increased binding with CDK2, resulting in a marked decrease in CDK2 and cyclin E kinase activity.

Cyclin D1 is involved in cell cycle during early G_1 phase (23). In controlled cell growth, association of cyclin D1 with CDK4 or CDK6 leads to phosphorylation of RB; hyperphosphorylated RB leads to its release from E_2F (33). The free E_2F then activates c-*myc*, resulting in cell proliferation by progression via G_1 (34). However, overexpression of cyclin D1 is associated with various cancers and tumor-derived cell lines, explaining their uncontrolled growth (35). One of the aspects of cyclin D1 overexpression in cells is a shorten G_1 phase, resulting in a more rapid entry into S phase and increased proliferation (35). Based on these and other studies (34–36), a significant decrease in protein levels of cyclin D1, CDK4, and CDK6 by silibinin suggests that silibinin should be a useful agent for the intervention of malignancies overexpressing cyclin D1, CDK4, and/or CDK6. The observed inhibitory effects of silibinin on cyclin D1, CDK4, and CDK6 in

LNCaP cells are of particular significance for the intervention of hormone-refractory PCA because cyclin D1 is strongly associated with androgen-stimulated growth of LNCaP cells (37). Cyclin D1 is also constitutively expressed in androgen-independent human PCA PC3 and DU145 cells, but it is significantly lower in LNCaP cells grown without serum (38). In a recent study, overexpression of cyclin D1 in LNCaP cells was shown to increase cell growth and tumorigenicity in nude mice (39). Consistently, we found that LNCaP cells grown in cFBS arrest mostly in G_1 phase, which is reversed by DHT. This finding suggests the involvement of androgen-mediated growth after the release of cells from G_1 arrest because of a significant decrease in cyclin D1 in the absence of androgen. Similarly, silibinin treatment of LNCaP cells grown in serum or cFBS + DHT also showed a G_1 arrest together with a decrease in serum- and androgen-stimulated PSA levels and cell growth inhibition. These results suggest that observed effects of silibinin are those mediated via AR in terms of PSA levels, cell growth, cell cycle progression, as well as modulation of cyclin D1 and associated CDKs. In support of this suggestion, we recently have shown that treatment of human PCA DU145 cells with silymarin does not involve alterations in cyclin D1 for G_1 arrest (40). More detailed studies are in progress to support the involvement of AR in the inhibitory effects of silibinin.

p53 is an important tumor-suppressor gene, and mutations in p53 are the most commonly observed genetic lesions in human tumors (41). In response to genotoxic stress, p53 induces Cip/p21, resulting in a G_1 arrest (42). However, activation of Cip1/p21 also occurs independent of p53 as observed by transforming growth factor β stimulation during differentiation or upon cellular senescence (43). In each case, up-regulation of Cip1/p21 correlated with an arrest in cell growth, suggesting that it plays a fundamental role in the decision fork between cell proliferation, differentiation, and death. For example, inhibition of Cip1/p21 expression through transfection of Cip1/p21 antisense oligonucleotides was shown to block growth factor-induced differentiation of SH-SY5Y neuroblastoma cells and resulted in their death (44). Cip1/p21 induction also is shown in a variety of cell differentiation, including myogenic, keratinocytic, promyelocytic (HL-60), and human melanoma cells (45–47); Kip1/p27 also has been reported to be involved in cell differentiation (48). Consistently, we observed that silibinin-caused induction of Cip1/p21 was p53-independent and that, together with resultant G_1 arrest, did not induce apoptosis in LNCaP cells. Because treatment of LNCaP cells with silibinin showed neuroendocrine differentiation like morphologic changes and increased K8 & K18 and chromogranin A levels, induction of both Cip1/p21 and Kip1/p27 is likely to be involved with cell cycle exit that is associated with differentiation.

Together, the central finding in the present study is that silibinin, an active constituent of milk thistle, inhibits both serum- and androgen-stimulated PSA protein levels in LNCaP cells concomitant with cell growth inhibition via a G_1 arrest in cell cycle progression. The silibinin-treated LNCaP cells that are unable to grow follow a differentiation pathway as evidenced by neuroendocrine-like morphology, elevated prostate tissue-differentiation markers K8 & K18 and chromogranin A, and altered cell cycle-regulatory molecules. More detailed mechanistic studies are in progress to identify and define the effect of silibinin on the growth-stimulatory signals in hormone-refractory prostate carcinoma cells at molecular levels and to assess the inhibitory effect of silibinin on human PCA tumor xenograft growth in nude mice. In summary, however, based on the present findings, we conclude that silibinin has strong potential to be developed as an antiproliferative differentiating agent for the intervention of hormone-refractory human prostate cancer.

1. Wingo, P. A., Landis, S. & Ries, L. A. G. (1997) *CA Cancer J. Clin.* **47**, 239–242.
2. Parker, S. L., Tong, T., Bolden, S. & Wingo, P. A. (1997) *CA Cancer J. Clin.* **47**, 5–27.
3. Shimizu, H., Ross, R. K., Bernstein, L., Yatani, R., Henderson, B. E. & Mack, T. M. (1991) *Br. J. Cancer* **63**, 963–966.
4. Anderson, K. E., Rosner, W., Khan, M. S., New, M. I., Pang, S., Wissel, P. S. & Kappas, A. (1987) *Life Sci.* **40**, 1761–1768.
5. Aquilina, J. W., Lipsky, J. J. & Bostwick, D. G. (1997) *J. Natl. Cancer Inst.* **89**, 689–696.
6. Thompson, I. M., Coltman, C. A., Brawley, O. W. & Ryan, A. (1995) *Semin. Urol.* **13**, 122–129.
7. Culig, Z., Hobisch, A., Hittmair, A., Peterziel, H., Cato, A. C. B., Bartsch, G. & Klocker, H. (1998) *Prostate* **35**, 63–70.
8. Wang, L. G., Liu, X. M., Kreis, W. & Budman, D. R. (1997) *Cancer Res.* **57**, 714–719.
9. Stamey, T. A. & Kabalin, J. N. (1989) *J. Urol.* **141**, 1070–1075.
10. Cadeddu, J. A., Pearson, J. D., Partin, A. W., Epstein, J. I. & Carter, H. B. (1993) *Urology* **42**, 383–389.
11. Brausi, M., Jones, W. G., Fossa, S. D., de Mulder, P. H., Droz, J. P., Lentz, M. A., van Glabbeke, M. & Pawinski, A. (1995) *Eur. J. Cancer* **31A**, 1622–1626.
12. Adlercreutz, H., Fotsis, T., Bannwart, C., Wahala, K., Makela, T., Brunow, G. & Hase, T. (1986) *J. Steroid Biochem.* **25**, 791–797.
13. Morton, M. S., Chan, P. S. F., Cheng, C., Blacklock, N., Matos-Ferreira, A., Abranches-Monteiro, L., Correia, R., Lloyd, S. & Griffiths, K. (1997) *Prostate* **32**, 122–128.
14. Sun, X.-Y., Plouzek, C. A., Henry, J. P., Wang, T. T. Y. & Phang, J. M. (1998) *Cancer Res.* **58**, 2379–2384.
15. Wagner, V. H., Diesel, P. & Seitz, M. (1974) *Arzneim.-forsch.* **24**, 466–471.
16. Schandalik, R. & Perucca, E. (1994) *Drugs Exp. Clin. Res.* **20**, 37–42.
17. Luper, S. (1998) *Altern. Med. Rev.* **3**, 410–421.
18. Katiyar, S. K., Korman, N. J., Mukhtar, H. & Agarwal, R. (1997) *J. Natl. Cancer Inst.* **89**, 556–566.
19. Lahiri-Chatterjee, M., Katiyar, S. K., Mohan, R. R. & Agarwal, R. (1999) *Cancer Res.* **59**, 622–632.
20. Zi, X., Feyes, D. K. & Agarwal, R. (1998) *Clin. Cancer Res.* **4**, 1055–1064.
21. Ahmad, N., Feyes, D. K., Agarwal, R. & Mukhtar, H. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 6977–6982.
22. Elledge, S. J. & Haper, J. W. (1994) *Curr. Opin. Cell Biol.* **6**, 847–852.
23. Sherr, C. J. (1994) *Cell* **79**, 551–555.
24. White, E. (1995) *Genes Dev.* **10**, 1–15.
25. Haldar, S., Basu, A. & Croce, C. M. (1998) *Cancer Res.* **58**, 1609–1615.
26. Hsieh, T. C., Xu, W. & Chiao, J. W. (1995) *Exp. Cell Res.* **218**, 137–143.
27. Bang, Y. J., Pirnia, F., Fang, W. G., Kang, W. K., Sartor, A., Whitesell, L., Ha, M. J., Tsokos, M., Sheahan, M. D., Nguyen, P., et al. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 5330–5334.
28. Lee, C., Sutkowski, D. M., Sensibar, J. A., Zelner, D., Kim, I., Amsel, I., Shaw, N., Prins, G. S. & Kozlowski, J. M. (1995) *Endocrinology* **136**, 796–803.
29. Thalmann, G. N., Anezinis, P. E., Chang, S.-M., Zhau, H. E., Kim, E. E., Hopwood, V. L., Pathak, S., von Eschenbach, A. C. & Chung, L. W. K. (1994) *Cancer Res.* **54**, 2577–2581.
30. Ban, Y., Wang, M. C., Watt, K. W. K., Loor, R. & Chu, T. M. (1984) *Biochem. Biophys. Res. Commun.* **123**, 482–488.
31. Lijia, H. A. A. (1985) *J. Clin. Invest.* **76**, 1899–1903.
32. Lu, S., Tsai, S. Y. & Tsai, M.-J. (1997) *Cancer Res.* **57**, 4511–4516.
33. Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J.-Y, Bar-Sagi, D., Roussel, M. F. & Sherr, C. J. (1993) *Genes Dev.* **7**, 1559–1571.
34. Tam, S. W., Theodoras, A. M., Shay, J. W., Draetta, G. F. & Pagano, M. (1994) *Oncogene* **9**, 2663–2674.
35. Buckley, M. F., Sweeney, K. J. E., Hamilton, J. A., Sini, R. L., Manning, D. L., Nicholson, R. I., deFazio, A., Watts, C. K. W., Musgrove, E. A. & Sutherland, R. L. (1993) *Oncogene* **8**, 2127–2133.
37. Mueller, A., Odze, R., Jenkins, T. D., Shahsesfaei, A., Nakagawa, H., Inomoto, T. & Rustgi, A. K. (1997) *Cancer Res.* **57**, 5542–5549.
37. Chen, Y., Navone, N. M. & Conti, C. J. (1995) *Urol. Oncol.* **1**, 101–108.
38. Chen, Y., Robles, A. I., Martinez, L. A., Liu, F., Gimenez-Conti, I. B. & Conti, C. J. (1996) *Cell Growth Differ.* **7**, 1571–1578.
39. Chen, Y., Martinez, L. A., Lacava, M., Coghlan, L. & Conti, C. J. (1998) *Oncogene* **16**, 1913–1920.
40. Zi, X., Grasso, A. W., Kung, H.-J. & Agarwal, R. (1998) *Cancer Res.* **58**, 1920–1929.
41. Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C. C. (1991) *Science* **253**, 49–53.
42. Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J. & Reed, S. I. (1994) *Cell* **76**, 1013–1023.
43. Reynisdottir, I., Polyak, K., Iavarone, A. & Massague, J. (1995) *Genes Dev.* **9**, 1831–1845.
44. Poluha, W., Poluha, D. K., Chang, B., Crosbie, N. E., Schonhoff, C. M., Kilpatrick, D. L. & Ross, A. H. (1996) *Mol. Cell. Biol.* **16**, 1335–1341.
45. Guo, K., Wang, J., Andres, V., Smith, R. C. & Walsh, K. (1995) *Mol. Cell. Biol.* **15**, 3823–3829.
46. Missero, C., Calautti, E., Eckner, R., Chin, J., Tsai, L. H., Livingston, D. M. & Dotto, G. P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 5451–5455.
47. Jiang, H., Lin, J., Su, Z., Herlyn, M., Kerbel, R. S., Weissman, B. E, Welch, D. R. & Fisher, P. B. (1995) *Oncogene* **10**, 1855–1864.
48. Hengst, L. & Reed, S. I. (1996) *Science* **271**, 1861–1864.

Impairment of erbB1 Receptor-mediated and Fluid-phase Endocytosis, and Associated Mitogenic Signaling by Inositol Hexaphosphate in Human Prostate Carcinoma DU145 Cells: A Novel Approach for the Intervention of Prostate Cancer

Xiaolin Zi, Neehar Bhatia and Rajesh Agarwal*,+

Center for Cancer Causation and Prevention, AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214; and *University of Colorado Cancer Center, UCHSC, Denver, CO 80262

+To whom reprint request should be addressed. E-mail: agarwalr@amc.org

Abbreviations: AP2, plasma membrane clathrin-associated protein complex 2; EGFR (erbB1), epidermal growth factor receptor; ERK1/2, extracellular signal-regulated protein kinase 1/2; HRP, horseradish peroxidase; IGF-1, insulin like growth factor 1; IP6, inositol hexaphosphate; MAPK, mitogen activated protein kinase; NDF, neu differentiation factor; PCA, prostate cancer; PI3K, phosphatidylinositol 3-kinase; PIN, prostatic intraepithelial neoplasia; TGFα, transforming growth factor α.

ABSTRACT Endocytosis, a carefully orchestrated process, is required by cells for nutrition, down-regulation of surface receptors and maintenance of cell homeostasis. Enhanced endocytosis, however, has been associated with neoplastic transformation including advanced and androgen-independent prostate cancer (PCA) growth. We examined whether inositol hexaphosphate (IP6), a ubiquitous plant component constituting 0.4 to 6.4% (w/w) of most cereals, nuts, legumes, oil seeds and soybean, impairs erbB1 endocytosis and associated mitogenic signaling in androgen-independent human PCA DU145 cells. IP6 treatment of cells results in a significant to complete inhibition of TGFα-induced binding of plasma membrane clathrin-associated protein complex 2 (AP2) to erbB1 demonstrating the impairment of ligand-induced erbB1 receptor endocytosis. As observed by an inverse correlation in terms of an increase in the levels of activated erbB1 with the decrease in its binding with AP2 following ligand treatment, IP6 inhibited erbB1 receptor endocytosis independent of its effect on ligand-caused activation of erbB1. This effect of IP6, however, resulted in a highly significant inhibition of Shc activation and Shc-erbB1 binding. In other studies, IP6 also resulted in a highly significant inhibition of fluid-phase endocytosis by inhibiting PI3K-AKT signaling pathway as an upstream response. These effects of IP6 also resulted in a highly significant inhibition of mitogenic signaling mediated by MAPK ERK1/2, and produced biological response in terms of both anchorage-dependent and -independent growth inhibition of DU145 cells. Together, these results identify a novel molecular approach, the impairment of erbB1 endocytosis and associated mitogenic signaling, for the intervention of PCA by IP6.

Prostate cancer (PCA) is the most invasive and frequently diagnosed malignancy, and second leading cause of cancer deaths in US' males (1,2). Induction of PCA is viewed as a multistage process, involving progression from small, latent carcinomas of low histologic grade, to large, metastatic carcinomas of higher grade (2-5). The widely accepted risk factors for PCA are age, race, ethnicity, dietary habits, and androgen secretion and metabolism (2-5). Epidemiological data have revealed that environmental and behavioral factors are more important than genetic factors in determining overall cancer frequency among populations (6). Consistently, diet and androgen play a major role in the pathogenesis and promotion of PCA (2-5). For example, PCA rarely occurs in eunuchs or men with a deficiency in 5α-reductase, the enzyme that converts testosterone to its active metabolite dihydrotestosterone (7-9). Since the growth and development of PCA is initially androgen-dependent, androgen deprivation has been extensively explored as a strategy for PCA prevention and therapy (7). PCA patients treated with androgen deprivation therapy often have remission of their PCA, however, tumor re-growth occurs which is largely due to progression of initially androgen-dependent PCA cells to tumor cells that do not depend on androgen for their proliferation (8).

In addition to the loss of androgen-dependence due to lack of androgen receptor and/or its function (10), functional autocrine and paracrine growth factor/growth factor receptor interactions are major contributors to the multifactorial mechanisms of androgen-independence in PCA (11-13). Advanced and metastatic human PCA cells express high levels of EGFR (or erbB1) and TGFα (11-13), and aberrant expression of erbB family members (e.g. erbB1, erbB2 and erbB3) is shown with high frequency in PIN and in invasive PCA, both primary and metastatic (14-19). In fact, erbB family receptors are one of the few potential surrogate endpoint genetic markers which are being employed for the screening of interventive agents against PCA in short-term phase II clinical trials

(14-19). They are also extensively explored as major potential molecular target for PCA intervention, specifically in case of androgen independent PCA (reviewed in ref. 20).

Activation of erbB1 by its ligand includes receptor dimerization, activation of intrinsic receptor tyrosine kinase activity, autophosphorylation of the receptor at carboxyl terminus, and tyrosine phosphorylation of and/or association with intracellular signaling molecules such as Shc, PI3K, etc. (19-22). The binding of ligand to receptor results in a rapid disappearance of receptors from the cell surface. Receptor down-regulation is due to the ligand-accelerated endocytosis and degradation of erbB1 (23,24). Morphological studies suggest that ligand increases receptor endocytosis by promoting receptor clustering into clathrin-coated pits on the plasma membrane (the formation of clathrin-coated pits is the first step in endocytosis) which is followed by receptor internalization into clathrin-coated vesicles; there are two types of endocytosis: receptor-mediated and fluid-phase (25).

The ligand-dependent acceleration of receptor internalization is the rate-limiting step in receptor down-regulation and activation (26,27). The internalization process of receptor occurs through receptor-mediated endocytosis, where plasma membrane-coated pits function as sorting organelles selectively recruiting receptors that contain internalization sequences or "codes" within their cytoplasmic domains (26,27). A main structural component of coated pits is the clathrin lattice anchored to cytoplasmic surface of the membrane by associated protein complexes or adoptors AP2 (28). AP2 is the most ubiquitous of the associated proteins found in coated vesicles derived from the plasma membrane (29), and has been shown to specifically interact with erbB family members (22,30). In addition to receptor-mediated endocytosis involving initial binding of activated receptor with AP2 for its internalization, the other step is fluid-phase endocytosis mediated via PI3K-AKT-Rab5 pathway (31-35).

Together, these studies suggest that erbB receptor endocytosis signaling and mitogenic and anti-apoptotic pathways associated with them play a major role in the growth and proliferation of neoplastic cells including PCA. This suggestion is supported by the recent studies where for the first time we observed that both erbB1 receptor-mediated and fluid-phase endocytosis, and associated mitogenic and anti-apoptotic signaling are operational in human prostate carcinoma cells in the order of DU145>PC3>LNCaP (36). Taken together, we reasoned that erbB1 receptor endocytosis and associated mitogenic and anti-apoptotic signaling are obligatory steps in the progression of human PCA from low-grade androgen-dependent to high-grade, advanced and androgen-independent PCA with enhanced metastatic potential (36), and that the agents which impair receptor endocytosis and mitogenic signaling mediated by it will be useful for the intervention of human PCA.

Traditional Asian diets and those of vegetarians, are not only high in starch and fiber, they are also rich in many bio-active compounds which are receiving increasing attention for the prevention and intervention of a wide variety of human cancers (20,37-43). Based on epidemiological data, consumption of high-fiber diet has been associated with a lowering of breast, colon and prostate cancers (reviewed in ref. 44). Specifically, the only types of high-fiber diets which have been consistently associated with a reduction of colon and breast cancers are the cereals and legumes with high IP6 (44). As a ubiquitous plant component, IP6 (also known as phytic acid) constitutes 0.4 to 6.4% (w/w) of most cereals, nuts, legumes, oil seeds and soybean (44,45). Several studies in recent years have shown the chemopreventive and anti-carcinogenic effects of IP6 against different cancers of epithelial and mesenchymal cell origin in both *in vivo* and *in vitro* models (reviewed in refs. 44,46-49). The prevention studies with IP6 include protection against colon (44,46,47), mammary (50,51), liver (52), lung (53) and skin (44) tumorigenesis. IP6 has also been shown to inhibit the growth of mouse fibrosarcoma FSA-1 cell tumor xenograft in nude mice, and to

reduce the number of metastatic lung colonies together with an improvement in host survival (54). With regard to PCA, it has been shown that IP6 inhibits the cell growth and induces differentiation of human prostate carcinoma PC3 cells (55). In addition, IP6 is involved in various signal transduction pathways, and it binds to clathrin assembly protein AP2 (56) and inhibits PI3K (57), the two essential components in receptor-mediated and fluid-phase endocytosis, respectively.

Together, we reasoned that IP6 could impair both AP2- and PI3K-mediated endocytosis, and thereby associated cellular mitogenic responses in PCA as a novel approach for the intervention of this deadly malignancy. Indeed, the data obtained provide convincing evidence that IP6 impairs both receptor-mediated and fluid-phase endocytosis by inhibiting a) AP2 binding to erbB1 which leads to the impairment of erbB1-Shc-MAPK signaling pathway, and b) PI3K-AKT signaling pathway. The observed inhibitory effects of IP6 on endocytosis and mitogenic signaling also resulted in the inhibition of prostate carcinoma DU145 cell growth.

MATERIALS AND METHODS

Materials. Human prostate carcinoma DU145 cell line was obtained from American Type Culture Collection (Rockville, MD). IP6, HRP and wortmannin were from Sigma-Aldrich Chemical Co. (St. Louis, MO). Human TGFα, and all other cell culture materials were from Life Technologies, Inc. (Gaithersburg, MD). IGF-I, anti-erbB1 (EGFR), anti-Shc, anti-PI3K, anti-AKT, anti-phospho-AKT and anti-phosphotyrosine antibodies were from Upstate Biotechnology (Lake Placid, NY). NDF was from Neomakers (Fremont, CA). Anti-AP2 antibody was from Affinity Bioreagents Inc. (Denver, CO). Anti-phospho-MAPK and anti-MAPK antibodies were from New England Biolabs (Boston, MA). Rabbit anti-mouse immunoglobulin- and goat anti-rabbit

immunoglobulin-HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). ECL detection system was from Amersham Corp. (Arlington Heights, IL).

Cell Culture and Treatments. Cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin under standard culture conditions in 100 mm dishes until 70% confluency. Cells were then starved in serum free medium for 36 hrs and during the last 2 hrs of starvation, treated with vehicle alone or varying concentrations (0.25 to 2 mM) of IP6 or 200 ng/ml wortmannin. At the end of these treatments, cultures were added with either PBS or ligand TGFα (100 ng/ml medium), and incubated for 10 min at 37°C. After washing with PBS, cell lysates were prepared as detailed recently (20).

Immunoprecipitation and Immunoblotting. For Immunoprecipitation, cell lysates (200-500 μg protein) were clarified by protein A/G agarose for 1 hr, and then incubated with primary antibody directed against erbB1, Shc, PI3K or AKT for 4 hrs followed by addition of protein A/G agarose and overnight incubation at 4°C with rocking. Immunocomplexes were washed three times with lysis buffer (20). For immunoblotting, immunocomplexes or cell lysates (20-80 μg protein) were denatured in sample buffer, proteins were separated on SDS-PAGE (8 or 12% gel) and transferred on to nitrocellulose membranes. The membranes were blocked with blocking buffer at room temperature for 1 hr, then incubated with appropriate primary antibody directed against AP2, phosphotyrosine, erbB1, Shc, PI3K, AKT, phospho-AKT, MAPK or phospho-MAPK overnight followed by appropriate secondary antibody, and developed by ECL kit (20).

Fluid-Phase Endocytosis Assay. Fluid-phase endocytosis assay was performed as reported recently (58). Briefly, cultures in 35 mm dishes at 70-80% confluency were washed three times with serum-free α-MEM, and treated with varying doses of IP6 or 200 ng/ml wortmannin in α-MEM for 2 hrs at 37°C. HRP endocytosis was initiated by the addition of 2 mg/ml HRP and 1% (w/v) bovine serum albumin at 37°C for another 1 hr. To estimate HRP uptake, the cells were washed three times with PBS, trypsinized on ice for 20 min, washed two times with PBS, and lysed in 500 μl of lysis buffer (58). Cell lysates were assayed for HRP activity (14), and protein concentration was determined by Bio-Rad DC protein assay according to the manufacturer's instructions.

Cell Growth and Soft Agar Colony Formation Assays. For cell growth, DU145 cells were plated at a density of 0.5×10^5 cells per 60 mm plate. On day 2, cells were fed with fresh medium and left untreated, or treated with IP6 at the doses of 0.25, 0.5, 1 and 2 mM (final concentration) dissolved in double distilled water. The cultures were fed with fresh medium with or without same concentrations of IP6 every alternate day up to the end of the experiment. Each treatment and time point had four plates. At days 1 to 5 after these treatments, cells were trypsinized and counted as described recently (20), and cell viability was assessed using Trypan blue dye exclusion method.

For soft agar colony formation, DU145 cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin as detailed above. Soft agar colony formation assay was performed using 6-well plates as described recently (20). Briefly, each well contained 2 ml of 0.5% agar in medium as bottom layer, 1 ml of 0.38% agar in medium and 1,000 cells as feeder layer, and 1 ml of 0.38% agar in medium with different doses of IP6 as top layer. Each treatment had three wells. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The number of

colonies was determined by counting them under an inverted phase-contrast microscope at x100 magnification; a colony was counted as more than 10 cells.

RESULTS

IP6 Impairs erbB1 Receptor-mediated Endocytosis by Inhibiting the Binding of AP2 to erbB1 in DU145 Cells. Endocytosis, a carefully orchestrated process, is required by cells for nutrition, down-regulation of surface receptors and maintenance of cell homeostasis (59). Some endocytosis proteins have been reported in human cancers, and enhanced endocytosis is associated with neoplastic transformation (60). The erbB family of receptors are one of the main contributors to hormone independent growth, proliferation and metastasis of PCA (11-19), and both erbB1 receptor-mediated and fluid-phase endocytosis and associated mitogenic responses are operational in human PCA cells which plausibly play a potential role in advanced and androgen-independent PCA growth (36). Therefore, we focused our efforts in this study to assess the impairment of erbB1-mediated endocytosis and associated signaling by IP6 in human prostate carcinoma DU145 cells. Two separate approaches were employed to assess the effect of IP6 on receptor-mediated and fluid-phase endocytosis, and define the signaling pathways impaired following the effect of IP6 on endocytosis.

Advanced and metastatic human PCA cells such as DU145 and PC3 lack androgen receptor and express high levels of erbB1 together with TGFα leading to an autonomous growth of cancer cells via an autocrine feedback loop (11-13,20). These cells, therefore, represent a valuable system to investigate erbB1-mediated endocytosis signaling and its impairment by preventive agent under test. Employing DU145 cells, first we explored the effect of IP6 on receptor-mediated endocytosis process by analyzing the binding of AP2 to erbB1; AP2 has been shown to specifically interact with erbB1 for the receptor endocytosis (23). As shown in Fig. 1 (lane 1), immunoprecipitation with anti-

erbB1 antibody and blotting with anti-AP2 antibody clearly showed that 36 hrs of serum starvation of DU145 cells resulted in a diminished binding of AP2 to erbB1. Treatment of starved cells with TGFα (100 ng/ml) for 10 min, however, showed a very strong binding of AP2 with erbB1 (Fig. 1, lane 2) providing a convincing evidence of erbB1 receptor-mediated endocytosis process in human PCA DU145 cells. Pretreatment of cultures with 0.25, 0.5 and 1 mM doses of IP6 during last 2 hrs of starvation followed by treatment with TGFα at the same dose and time resulted in a dose-dependent decrease in the binding of AP2 to erbB1 (Fig. 1, lanes 3-5). So much so, the highest dose (1 mM) of IP6 used in the study showed complete inhibition in the binding of AP2 to erbB1 (Fig. 1, lane 5). A further increase in IP6 dose to 2 mM showed similar inhibitory effect as with 1 mM dose (data not shown). Treatment of serum starved cultures with a PI3K inhibitor, wortmannin, at 200 ng/ml dose followed by TGFα did not show any change in AP2 binding to erbB1 (Fig. 1, lane 6) suggesting that erbB1 receptor-mediated endocytosis signaling is a PI3K independent process.

Impairment of erbB1 Receptor-mediated Endocytosis by IP6 Does Not Affect erbB1 Activation in DU145 Cells. Based on the data showing a dose-dependent decrease in ligand-induced binding of AP2 to erbB1 by IP6, we next assessed the involvement of erbB1 activation in this process. As shown in Fig. 2 (top panel), serum starvation of cells for 36 hrs resulted in a complete inactivation of erbB1 (lane 1), however, as expected, treatment of starved cultures with TGFα for 10 min resulted in a marked activation of erbB1 receptor (Fig. 2, lane 2). Pretreatment of cultures with 0.25, 0.5, 1 and 2 mM doses of IP6 for 2 hrs followed by ligand treatment under identical conditions resulted in a further increase in erbB1 activation (tyrosine phosphorylation) in a dose-dependent manner (Fig. 2, lanes 3-6). Immunoblotting the membrane with anti-erbB1 antibody indicated no change in erbB1 protein expression (Fig. 2, lower panel) suggesting that the observed increase in

erbB1 activation by IP6 was not due to an increase in erbB1 protein. When erbB1 activation data

were compared with the binding of AP2 to erbB1, the observed IP6 dose-dependent increase in the

levels of activated erbB1 was inversely related with the decrease in the binding of AP2 to erbB1

(Fig. 2 *versus* Fig. 1). Together, these data suggest that the observed increase in erbB1 activation by

IP6 is due to an impairment of erbB1 receptor-mediated endocytosis process where a lack of binding

with AP2 leads to the activated erbB1 receptor at the cell surface instead of it getting internalized

and degraded by a stepwise process (22,23). These results also suggest that IP6 impairs receptor-

mediated endocytosis by targeting its effect on AP2 binding with erbB1 in an erbB1 activation

independent manner.

Impairment of erbB1 Receptor-mediated Endocytosis by IP6 Inhibits erbB1-mediated

Mitogenic Signaling by Inhibiting Shc Activation and Shc-erbB1 Binding in DU145 Cells.

Years of research has established the current paradigm that activation of membrane receptor tyrosine

kinases including erbB1 results in recruitment of src homology-2 (SH2) domain containing proteins

including adapters such as Shc that associate with guanine-nucleotide exchange factors for Ras

ultimately leading to MAPK activation for mitogenic responses (19,20). Ligand-induced activation

of erbB family members also associates with PI3K activation followed by that of survival factor

AKT, a signaling pathway also associated with fluid-phase endocytosis (19-22,31-35). Based on

these established signaling pathways, next we assessed the effect of receptor-mediated endocytosis

impairment on erbB1-mediated immediate down-stream signaling pathway involving Shc activation.

In contrast to erbB1 activation data, treatment of cultures with different doses of IP6 for 2 hrs prior

to the addition of TGFα showed a significant decrease in the activation (tyrosine phosphorylation) of

Shc protein (Fig. 3A, lanes 3-6). As a control, serum starvation of cells for 36 hrs led to a complete

diminution of tyrosine phosphorylated 46 and 52 kDa Shc proteins (Fig. 3A, lane 1), however, treatment of starved cultures with TGFα showed a strong activation of 46 kDa Shc protein and a weak activation of 52 kDa Shc protein as evidenced by a reactivity of immunoprecipitated Shc to anti-phosphotyrosine (Fig. 3A, lane 2). The observed inhibitory effect of IP6 on Shc activation was not due to a change in Shc protein levels (Fig. 3B). Shc proteins contain SH2 domain that binds to phosphotyrosine-containing sequences, including erbB1, upon ligand activation. As shown in Fig. 3C, compared to a strong binding in TGFα alone treated sample (lane 2), IP6 treatment at various doses resulted in a highly significant decrease in the binding of Shc to erbB1 (Fig. 3C, lanes 3-6). This observation further suggests an inhibition of erbB1-mediated down stream signaling following impairment of receptor-mediated endocytosis by IP6.

IP6 Also Inhibits Fluid-phase Endocytosis in DU145 Cells. Further studies were performed to explore the effect of IP6 on fluid-phase endocytosis, and the signaling pathway associated with this process. To determine the effect of IP6 on fluid-phase endocytosis in DU145 cells, HRP uptake experiments were carried out as reported recently (58). As shown in Fig 4, compared to control, IP6 treatment resulted in a dose-dependent decrease in the HRP uptake in DU145 cells as measured by HRP activity in terms of its binding with cellular proteins (58). The lowest dose (0.25 mM) of IP6 used in this study, showed only 19% inhibition, though statistically significant ($P < 0.05$, Student's t test), of fluid-phase endocytosis (Fig. 4). Much higher inhibition, however, was observed at 0.5, 1 and 2 mM doses of IP6 accounting for 38, 42 and 52% inhibition ($P < 0.001$, Student's t test), respectively (Fig. 4). Treatment of cells with a PI3K inhibitor, wortmannin, also showed similar decrease (44% inhibition, $P < 0.001$, Student's t test) in fluid-phase endocytosis (Fig. 4). Comparing the data obtained with wortmannin showing inhibition of fluid-

phase endocytosis with those where it does not change the binding of AP2 to erbB1 in receptor-mediated endocytosis study (Fig. 1, lane 6), it can be concluded that fluid-phase endocytosis involves a PI3K-mediated pathway in DU145 cells, whereas receptor-mediated endocytosis does not.

Inhibition of Fluid-phase Endocytosis by IP6 is Mediated by Impairment of PI3K-AKT Pathway in DU145 Cells. Based on the inhibitory effect of IP6 on fluid-phase endocytosis, and the similar response by PI3K inhibitor wortmannin, further studies were performed to delineate the involvement of PI3K-AKT pathway in this process. As shown in Fig. 5 (top panel), immunoprecipitation of PI3K and blotting with anti-phosphotyrosine antibody clearly showed that treatment of serum starved cultures with TGFα results in a marked activation (tyrosine phosphorylation) of only 110 kDa PI3K protein (Fig. 5, lane 2). However, pre-treatment of cells with IP6 resulted in a significant inhibition of PI3K activation in a dose-dependent manner (Fig. 5, lanes 3-5). The observed effect of IP6 was a complete inhibition of p110 PI3K tyrosine phosphorylation at 0.5 mM and higher doses (Fig. 5, lanes 4 and 5). Parallel to IP6, wortmannin (at 200 ng/ml dose), also caused the complete inhibition of p110 PI3K tyrosine phosphorylation (Fig. 5, lane 6). Serum starvation of DU145 cells for 36 hrs did not show any tyrosine phosphorylation band for both p110 and p85 PI3K subunits (Fig. 5, lane 1). The observed activation of p110 PI3K and its inhibition by IP6 was not due to a change in either of p110 and p85 PI3K protein levels (Fig. 5, bottom panel). The results obtained in the present study for the activation of p110 PI3K subunit by TGFα, were in contrast with those observed by us in another study showing that treatment of serum starved DU145 cells with IGF-I or NDF results in the activation of p85 PI3K subunit (36). It is important to emphasize here that PI3K is activated by: a) its p85 subunit that binds to tyrosine kinase which is autophosphorylated at the sequence YXXM, a specific SH2 domain phosphotyrosine

binding sequence, or b) its 110 kDa subunit that binds to the ras effector domain in a GTP-dependent manner (33,61). A differential ligand-induced activation of PI3K p110 and p85 subunits observed by us in the present study and another study (36), suggests that in DU145 cells, TGFα activates PI3K class which does not interact with SH2-domain-containing adoptors but contains an amino-terminal ras-binding site, and therefore interacts with ras proteins in a GTP-dependent manner. More detailed mechanistic studies are in progress to further address this pathway. However, to the best of our knowledge, ours is the first report showing that TGFα activates 110 kDa subunit of PI3K in DU145 cells, and that a cancer chemopreventive agent IP6 inhibits this activation.

AKT is an immediate downstream target of PI3K, and becomes serine-threonine phosphorylated *in vivo* in a PI3K-sensitive manner (35,62). AKT has been linked to diversified cellular processes including cell survival by suppressing apoptosis via phosphorylation of BAD (63). Based on our data showing that IP6 inhibits ligand-induced activation of PI3K, we next assessed its effect on AKT phosphorylation. As shown in Fig. 6 (top panel), compared to no reactivity of immunoprecipitated sample from serum starved cells towards phospho-AKT antibody, treatment of starved cultures with TGFα resulted in a strong activation of AKT (lanes 1 and 2, respectively). However, pre-treatment of cells with IP6 resulted in a significant to complete inhibition of TGFα-induced AKT activation in a dose-dependent manner (Fig. 6, lanes 3-5). Similar to IP6, PI3K inhibitor wortmannin also showed a complete inhibition of ligand-induced AKT activation (Fig. 6, lane 6). The observed changes in the levels of activated AKT were not due to a change in AKT protein levels in different treatment samples (Fig. 6, bottom panel). Several recent studies have shown a direct involvement of PI3K-AKT pathway in fluid-phase endocytosis by regulating Rab5 which is active in GTP form and is rate-limiting factor for endocytosis (33,34); specifically ras/PI3K is connected to the activation of AKT which is a key regulator of fluid-phase endocytosis (33,34).

Corroborative to our present finding that TGFα activates 110 kDa PI3K subunit in DU145 cells, as compared to p85 PI3K by IGF-1 and NDF found in another study by us (36), TGFα also showed strongest effect in terms of fluid-phase endocytosis of HRP compared to that by IGF-I and NDF (36). Together, these results further support the argument regarding the involvement of ras/PI3K-AKT pathway in fluid-phase endocytosis, and enhance our reasoning that IP6 inhibits fluid-phase endocytosis via inhibition of PI3K-AKT pathway.

Inhibition of erbB1 Receptor-mediated and Fluid-phase Endocytosis by IP6 Impairs MAPK ERK1/2 Activation in DU145 Cells. Via several different cytoplasmic signaling pathways, activation of erbB1 ultimately activates MAPK ERK1/2 which localizes to nucleus and activates transcription factors for cell growth and proliferation (19-22). Paralleled with the inhibitory effect of IP6 on Shc activation, Shc binding to erbB1 and PI3K-AKT activation, IP6 also inhibited TGFα-induced MAPK ERK1/2 activation in a dose-dependent manner with no change in their protein levels (Fig. 7). Together, these data provide convincing evidence that IP6 treatment of DU145 cells results in the impairment of receptor-mediated and fluid-phase endocytosis and associated signaling which leads to the inhibition of MAPK signaling pathway as a down-stream effect.

IP6 Inhibits Both Anchorage-dependent and -independent Growth of DU145 Cells. To assess whether impairment of erbB1 endocytosis and the mitogenic signaling associated with it by IP6 produces biological effects which occur at similar doses, we next assessed the effect of IP6 on anchorage-dependent and -independent growth of DU145 cells. In terms of anchorage-dependent cell growth, as shown by data in Fig. 8A, the treatment of cells with IP6 resulted in a significant inhibition of cell growth in both dose- and time-dependent manner. Compared to control, treatment

of cells at 0.25 mM dose of IP6 showed more than 50% inhibition (p < 0.001, Student's test) of cell

growth at day 4 of treatment (Fig. 8A). A much higher cell growth inhibition was observed at the

dose of 0.5 mM IP6 during the entire treatment time, and accounted for almost 80% inhibition

(p<0.0001, Student's test) at day four of treatment (Fig. 8A). At 1 and 2 mM doses of IP6, no cell

growth was observed after one day of treatment throughout the study (Fig. 8A). The Trypan blue

dye exclusion assay indicated that the cell growth inhibitory effects of IP6 were not due to

cytotoxicity (data not shown). Based on the results showing that IP6 inhibits anchorage-dependent

growth of DU145 cells, we next assessed the effect of IP6 on anchorage-independent growth of

DU145 cells by soft agar colony formation assay. As many as 54.5 ± 3.6 (mean $\pm$ SE of three

independent plates) colonies/1000 cells (per plate) were counted in controls after 10 days of initial

seeding (data not shown). Treatment of cells with IP6 resulted in a significant inhibition in soft agar

colony formation of DU145 cells in a dose-dependent manner (Fig. 8B). The lower doses of IP6 (0.1

and 0.2 mM) showed almost no inhibition, however, ~30 and 50% inhibition ($P < 0.05$ & 0.001,

Student's t test) was evident at 0.4 and 0.5 mM doses, respectively (Fig. 8B). The highest dose of

IP6 (4 mM) used in this assay, showed almost 75% inhibition ($P < 0.001$, Student's t test) in number

of colonies per plate.

DISCUSSION

The central finding in the present study is that IP6 impairs both receptor-mediated and fluid-phase

endocytosis which result in the inhibition of mitogenic signals associated with growth and

proliferation of human prostate carcinoma DU145 cells. The results obtained suggest a novel

molecular pathway for the intervention of advanced and androgen-independent human PCA by IP6.

These results are specifically significant since the major thrust in PCA control has been to design and

develop molecular mechanism-based intervention approaches for this deadly malignancy (14,15). For example, the major emphasis of the National Cancer Institute, NIH has been to develop SEBs for early detection, risk assessment and intervention of PCA; special emphasis has also been placed on those markers which relate to the progression of microscopic to clinically relevant PCA that could be explored in intervention trials (14,15). As found in a significant number of routine contemporary needle biopsies without cancer, high-grade PIN is the most likely precursor of PCA, and therefore PIN has been extensively used as a suitable endpoint biomarker for PCA intervention in clinical trials (14,15). It is important to emphasize here that aberrant expression of erbB family members has been shown in SEB, human PIN; erbB family members are one of the few potential surrogate endpoint genetic markers which are being employed for the screening of interventive agents against PCA in short-term phase II clinical trials (14-18); and erbB family members are also extensively explored as major potential molecular target for PCA intervention, specifically in case of androgen independent PCA (20). In view of these efforts, the results of the present study showing that a naturally occurring phytochemical, IP6, impairs erbB1 receptor endocytosis and associated mitogenic signaling in androgen-independent human PCA DU145 cells, could have direct implications in the intervention of advanced and androgen-independent human PCA.

The erbB and other receptor- and non-receptor-mediated signaling cascades activate MAPKs which are a family of signaling molecules defined as the ultimate cytoplasmic targets in signaling cascades (reviewed in refs. 64-68). Following their activation, MAPKs translocate to the nucleus where they activate transcription factors for cell growth, proliferation and differentiation (64-68). These studies suggest that growth factors and receptors associated with PCA progression regulate cell growth mostly through the activation of MAPKs. Indeed, very recently, it has been shown that MAPK ERK1/2 is constitutively very active in human PCA DU145 cells; and that epidermal growth

factor, IGF-1 and protein kinase A activator significantly activate MAPK ERK1/2 in both LNCaP and DU145 human PCA cells via erbB1 receptor (69). An increase in the activation of MAPK ERK1/2 signaling has also been reported very recently as human PCA progresses to a more advanced and androgen-independent malignancy (70). Consistent with the involvement of activated MAPK ERK1/2, possibly via erbB1 autocrine loop, in the progression of advanced and androgen-independent human PCA, in the present study, we observed that impairment of erbB1 endocytosis by IP6 also results in the inhibition of MAPK ERK1/2 activation in DU145 cells. Whereas observed inhibitory effect of IP6 on MAPK ERK1/2 activation could be via impairment of erbB1-Shc-ras/raf- and/or erbB1-PI3K-pathways, more studies are in progress to further define the specific signaling pathway affected by IP6 in inhibiting MAPK ERK1/2 activation. The specificity of IP6 in inhibiting PI3K followed by AKT activation and fluid-phase endocytosis, also needs to be further explored. For example, whereas it can be argued that the inhibitory effect of IP6 on PI3K activation observed in the present study is due to the impairment of ligand-induced erbB1 receptor endocytosis in DU145 cells, direct inhibition of PI3K by IP6 has also been reported recently in an *in vitro* assay (57). The results of this study also showed that IP6 significantly inhibits tumor promoter-induced cell transformation, AP-1 activity, PI3K activity and MAPK ERK1/2 activation in JB6 cells (57). Consistent with the effects of IP6 reported in previous study, in the present study, we have identified additional up-stream molecular signaling events which are impaired by IP6 as a plausible cause for its down-stream inhibition of PI3K and MAPK ERK1/2 followed by AP-1 activation and cell transformation.

In summary, based on the data reported in the present study, together with earlier studies showing cancer preventive and anti-carcinogenic effects of IP6 in several tumor models, we suggest that the usefulness of IP6 should be explored as a dietary interventive agent against human PCA. The

dietary intervention of PCA by IP6 could be of particular significance since diet is one such factor that varies significantly from country to country, and has been estimated to account for up to 35% of overall cancer rate differences (71). For example, the incidence of prostate, breast, and colon cancers is lower in Asian countries than in West including the United States; and people living in Japan, China, Korea and other Asian countries, are four to ten times less likely to be diagnosed with and die from prostate and breast cancers than those in the United States (1). Particularly in PCA, clinical incidence of this malignancy is low in Asian men, and highest in African-Americans and Scandinavians (1,72). However, once moved to the United States, the incidence and mortality rate due to PCA are increased in Asian men approximating those of Americans (72). The epidemiological studies suggest that dietary and environmental factors are the major causes for an increase in PCA in the United States men as well as in migrating Asians (1,72). As low-fat and/or high-fiber diet significantly affects sex hormone metabolism in men (73), in Japan and some other Asian countries, despite the same incidence of latent small or non-infiltrating prostatic carcinomas, the incidence of clinical PCA and the mortality rate associated with it is low (72). This could, at least partly, be explained by a diet-related lowering of this malignancy (73,74). IP6 is also a ubiquitous plant component constituting 0.4 to 6.4% of most cereals, nuts, legumes, oil seeds and soybean, and is rich in dietary fibers. A dietary habit consuming food rich in IP6 could be beneficial for the intervention of PCA in particular and other human malignancies in general.

1. Cancer Facts & Figures, 1998, American Cancer Society, Inc., 1999.

2. Godley, P.A., Campbell, M.K., Gallagher, P., Martinson, F.E.A., Mohler, J.L. & Sandler, R.S. (1996) *Cancer Epidemiol. Biomarkers Prevention* **5,** 889-895.

3. Ross, R.K. & Henderson, B.E. (1994) *J. Natl. Cancer Inst.* **86,** 252-254.

4. Giovannucci, E., Rimm, E.B., Colditz, G.A., Stampfer, M.J., Ascherio, A., Chute, C.C. & Willett, W.C. (1993) *J. Natl. Cancer Inst.* **85,** 1571-1579.

5. Wynder, E.L., Rose, D.P. & Cohen, L.A. (1994) *Nutrition and Cancer* **22,** 1-10.

6. Fournier, D.B., Erdman Jr, J.W. & Gordon, G.B. (1998) *Cancer Epidemiol. Biomarkers Prevention* **7,** 1055-1065.

7. Aquilina, J.W., Lipsky, J.J. & Bostwick, D.G. (1997) *J. Natl. Cancer Inst.* **89,** 689-696.

8. Umekita, Y., Hiipakka, R.A., Kokontis, J.M. & Liao, S. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11802-11807.

9. Ripple, M.O., Henry, W.F., Rago, R.P. & Wilding, G. (1997) *J. Natl. Cancer Inst.* **89,** 40-48.

10. Tilley, W.D., Wilson, C.M., Marcelli, M. & McPhaul, M.J. (1990) *Cancer Res.* **50,** 5382-5386.

11. Hofer, D.R., Sherwood, E.R., Bromberg, W.D., Mendelsohn, J., Lee, C. & Kozlowski, J.M. (1991) *Cancer Res.* **51,** 2780-2785.

12. Fong, C.J., Sherwood, E.R., Mendelsohn, J., Lee, C. & Kozlowski, J.M. (1992) *Cancer Res.* **52,** 5887-5892.

13. Peng, D., Fan, Z., Lu, Y., DeBlasio, T., Scher, H. & Mendelsohn, J. (1996) *Cancer Res.* **56,** 3666-3669.

14. Bostwick, D.G. & Aquilina, J.W. (1996) *J. Cell. Biochem. Suppl.* **25,** 156-164.

15. Karp, J.E., Chiarodo, A., Brawley, O. & Kelloff, G.J. (1996) *Cancer Res.* **56,** 5547-5556.

16. Bostwick, D.G. (1994) *J. Natl. Cancer Inst.* **86,** 1108-1110.

17. Myers, R.B., Srivastava, S., Oelschlager, D.K. & Grizzle, W.E. (1994) *J. Natl. Cancer Inst.* **86,** 1140-1145.

18. Pretlow, T.G., Pelley, R.J. & Pretlow, T.P. (1994) *In* T.G. Pretlow and T.P. Pretlow (eds.), Biochemical and Molecular Aspects of Selected Cancers, pp. 169-237. San Diego: Acad. Press, 1994.

19. Grasso, A.W., Wen, D., Miller, C.M., Rhim, J.S., Pretlow, T.G. & Kung, H.-J. (1997) *Oncogene* **15,** 2705-2716.

20. Zi, X., Grasso, A.W., Kung, H.J. & Agarwal, R. (1998) *Cancer Res.* **58,** 1920-1929.

21. Goldman, R., Levy, R.B., Peles, E. & Yarden, Y. (1990) *Biochemistry* **29,** 11024-11028.

22. Earp, H.S., Dawson, T.L., Li, H. & Yu, H. (1995) *Breast Cancer Res. Treatment* **35,** 115-132.

23. Sorkin, A., McKinsey, T., Shih, W., Kirchausen, T. & Carpenter, G. (1995) *J. Biol. Chem.* **270,** 619-625.

24. Chen, W.S., Lazar, C.S., Lund, K.A., Welsh, J.B., Chang, C.P., Walton, G.M., Der, C.J., Wiley, H.S., Gill, G.N. & Rosenfeld, M.G. (1989) *Cell* **59,** 33-43.

25. Lamaze, C., Baba, T., Redelmeier, T.E. & Schmid, S.L. (1993) *Mol. Biol. Cell* **4,** 715-727.

26. Pearse, B. & Robinson, M.S. (1990) *Annu. Rev. Cell Biol.* **6,** 151-171.

27. Trowbridge, I.S. (1991) *Curr. Opin. Cell Biol.* **3,** 634-641.

28. Chin, D.J., Straubinger, R.M., Acton, S., Nathke, I. & Brodsky, F.M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9289-9293.

29. Beck, K.A., Chang, M., Brodsky, F.M. & Ken, J.H. (1992) *J. Cell Biol.* **119,** 787-796.

30. Sorkin, A. & Carpenter, G. (1993) *Science* **261,** 612-615.

31. Novick, P. & Zerial, M. (1997) *Curr. Biol.* **9,** 496-504.

32. Li, G., D'Souza-Schorey, C., Barbieri, M.A., Cooper, J.A. & Stahl, P.D. (1997) *J. Biol. Chem.* **272,** 10337-10340.

33. Barbieri, M.A., Kohn, A.D., Roth, R.A. & Stahl, P.D. (1998) *J. Biol. Chem.* **273,** 19367-19370.

34. Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.-M., Brech, A., Callaghan, J., Toh, B.-H., Murphy, C., Zerial, M. & Stenmark, H. (1998) *Nature* **394,** 494-498.

35. Burgering, B.M. & Coffer, P.J. (1995) *Nature* **376,** 599-602.

36. Zi, X. & Agarwal, R. (1999) *Proc. Am. Assoc. Cancer Res.* **40,** 614.

37. Birt, D.F., Pelling, J.C., Nair, S. & Lepley, D. (1996) *Prog. Clin. Biol. Res.* **395,** 223-234.

38. Morse, M.A. & Stoner, G.D. (1993) *Carcinogenesis* **14,** 1737-1746.

39. Conney, A.H., Lou, Y.R., Xie, J.G., Osawa, T., Newmark, H.L., Liu, Y., Chang, R.L. & Huang, M.T. (1997) *Proc. Soc. Exp. Biol. Med.* **216,** 234-245.

40. Hong, W.K. & Sporn, M.B. (1997) *Science* **278,** 1073-1077.

41. Wattenberg, L.W. (1997) *Proc. Soc. Exp. Biol. Med.* **216,** 133-141.

42. Ames, B.N., Gold, L.S. & Willett, W.C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5258-5265.

43. Yang, C.S. & Wang, Z.Y. (1993) *J. Natl. Cancer Inst.* **85,** 1038-1049.

44. Shamsuddin, A.M., Vucenik, I. & Cole, K.E. (1997) *Life Science* **61,** 343-354.

45. Harland, B.F. & Oberleas, D. (1987) *World Rev. Nutr. Diet* **52,** 235-259.

46. Shamsuddin, A.M., Elsayed, A. & Ullah, A. (1988) *Carcinogenesis* **9,** 577-580.

47. Ullah, A. & Shamsuddin, A.M. (1990) *Carcinogenesis* **11,** 2219-2222.

48. Pretlow, T.P., O'Riordan, M.A., Somich, G.A., Amini, S.B. & Pretlow, T.G. (1992) *Carcinogenesis* **13,** 1509-1512.

49. Sakamoto, K., Venkatraman, G. & Shamsuddin, A.M. (1993) *Carcinogenesis* **14,** 1815-1819.

50. Vucenik, I., Yang, G. & Shamsuddin, A.M. (1995) *Carcinogenesis* **16,** 1055-1058.

51. Hirose, M., Hoshiya, T., Akagi, K., Futakuchi, M. & Ito, N. (1994) *Cancer Lett.* **83,** 149-156.

52. Hirose, M., Ozaki, K., Takaba, K., Fukushima, S., Shirai, T. & Ito, N. (1991) *Carcinogenesis* **12,** 1917-1921.

53. Estensen, R.D. & Wattenberg, L.W. (1993) *Carcinogenesis* **14,** 1975-1977.

54. Vucenik, I., Tomazic, V.J., Fabian, D. & Shamsuddin, A.M. (1992) *Cancer Lett.* **65,** 9-13.

55. Shamsuddin, A. & Yang, G.-Y. (1995) *Carcinogenesis* **16,** 1975-1979.

56. Voglmaier, S.M., Keen, J. H., Murphy, J. E., Ferris, C. D., Prestwich, G. D., Snyder, S. H. & Theibert, A. B. (1992) *Biochem. Biophys. Res. Commun.* **187,** 158-163.

57. Huang, C., Ma, W.-Y., Hecht, S. & Dong, Z. (1997) *Cancer Res.* **57,** 2873-2878.

58. Li, G. & Stahl, P. D. (1993) *J. Biol. Chem.* **268,** 24475-24480.

59. Li, G., D'Souza-Schorey, C., Barbieri, M. A., Roberts, R. L., Klippel, A., Williams, L. T. & Stahl, P. D. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10207-10211.

60. Floyd, S. & De Camilli, P. (1998) *Trends Cell Biol.* **8,** 299-301.

61. Vanhaesebroech, B., Leevers, S .J., Panayotou, G. & Waterfield, M. D. (1997) *Trends Biochem. Sci.* **22,** 267-272.

62. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J. & Tsichlis, P. (1998) *Oncogene* **17,** 313-325.

63. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. & Greenberg, M. E. (1997) *Cell* **91,** 231-241.

64. Levitzki, A. & Gazit, A. (1995) *Science* **267,** 1782-1788.

65. Canman, C.E. & Kastan, M.B. (1996) *Nature* **384,** 213-214.

66. Groom, L.A., Sneddon, A.A., Alessi, D.R., Dowd, S. & Keyse, S.M. (1996) *EMBO J.* **15,** 3621-3632.

67. Whitmarsh, A.J., Cavanagh, J., Tournier, C., Yasuda, J. & Davis, R.J. (1998) *Science* **281,** 1671-1674.

68. Hoffmeyer, A., Grosse-Wilde, A., Flory, E., Neufeld, B., Kunz, M., Rapp, U.R. & Ludwig, S. (1999) *J. Biol. Chem.* **274,** 4319-4327.

69. Putz, T., Culig, Z., Eder, I.E., Nessler-Menardi, C., Bartsch, G., Grunicke, H., Uberall, F. & Klocker, H. (1999) *Cancer Res.* **59,** 227-233.

70. Gioeli, D., Mandell, J.W., Petroni, G.R., Frierson Jr, H.F. & Weber, M.J. (1999) *Cancer Res.* **59,** 279-284.

71. Doll, R. & Peto, R. (1981) *J. Natl. Cancer Inst.* **66,** 1193-1308.

72. Shimizu, H., Ross, R.K., Bernstein, L., Yatani, R., Henderson, B.E. & Mack, T.M. (1991) *Br. J. Cancer* **63,** 963-966.

73. Anderson, K.E., Rosner, W., Khan, M.S., New, M.I., Pang, S., Wissel, P.S. & Kappas, A. (1987) *Life Sci.* **40,** 1761-1768.

74. Greco, K.E. & Kulawiak, L. (1994) *Oncol. Nurs. Forum* **21,** 1504-1511.

Figure Legends

Fig. 1. IP6 impairs erbB1 receptor-mediated endocytosis by inhibiting the binding of AP2 to

erbB1 in DU145 cells. Cells at 70-80% confluency were serum starved for 36 hrs, and during the last

two hrs of starvation were either treated with vehicle or 0.25, 0.5 or 1 mM IP6, or 200 ng/ml

wortmannin. At the end of these treatments, cells were added with PBS or TGFα (100 ng/ml) for 10

min at 37°C, and cell lysates were prepared as detailed in Methods. erbB1 was immunoprecipitated

using anti-erbB1 antibody, and then immunoprecipitates were subjected to SDS-PAGE followed by

Western blotting. Membrane was probed with anti-AP2 antibody followed by peroxidase-conjugated

appropriate secondary antibody, and visualized by the ECL detection system. The treatment in each

lane is as marked in the figure.

Fig. 2. Impairment of erbB1 receptor-mediated endocytosis by IP6 does not affect erbB1

activation in DU145 cells. Cells at 70-80% confluency were serum starved for 36 hrs, and during the

last two hrs of starvation were either treated with vehicle or 0.25, 0.5, 1 or 2 mM IP6. At the end of

these treatments, cells were added with PBS or TGFα (100 ng/ml) for 10 min at 37°C, and cell

lysates were prepared as detailed in Methods. erbB1 was immunoprecipitated using anti-erbB1

antibody, and then immunoprecipitates were subjected to SDS-PAGE followed by Western blotting.

The membrane was probed with anti-phosphotyrosine (*upper panel*) or anti-EGFR (*lower panel*)

antibody followed by peroxidase-conjugated appropriate secondary antibody, and visualized by the

ECL detection system. The treatment in each lane is as marked in the figure.

Fig. 3. Impairment of erbB1 receptor-mediated endocytosis by IP6 inhibits erbB1-mediated

mitogenic signaling by inhibiting Shc activation and Shc-erbB1 binding in DU145 cells. Cells at 70-

80% confluency were serum starved for 36 hrs, and during the last two hrs of starvation were either treated with vehicle or 0.25, 0.5, 1 or 2 mM IP6. At the end of these treatments, cells were added with PBS or TGFα (100 ng/ml) for 10 min at 37°C, and cell lysates were prepared as detailed in Methods. Shc was immunoprecipitated using anti-Shc antibody, and then immunoprecipitates were subjected to SDS-PAGE followed by Western blotting. The membrane was probed with anti-phosphotyrosine (*panel A*), anti-Shc (*panel B*) or anti-erbB1 (*panel C*) antibody followed by peroxidase-conjugated appropriate secondary antibody, and visualized by the ECL detection system. The treatment in each lane is as marked in the figure.

Fig. 4. IP6 also impairs fluid-phase endocytosis in DU145 cells. Cultures in 35mm dishes at 70-80% confluency were washed with α–MEM three time and treated with 0, 0.25, 0.5, 1 or 2 mM IP6 or 200 ng/ml wortmannin in α-MEM for 2 hrs at 37°C. Thereafter, cultures were added with 2 mg/ml horseradish peroxidase and 1% bovine serum albumin, and incubated for another hour at 37°C. The HRP uptake was determined as detailed in Methods. The data shown are mean ± SE of three independent experiments, each done in duplicate.

Fig. 5. Inhibition of fluid-phase endocytosis by IP6 is mediated by impairment of PI3K-AKT pathway in DU145 cells: effect on PI3K activation. Cells at 70-80% confluency were serum starved for 36 hrs, and during the last two hrs of starvation were either treated with vehicle or 0.25, 0.5 or 1 mM IP6, or 200 ng/ml wortmannin. At the end of these treatments, cells were added with PBS or TGFα (100ng/ml) for 10 min at 37°C, and cell lysates were prepared as detailed in Methods. PI3K was immunoprecipitated using anti-PI3K antibody, and then immunoprecipitates or total cell lysates were subjected to SDS-PAGE followed by Western blotting as described in Methods.
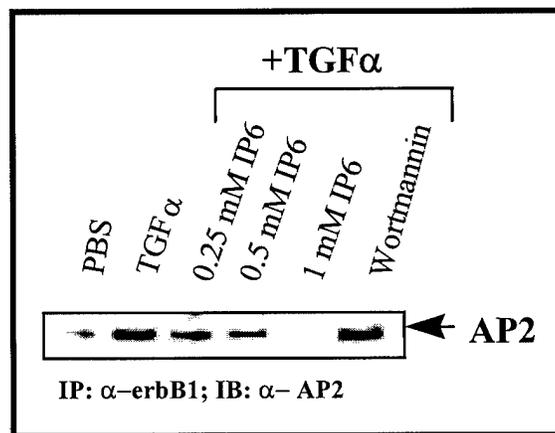
Immunoprecipitated PI3K following blotting was probed with anti-phosphotyrosine (*upper panel*), and cell lysates following blotting were probed with anti-PI3K (*lower panel*) antibodies. Following primary antibody, membranes were incubated with peroxidase-conjugated appropriate secondary antibody, and visualized by the ECL detection system. The treatment in each lane is as marked in the figure.

Fig. 6. Inhibition of fluid-phase endocytosis by phytic acid is mediated by impairment of PI3K-AKT pathway in DU145 cells: effect on AKT activation. Cells at 70-80% confluency were serum starved for 36 hrs, and during the last two hrs of starvation were either treated with vehicle or 0.25, 0.5 or 1 mM IP6, or 200 ng/ml wortmannin. At the end of these treatments, cells were added with PBS or TGFα (100ng/ml) for 10 min at 37°C, and cell lysates were prepared as detailed in Methods. AKT was immunoprecipitated using anti-AKT antibody, and then immunoprecipitates or total cell lysates were subjected to SDS-PAGE followed by Western blotting as described in Methods. Immunoprecipitated AKT following blotting was probed with anti-phospho AKT (*upper panel*), and cell lysates following blotting were probed with anti-AKT (*lower panel*) antibodies. Following primary antibody, membranes were incubated with peroxidase-conjugated appropriate secondary antibody, and visualized by the ECL detection system. The treatment in each lane is as marked in the figure.
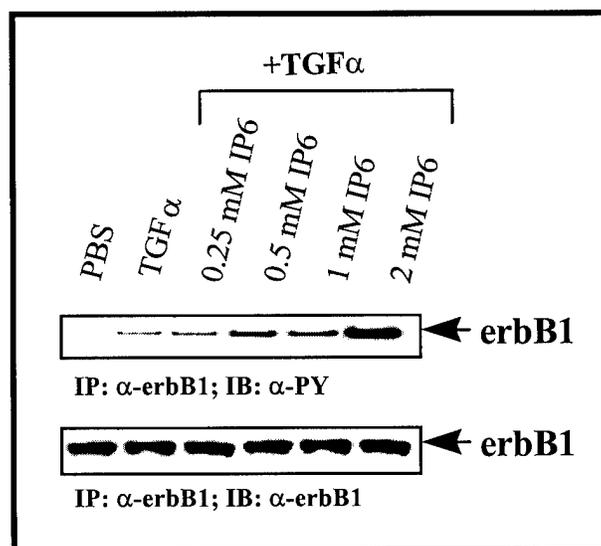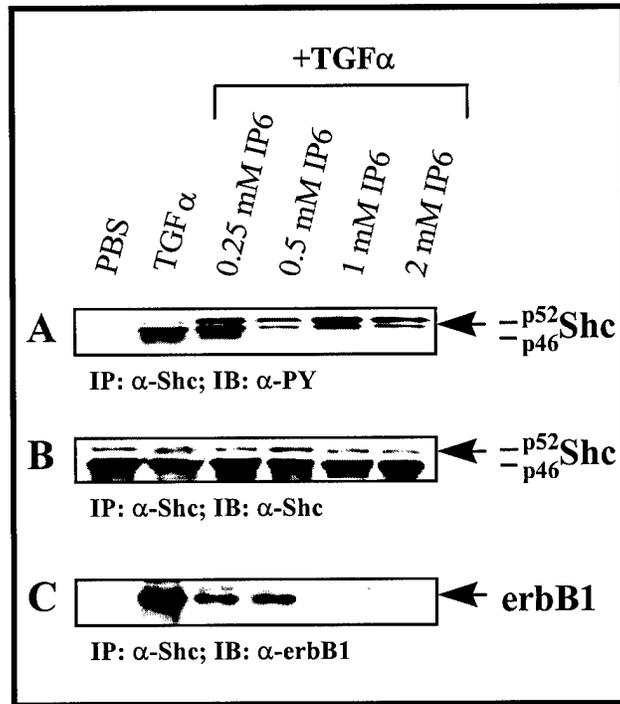
Fig. 7. Inhibition of erbB1 receptor-mediated and fluid-phase endocytosis by IP6 impairs MAPK ERK1/2 activation in DU145 cells. Cells at 70-80% confluency were serum starved for 36 hrs, and during the last two hrs of starvation were either treated with vehicle or 0.25, 0.5, 1 or 2 mM IP6. At the end of these treatments, cells were added with PBS or TGFα (100 ng/ml) for 10 min at

37°C, and cell lysates were prepared as detailed in Methods. Total cell lysates were subjected to

SDS-PAGE followed by Western blotting as described in Methods, and membranes were probed

with anti-phospho MAPK (*upper panel*) or anti-MAPK (*lower panel*) antibody. Following primary

antibody, membranes were incubated with peroxidase-conjugated appropriate secondary antibody,

and visualized by the ECL detection system. The treatment in each lane is as marked in the figure.

Fig. 8. IP6 inhibits both anchorage-dependent and -independent growth of DU145 cells. For

anchorage-dependent cell growth (A), cells were plated at 0.5 x 10^5 cells/ 60-mm plate, and on day 2

were treated with water or indicated doses of IP6. Total number of cells were counted for varying

time periods (1-5 days). The cell growth data shown are mean $\pm$ SE of four independent plates; each

sample counted in duplicate. For anchorage-independent cell growth (B), soft agar colony formation

assay was performed using 6-well plates as detailed in Methods. The number of colonies was

determined under an inverted phase-contrast microscope at x100 magnification; a group of more than

10 cells was counted as a colony. The data shown are means $\pm$ SE of three independent wells at

optimum time of 10 days from the start of cell seeding; the experiment was repeated once with
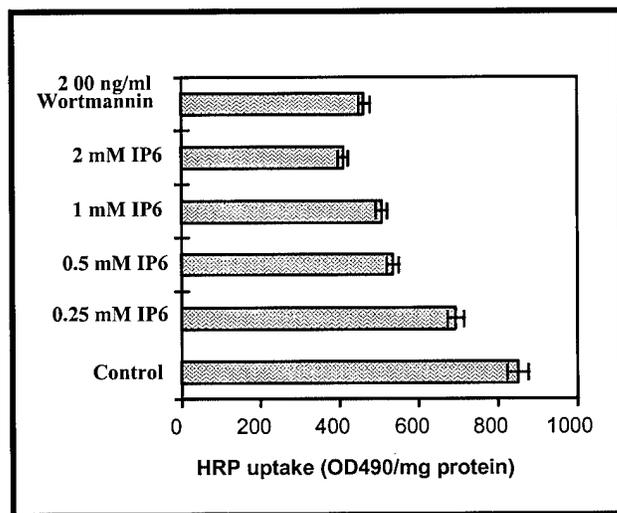
similar results.

+TGFα

PBS
TGFα
0.25 mM IP6
0.5 mM IP6
1 mM IP6
Wortmannin

◀— AP2

IP: α–erbB1; IB: α– AP2

Zi et al, Figure 1

+TGFα

PBS | TGFα | 0.25 mM IP6 | 0.5 mM IP6 | 1 mM IP6 | 2 mM IP6

← erbB1

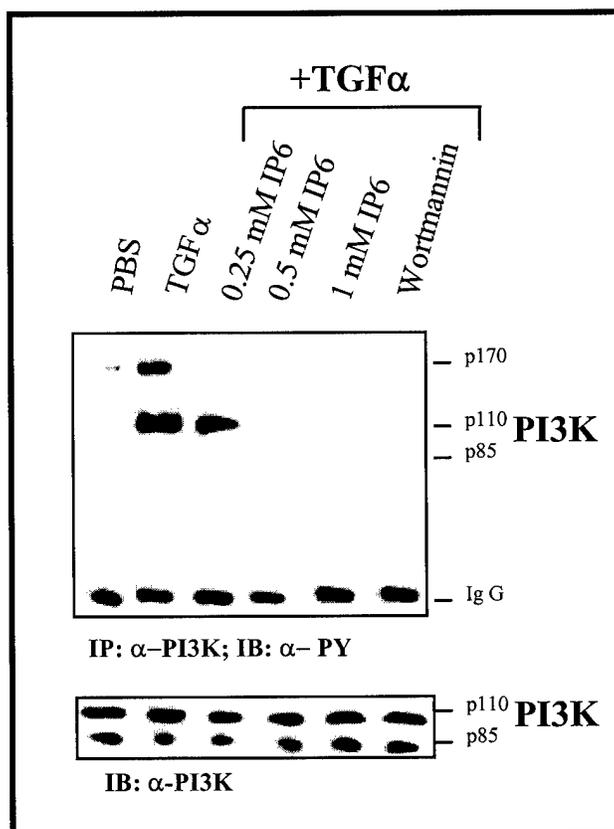IP: α-erbB1; IB: α-PY
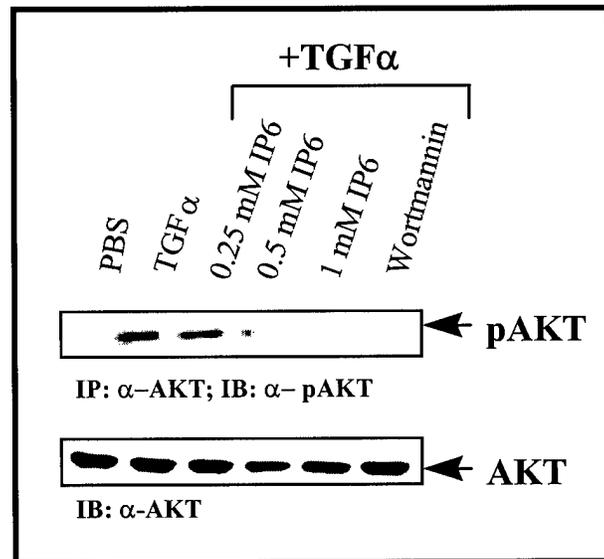
← erbB1

IP: α-erbB1; IB: α-erbB1

Zi et al, Figure 2

Zi et al, Figure 3
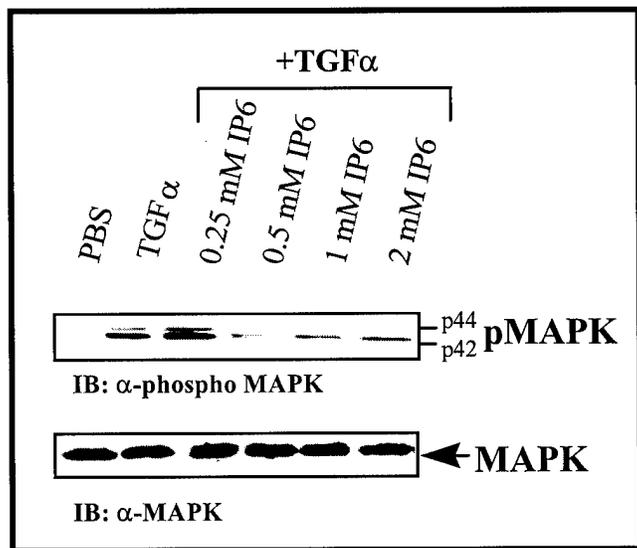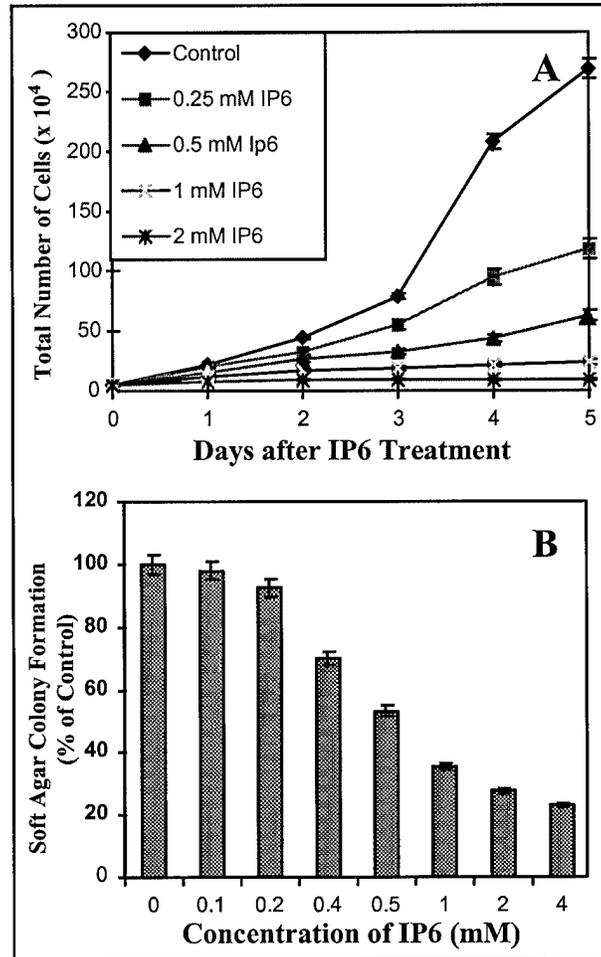
Zi et al, Figure 4

Zi et al, Figure 5

Zi et al, Figure 6

Zi et al, Figure 7

Zi et al, Figure 8

INTERNATIONAL CONFERENCE
ON DIET AND PREVENTION OF CANCER

with special emphasis on chemoprevention of cancer

May 28 – June 2, 1999

Tampere, Finland

ABSTRACT BOOK

Diet and Prevention of Cancer

Final Program

Saturday 29th of May

8.30-9.00 All posters are mounted. They may stay during the whole conference.

Breast Cancer (Chairman Pirkko Vihko)

9.00-9.25	P. K. Siiteri (USA)	
	Breast cancer, estrogens, phytoestrogens and intracellular metabolism	S1.1

9.30-9.55	L. Jones (USA)	
	Diet, hormones and breast cancer	S1.2

10.00-10.25	C. A. Lamartiniere (USA)	
	Genistein: Chemoprevention, in vivo mechanisms of action, potential for toxicity and bioavailability	S1.3

10.30-11.00   Coffee break

11.00-11.25	Ann R. Kennedy (USA)	S1.4
	Prevention of cancer with the Bowman-Birk inhibitor	

11.30-11.55	Gertraud Maskarinec, L. Meng, A. Franke, et al. (USA)	
	Soy intake and mammographic densities among multi-ethnic women	S1.5

12.00-12.25	L.A. Cohen (USA)	
	Dietary fiber and experimental mammary cancer	S1.6

12.30-13.30   Lunch

13.30-14.30   Poster Session: Breast and Prostate Cancer (Authors present)

Prostate cancer (Chairman Pentti K. Siiteri)

14.30-14.55	Pirkko Vihko (Finland)	
	17ß-hydroxysteroid dehydrogenases, phytoestrogens and estrogen and androgen metabolism	S2.7

15.00-15.25   R. Agarwal (USA)
Prostate cancer prevention by an antioxidant silymarin   S2.8

15.30-15.55   A. Constantinou, X. Xu, Y. Wang et al. (USA)
Chemoprevention of prostate cancer by lycopene and its metabolites: Analytical
and mechanistic studies   S2.9

16.00-16.30   Coffee break

16.30-16.55   M. Morton, O. Arisaka, A. Miyake, et al. (UK)
Dietary soya and prostate cancer prevention   S2.10

Mechanisms 1

17.00-17.25   Albena Dinkova-Kostova and P. Talalay (USA)
Protection against carcinogenesis by dietary constituents: Curcumin and its
analogues as phase 2 enzyme inducers   S3.11

Sunday 30th of May

Mechanisms 2 (Chairman Michael Gould)

9.00-9.25   J. Weisburger (USA)
Effective cancer prevention based on understanding of mechanisms   S4.12

9.30-9.55   T. Fotsis, M.S. Pepper, E. Aktas et al.(Greece)
Phytoestrogens and inhibition of angiogenesis   S4.13

10.00-10.25   J.E. Trosko (USA)   S4.14
Modulation of cell-cell communication in the cause and prevention of cancer

10.30-11.00   Coffee break

11.00-11.25   P.R. Harrison, J. Brown, J.O'Prey et al.
Mechanisms of cancer prevention by dietary flavonoids   S4.15

11.30-11.55   J.-K. Lin, Y.-C. Liang, S.-Y. Lin-Shiau (Taiwan)
Action mechanisms of cancer chemopreventative polyphenols in green, oolong
and black teas   S4.16

Rajesh Agarwal, Ph.D.
AMC Cancer Research Center, Denver, CO 80214, USA

Prostate cancer (PCA) is the most common invasive malignancy and leading cause (after lung) of cancer deaths in the United States males. Since PCA is initially androgen-dependent, strategies are targeted toward androgen depletion for its control. However, tumor re-growth appears following this modality which is androgen-independent and involves autocrine growth factor/receptor interaction causing an autonomous loop for persistent PCA growth and metastasis. Recently, we showed that silymarin, a flavonoid antioxidant isolated from milk thistle, possesses exceptionally high to complete protective effects against experimentally-induced tumorigenesis. Since reduction in serum prostate specific antigen (PSA) levels has been proposed as an end point biomarker for androgen-dependent human PCA intervention, and impairment of erbB1-mediated mitogenic signaling could be useful for the intervention of androgen-independent PCA, we assessed the effect of silymarin on: a) PSA expression, and b) erbB1 activation and associated downstream events, in human prostate carcinoma LNCaP and DU145 cells, respectively. Studies were also performed to assess whether silymarin modulates cell cycle regulatory proteins and progression, leading to growth inhibition of human prostate carcinoma cells.

In LNCaP cells, silymarin treatment resulted in a significant decrease in both intracellular and secreted forms of PSA concomitant with a highly significant to complete inhibition of cell growth via a G1 arrest in cell cycle progression. Treatment of cells grown in charcoal-stripped serum and DHT showed that the observed effects of silymarin are those involving androgen-stimulated PSA expression and cell growth. Silymarin-induced G1 arrest was associated with a marked decrease in the kinase activity of CDKs and associated cyclins due to a highly significant decrease in cyclin D1, CDK4 and CDK6 levels, and an induction of Cip1/p21 and Kip1/p27 followed by their increased binding with CDK2. Silymarin treatment of cells did not result in apoptosis and changes in p53 and bcl2 levels. Conversely, it resulted in a significant neuroendocrine differentiation of LNCaP cells as an alternative pathway following Cip1/p21 induction and G1 arrest.

In DU145 cells, treatment of serum-starved cells with silymarin resulted in a significant inhibition of TGFα-mediated activation of erbB1, but no change in its protein levels. Silymarin treatment of cells also resulted in a significant decrease in tyrosine phosphorylation of an immediate down-stream target of erbB1, the adapter protein SHC, together with a decrease in its binding to erbB1. Silymarin treatment also resulted in a significant induction of CDKIs Cip1/p21 and Kip1/p27, concomitant with a significant decrease in CDK4 expression but no change in the levels of CDK2, CDK6, and associated cyclin E and cyclin D1, respectively. Cells treated with silymarin also showed an increased binding of CDKIs with CDKs, together with a marked decrease in the kinase activity of CDKs and associated cyclins. In additional studies, treatment of cells grown in 10% serum with anti-EGFR monoclonal antibody clone 225 (mAb 225) or different doses of silymarin also resulted in significant inhibition of constitutive tyrosine phosphorylation of both erbB1 and SHC, but no change in their protein levels. Furthermore, whereas silymarin treatment resulted in a significant increase in the protein levels of both Cip1/p21 and Kip1/p27, mAb 225 showed an increase only in Kip1/p27. These findings suggest that silymarin also inhibits constitutive activation of erbB1, and that the observed effect of silymarin on an increase in CDKI protein levels is mediated via inhibition of erbB1 activation only in the case of Kip1/p27; however, additional pathways independent of inhibition of erbB1 activation are possibly responsible for the silymarin-caused increase in Cip1/p21 in DU145 cells. In other studies, silymarin treatment also induced a G1 arrest in the cell cycle progression of DU145 cells, and resulted in a highly significant to complete inhibition of both anchorage-dependent and -independent growth of DU145 cells in a dose- and time-dependent manner.

Taken together, these results suggest that silymarin may exert a strong anti-carcinogenic effect against both androgen-dependent and -independent human PCA, and that these effects are likely to involve inhibition of PSA expression and impairment of erbB1-SHC-mediated signaling pathway, induction of CDKIs, and a resultant G1 arrest.

DO NOT FOLD

Appendix 4

Abstract Form

91st

ANNUAL MEETING
April 1-5, 2000

The Moscone Center • San Francisco, CA

Deadline: November 1, 1999
Enclose:
- Original abstract form. (This form must be filled out and signed completely.)
- Check for US$40 payable to AACR, Inc. OR authorization to charge US$40 to credit card (see Item 8 below).
- A completed and signed original of the Copyright Transfer/Disclosure form.

1. Category and Subclassification
(see pages 10-12): Type the five-character code in the blocks provided.

| P | R | 1 | - | 0 | 4 |

A FLAVONOID ANTIOXIDANT SILIBININ INHIBITS TGFα EXPRESSION AND ITS BINDING TO erbB1 RESULTING IN IMPAIRMENT OF LIGAND/RECEPTOR AUTOCRINE GROWTH LOOP IN HUMAN PROSTATE CARCINOMA CELLS. Y. Sharma, and R. Agarwal. *AMC Cancer Research Center, Denver, CO 80214*

Oncogenic potential of advanced and androgen-independent prostate cancer (PCA) is causally associated with a ligand/receptor autocrine growth loop, e.g. interaction of transforming growth factor α (TGFα) and epidermal growth factor receptor (erbB1). We rationalized that targeting this pathway would be useful for PCA intervention, and showed recently that a flavonoid antioxidant silymarin inhibits erbB1 activation followed by a G1 arrest and inhibition of PCA cell growth. Here we did more studies to define cause and effect relationship for the observed effect of silymarin at membrane receptor level. Treatment of human prostate carcinoma LNCaP and DU145 cells with silibinin (major isoform of silymarin) at 50-150 μg/ml doses (2 hrs) resulted in 20-75% inhibition of TGFα binding to erbB1 and 12-95% inhibition in its internalization. This effect of silibinin resulted in a strong decrease in constitutive activation of erbB1 followed by ERK1/2. Silibinin treatment (at same doses) also resulted in 70-80% inhibition of cellular TGFα expression and up to 40% inhibition in its release. These effects of silibinin also corroborated with a strong inhibition (70-80%) of LNCaP and DU145 cell growth. These data show that as an initial step, silibinin inhibits ligand binding to erbB1 that impairs its internalization causing inhibition of erbB1 and ERK1/2 activation. As this signaling causes activation of transcription factors for cell growth, its inhibition possibly results in transcriptional and translational changes such as a decrease in TGFα expression, as observed in silibinin treated cells. Together these effects of silibinin lead to an impairment of ligand/receptor autocrine growth loop in PCA. (Supported by DAMD17-98-1-8588).

Type abstract within black lines. See sample abstract.

2. Abstract is SPONSORED by: Assoc. Mem. Applicant Member No.
(See Directory of Members for Member No.)

YOGESH SHARMA Name
(Please Print)

AMC CANCER RESEARCH CENTER Address
1600 PIERCE STREET
DENVER CO City, State
80214 Zip/Postal Code USA Country
303-239-3527 Telephone No. 303-239-3567 FAX No.
Sharmay@amc.org E-Mail Address

3. As the SPONSOR of this abstract and on behalf of all the authors, I hereby indicate my support for the data contained herein.

_____ Signature of SPONSOR

4. (Complete only if SPONSOR is an Associate Member) I, the undersigned Active or Corresponding Member in good standing, Emeritus Member, or Honorary Member, endorse the content of this abstract, for which the above-named Associate Member is sponsor and presenter. (See regulations applying to Associate Members on page 5.)

RAJESH AGARWAL Name
(Please Print)

_____ Signature 7979 Member No.

5. Eligibility for Young Investigator Awards (American and International)
☒ A. The PRESENTER of this abstract is a medical or graduate student, medical resident, or postdoctoral or clinical fellow.
☐ B. The PRESENTER meets the criteria in Box A above and is also a minority scientist as defined by the National Cancer Institute (see page 7).
☐ C. The PRESENTER of this abstract is a full-time faculty member at a Historically Black College or University (HBCU).

6. Abstract to be PRESENTED by: Assoc. Mem. Applicant Member No.
(If an AACR Member.)

YOGESH SHARMA Name
(Please Print)

AMC CANCER RESEARCH CENTER Address
1600 PIERCE STREET
DENVER CO City, State
80214 Zip/Postal Code USA Country
303-239-3527 Telephone No. 303-239-3567 FAX No.
Sharmay@amc.org E-Mail Address

7. As the PRESENTER and as an author of this abstract and on behalf of all the authors, I hereby give exclusive permission to the American Association for Cancer Research, Inc. to record my presentation at the 2000 Annual Meeting and to collect all revenue from subsequent audiocassette sales. I also acknowledge that I am enclosing a completed, signed original of the Copyright Transfer/Disclosure Form.

_____ Signature of PRESENTER

8. Payment of US$40 Abstract Submission Fee.
☒ Check is enclosed. Please list name of PRESENTER on the check.
☐ I authorize you to charge my credit card for the abstract submission fee.
☐ VISA ☐ MasterCard ☐ American Express

Card No. _____ Expiration Date

Cardholder Name (PRINT) _____ Signature