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**THE ANTITUMOR AGENT, ARGLABIN-DMA, PREFERENTIALLY
INDUCES APOPTOSIS IN HUMAN COLON TUMOR CELLS**

by

Sung Wook Kwon
B.S. February 1992, Suwon University

A Dissertation Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY
May 2005

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ABSTRACT

THE ANTITUMOR AGENT, ARGLABIN-DMA, PREFERENTIALLY INDUCES APOPTOSIS IN HUMAN COLON TUMOR CELLS

Sung Wook Kwon
Old Dominion University, 2005
Director: Dr. Christopher J. Osgood

Arglabin-DMA, an analog of farnesyl pyrophosphate (FPP), reportedly inhibits farnesyltransferase (FTase) directly by competitively blocking the binding of Ras protein and its posttranslational modification, as suggested in previous studies. But, the mechanisms by which Arglabin-DMA inhibits tumor growth *in vivo* and *in vitro* are still relatively poorly characterized. To determine the mechanism by which this drug inhibits tumor growth, the effects of Arglabin-DMA in two human colon tumor cell lines (mutant *K-ras* HCT 116 and wild-type *ras* HT-29) were explored on cell proliferation, apoptosis, and cell cycle kinetics *in vitro*. In cell viability studies, we showed that Arglabin-DMA had striking morphological and physiological effects on the two human colon tumor cell lines, possibly more so than those of other anticancer drugs. Also, Arglabin-DMA exhibited less harm to normal cells (Hs27) which retained their potential for cell growth. An add-back experiment showed that Arglabin-DMA had no effect on the isoprenoid biosynthetic pathway. The drug not only affects the mutant *K-ras* human colon tumor cell line, but also the wild-type *ras* human colon tumor cell line. It may therefore inhibit one or more non-Ras proteins to exert its antitumor effects. Gel electrophoresis, TUNEL assay, Annexin V assay, apoptosis dye-uptake assay, and morphological criteria were

used to characterize apoptosis. Adherent cells and freely floating detached cells in Arglabin-DMA treatment were treated as two distinct populations. We demonstrated that the detached cells caused by Arglabin-DMA exposure exhibited increased apoptosis in a p53-independent manner. Cell cycle effects were studied using flow cytometry. After Arglabin-DMA was added, the proportion of the two human colon tumor cells in G₂/M phase increased, indicating a block in either G₂ or M phase. We conclude that Arglabin-DMA has specific cytotoxic effects in two human colon tumor cell lines, and less cytotoxicity to normal cells. It induces arrest at the G₂/M phase of the cell cycle. After treatment with Arglabin-DMA, rounded and detached cells enter apoptosis. This mechanism may be analogous to "anoikis," which is the induction of apoptosis in response to loss of cell contact. The utility of this drug in combating cancer remains an attractive, though complex possibility.

I dedicate this thesis to my mother and father, Ae-Ja Kim and Jung-Eui Kwon whose love and patience was a source of power that encouraged me to pursue and complete this goal.

ACKNOWLEDGMENTS

To all of the special people who contributed in a very meaningful way to my ability to devote to myself to academic study and this dissertation: I am eternally grateful for your support, friendship, and love.

I wish to extend my appreciation to Dr. Christopher J. Osgood, my committee chair. He deserves special thanks for having the faith in me that motivated me to excel. I am indebted to him for his invaluable guidance during all these years of my study. I am deeply indebted to Dr. Lloyd Wolfinbarger, Jr. also for all the technical and the professional guidance that he provided me throughout the research. They took time to not only know me as an advisee, but also as a person. Most importantly, I will always value the relationship with these two professors throughout my life.

Appreciation is also extended to Dr. Paul J. Homsher of the Department of Biological Science (emeritus), and to Dr. Mark S. Elliott and Dr. James H. Yuan of the Department of Chemistry at Old Dominion University for their willingness to serve as the members of dissertation committee. To these three professors, I express my gratitude for providing technical expertise, guidance, and support during this endeavor. Dr. Keith A. Carson, Dr. Andrew S. Gordon, Dr. Elizabeth C. Minor, and Dr. R. James Swanson were invaluable in bringing their considerable helping in equipment and facilities throughout the research.

I owe a debt of gratitude to the faculties and colleagues in the Center for Biotechnology at Old Dominion University. A debt of gratitude is owed to Bindu Ramachandran, Leila Masinaei, and Sittisak Honsawek who served as colleagues of mine and who acknowledged, understood, and guided me in my research and my writing. Also, these friends will be cherished for giving their understanding hearts, priceless sense of humor, and technical expertise. I am greatly thankful to Dr. Jorge A. Sosa-Melgarejo for his aid as an adviser and as a beneficial guide.

Also, I would like to express my appreciation to my colleagues in the LifeNet research laboratory (Bart Gaskins, Katrina Crouch, Alyce Linthurst Jones, Bryan Simkins, Davorka Softic, Christian Kuc, Dan Osborne, Allison O'neal, and Xiao-Fei Qin); they are all very good friends and colleagues.

In addition, I would like to acknowledge Carmony Hartwig and Mohammad Khatib who helped me to accomplish my goals.

Finally, I am grateful for the support of my family (mother, father, two elder sisters; Dae-Suk, and Hee-Kyung, and one elder brother; Dae-Kyung) who were always there when I needed them. I thank them for serving as consequential and honorary supporters of this manuscript, for their aid in this recent doctoral program, and especially for their valuable guidance in all the important events in my life.

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INTRODUCTION

1. CANCER AND RAS

Normal cells grow, divide, and die in an orderly fashion. The cells of most tissues divide only to replace worn-out or dying cells, or to repair injuries. However, cancer cells continue to grow and divide, exhibiting uncontrolled growth. They can spread to other parts of the body via the bloodstream, or lymph system. These cells accumulate and form tumors.

Different types of cancers, named for the organ or type of cell in which they originated, vary in their rates of growth, patterns of dispersal, and responses to different types of treatment. That is why people with cancer require treatment that is targeted towards their specific form of the disease. The number of treatment choices depends on the type of cancer, the stage of the cancer, and other individual factors such as age, health status, and personal preferences.

Presently, the three major types of treatment for cancer are surgery, radiation, and chemotherapy. Chemotherapy involves the use of drugs to kill tumor cells. The several classes of chemotherapeutic drugs act by various means, most frequently by inhibiting the ability of tumor cells to divide. Therapeutic drugs are commonly used in combination

The model for this dissertation is British Journal of Cancer.

to attack these tumors, as these cells are less able to withstand a variety of agents attacking in different ways. The compounds may be introduced into the body systemically, or they may be concentrated at the tumor site (Ampil *et al.*, 1999).

New approaches for chemotherapy were developed using the characteristic molecular abnormalities of cancers. Recently, researchers have studied the molecular defects that transform normal cells into malignant cells. They found that many of these defects consist of mutations in key classes of genes that are responsible in some way for the growth and replication of cells (Gatenby and Maini, 2002). These mutations alter the behavior of proteins encoded by growth-regulating genes and disrupt the functions that control cell division. The discovery of mutant genes is enabling pharmaceutical researchers to design new drugs that will act specifically on these disrupted genes, or their proteins. The defects targeted by molecular therapy are found in three classes of genes: oncogenes, which stimulate cell progression through the cell cycle; tumor suppressor genes restricting this growth; and genes governing the replication and repair of DNA. Most tumors possess mutations in one or more of these gene categories (Oloff, 1999).

A detailed understanding of the mechanisms by which genetic mutations appear to have increased cancerous risk to the cell is anticipated to result in mechanism-based cancer therapeutics. These therapies specifically target the underlying defects in cellular growth regulation. Compared to current chemotherapeutic agents, these alternative

therapeutics may prove to be much less toxic and more effective, thus possibly producing superior therapeutic results. One potential target is the Ras protein, which is mutationally activated in a large range of human tumors and is an important contributor to the neoplastic phenotype (Bos, 1998; Rowinsky *et al.*, 1999).

The Ras superfamily consists of over 150 small GTPase proteins. This superfamily contains monomeric proteins that bind GDP (guanine diphosphate) and GTP (guanine triphosphate) and may be found in the plasma membrane and nuclear envelope. Within the superfamily there are six subfamilies: Ras, Rho, Ran, Rab, Arf, and Kir/Rem. Although many Ras members can interact with the same effector molecules, the physiological roles of most Ras-like GTPases are not fully understood (Bos, 1997; Ehrhart *et al.*, 2002).

The distinct members of the Ras superfamily are involved in a wide diversity of cellular functions, the Ras and Rho groups being of special interest. Both the Ras and Rho families mediate key cellular processes in response to diverse stimuli such as cell growth, differentiation, apoptosis, lipid metabolism, cytoarchitecture, membrane trafficking, and transcriptional regulation. However, the negative aspect of these multifunctional proteins arises by way of point mutations, overexpression, or insensitivity to regulatory signals. This results from the ability of these GTPases to trigger specific signals, which lead to uncontrolled cell growth, enhanced angiogenesis, inhibition of

apoptosis, and genetic instability. Collectively, these mutations can result in tumor development (Rechavi *et al.*, 1989; Vermes and Haanen, 1994).

The members of Ras family, at least 13 at present, are characterized by extensive similarities in their effector domains (Lowy and Willumsen, 1993). Besides three Ras proteins (H-Ras, K-Ras, and N-Ras) that share about 85% sequence identity, two other Ras proteins (M-Ras and R-Ras), four Rap proteins (Rap1A, Rap1B, Rap2A, and Rap2B), two Ral proteins (RalA and RalB), TC21, and Rheb are classified in the Ras family. The other members, except the three Ras proteins (H-Ras, K-Ras, and N-Ras), share at least 40-50% amino acid identity with p21 Ras (Adjei, 2001; Beaupre and Kurzrock, 1999). The Ras family as a whole acts as molecular switches that regulate a wide range of cell functions, including proliferation and differentiation (Gibbs *et al.*, 1994; Moores *et al.*, 1991; Tamanai, 1993).

The Rho family of proteins share at least 30% sequence identity, and include several members: Rho A, Rho B, Rho C, Rho D, Rho E, Rho G, TC10, Rac 1A, Rac 1B, Rac 2, CDC42Hs, and G25K (Takai *et al.*, 1995). These proteins play critical roles in the regulation of the cytoskeleton as well as in signal transduction of different stimuli (Hernandez-Alcoceba *et al.*, 2000; Takai *et al.*, 2001).

Ras and Rho family members exhibit a carboxy-terminal CAAX motif, where a cysteine is followed by two aliphatic residues and one random amino acid. This motif is a target for processing by enzymes, which results in the addition of a carboxy-terminal

prenyl group. Either a farnesyl or a geranylgeranyl moiety is involved in anchoring Ras to membranes (Casey, 1995). Inhibitors of the enzymes involved in this prenylation process show promise as inhibitors of Ras function by blocking its localization to the plasma membranes (Ehrhardt *et al.*, 2002; Reuter *et al.*, 2000).

In isoprenoid biosynthesis in mammalian cells, the FPP (farnesyl pyrophosphate) comprises 15 carbon chains. Also two molecules of the 10-carbon GPP (geranyl pyrophosphate) combine to form the 20-carbon GGPP (geranylgeranyl pyrophosphate) (Adjei, 2001). GPP and FPP are intermediates in the synthesis of monoterpenes and sesquiterpenes. Assorted monoterpenes and sesquiterpenes posttranscriptionally down-regulate HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase activity, a key activity in the isoprenoid biosynthetic pathway (the mevalonate pathway). The isoprenoid-mediated suppression of mevalonate synthesis depletes tumor tissues of two intermediate products, GGPP and FPP, which are incorporated posttranslationally into growth control-associated proteins. Isoprenoids targeted to the inhibition of the isoprenylation of oncogenic forms of *ras* proteins may offer a novel approach to chemotherapy (Elson, 1995) (Figure 1).

Two mevalonic acid pathway intermediates, GGPP and FPP, are substrates for protein isoprenyl transferases. These enzymes catalyze the formation of thioester bonds between the carbon chains of isoprenyl pyrophosphates and cysteine residues located near the carboxy terminals of a variety of proteins that include the nuclear lamins and the

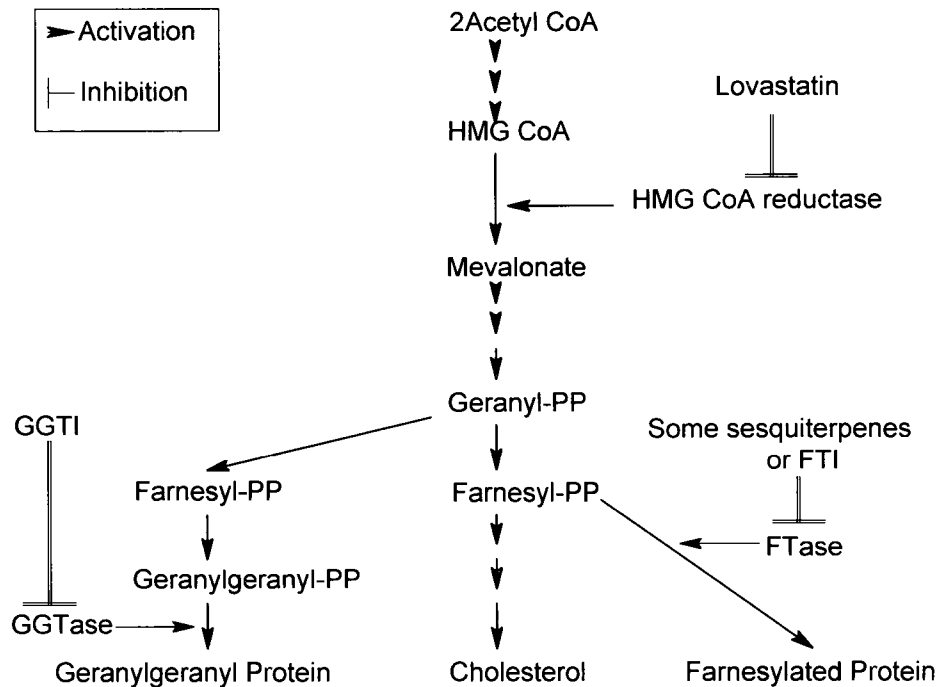
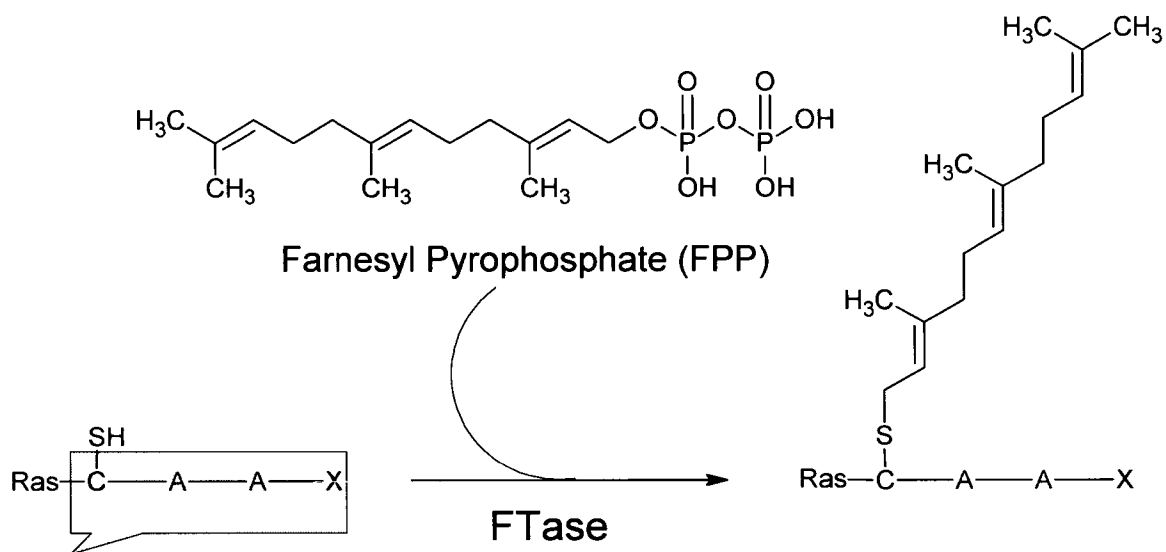


Figure 1 The isoprenoid biosynthetic pathway. Mevalonate is used as a precursor in metabolic labeling studies. Lovastatin blocks this pathway by the inhibition of HMG-CoA (3-hydroxy-3-methylglutanyl coenzyme A) reductase. GPP (Geranyl pyrophosphate), FPP (farnesyl pyrophosphate), and GGPP (geranylgeranyl pyrophosphate) are produced from mevalonate. FPP is responsible for the post-translational modification and membrane targeting of Ras protein, whereas GGPP is responsible for those of Rho family small GTP-binding protein. FTI (Farnesyltransferase inhibitor) or GGTI (Geranylgeranyltransferase Inhibitor) block farnesylation or geranylgeranylation to modify Ras or Rho. FPP is an intermediate in the synthesis of sesquiterpenes. Some sesquiterpenes like Arglabin-DMA inhibit farnesylation. (Elson, 1995; Holstein *et al.*, 2002; Maltese, 1990).

small and large G-proteins (Clarke, 1992). The development of inhibitors targeted to the Ras protein farnesyltransferase (FTase) offers an approach to the control of the growth of certain tumors (Goldstein and Brown, 1990).

The oncogenic *ras* genes in human cells have been identified, and are called H-*ras*, N-*ras*, and K-*ras*. These *ras* oncogenes encode four 21-kD proteins, called P21ras or Ras (H-Ras, N-Ras, and K-Ras4A and K-Ras4B, resulting from two alternatively spliced K-*ras* gene products) (Rowinsky *et al.*, 1999). Ras proteins consist of 188 or 189 amino acids that are highly conserved in the N and C termini. Most of the differences between these proteins occur in the near C-terminal hypervariable domain of about 25 amino acids, which is presumed to be responsible for their different functions (Adjei, 2001).

Ras is synthesized as a propeptide which undergoes a series of posttranslational lipid modifications that enable it to associate with the inner surface of the plasma membrane. The first step in posttranslational modification is mediated by FTase (farnesyl transferase), which transfers a farnesyl moiety from FPP to the cysteine moiety in the CAAX motif at the carboxyl terminus of Ras (Rowinsky *et al.*, 1999) (Figure 2). After farnesylation, CAAX proteins undergo cleavage of the AAX by CAAX protease. The exposed carboxyl group of the farnesyl-cysteine is carboxymethylated by a SAM (S-adenosyl-L-methionine) donor. In some Ras proteins, palmitoyl transferase catalyzes an additional modification of upstream cysteine residues by the fatty acid palmitate. These modifications enhance protein hydrophobicity and plasma membrane association (Adjei,



CAAX: C represents a cysteine residue; A represent aliphatic amino acids, usually valine, leusine, or isoleucine; and X is either methionine or serine

Figure 2 Ras posttranslational modification. The first step is mediated by farnesyltransferase (FTase), which transfers a farnesyl moiety from farnesyl pyrophosphate (FPP) to the cysteine moiety in the CAAX motif at the carboxyl terminus of Ras. FTase inhibitions block this enzymatic step (Rowinsky *et al.*, 1999).

2001; Gibbs and Oliff, 1997). A closely related enzyme, protein GGTase (geranylgeranyl transferase), recognizes the C-terminal CaaX motif present in proteins such as Rho A. After geranylgeranylation that transfers a geranylgeranyl moiety from GGPP to the cysteine moiety in the CAAX motif at the carboxyl terminus of target protein, these proteins undergo modifications that facilitate membrane association similar to Ras posttranslational lipid modifications (Tamanoi *et al.*, 2001) (Figure 3). Many mammalian proteins, besides the four forms of Ras, have a CAAX motif and are substrates for either FTase or GGTase (Rowinsky *et al.*, 1999) (Table 1).

Ras is activated in response to a variety of extracellular signals resulting in stimulation of tyrosine protein kinases, either directly or indirectly. Ras proteins function as molecular switches, and are determined by whether they are bound to GDP or GTP, conferred by the small GTPase (Ehrhardt *et al.*, 2002). The small GTPase, which cycles between active GTP-bound and inactive GDP-bound states, is activated by GEFs (guanine nucleotide exchange factors). GEFs include the SOS (son of sevenless) proteins and Cdc25. In the receptor tyrosine kinase cascade, the adaptor protein, Grb2 (growth factor receptor-binding protein), binds to activated receptors via its SH2 domain, then recruits GEFs to the cell membrane via its SH3 domain (Takai *et al.*, 2001). In its GTP-bound state, Ras interacts with several downstream effector pathways which contain multiple internal branch points. Raf is one of many effectors of Ras signaling. Other effectors include proteins Rac and Rho, and PI3K (phosphatidylinositol-3'-kinase), and MEKK (mitogen-activated protein kinase kinase kinase). The activation of the effector Raf phosphorylates MEK (mitogen-activated protein kinase kinase), which in turn

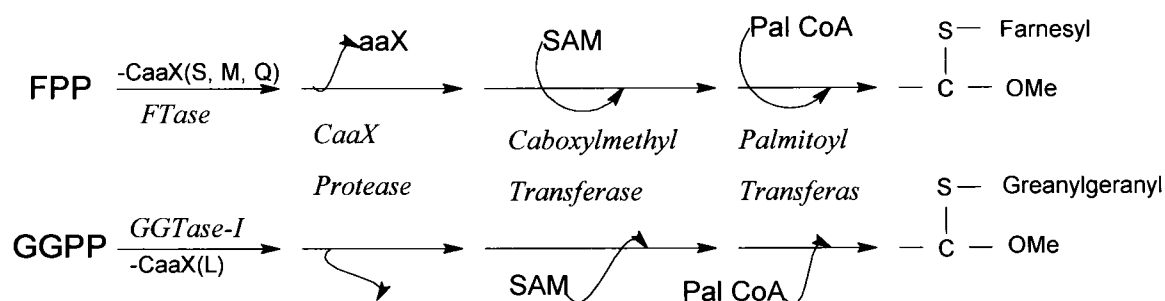


Figure 3 Posttranslational modifications of CaaX-containing proteins. FTase farnesylates CaaX substrates having C-terminal residues Ser, Met, and Gln (S, M, and Q). GGTase-I prefers CaaX substrates having a C-terminal Leu (L). (SAM, S-adenosyl-L-methionine; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate) (Gibbs and Oliff, 1997).

Table 1 Farnesylated or geranylgeranylated CAAX proteins

Farnesylated CAAX Protein(s)	Geranylgeranylated CAAX Protein(s)
H-ras, K4B-Ras, and N-ras	G-proteins γ -subunits
Lamins A and B	Rap 1
Rap2	Rho A, B, C, and G
Rho E	Cdc42
Pxf	Rac 1 and 2
Phosphorylase kinase α and β	R-Ras 1 and R-Ras 2/TC21
PRL-1/PTP CAAX 1 and 2	Ral A and B
Transducin γ	cGMP phosphodiesterase β
cGMP phosphodiesterase α	2'-3' ologo (A) synthetase 3'- phosphodiesterase
Rhodopsin kinase	Inosito-1,4,5-triphosphate 5- phosphatase type 1
YDJ1 homolog	
Inositol-1,4,5-triphosphate 5-phosphatase type 1	

Mammalian CAAX proteins that are known or likely to be prenylated. Data adapted from (Rowinsky *et al.*, 1999).

phosphorylates MAPK (mitogen-activated protein kinase), and ERK (extracellular signal-regulated kinase). Upon activation, MAPKs/ERKs stimulate transcription and proliferation. MEKK activates SEK1, which in turn phosphorylates another MAPK family member, JUN (jun amino-terminal kinase). It promotes apoptosis and proliferation. Activation of Rac and Rho also regulates cytoskeletal organization. PI3K signaling has been linked to a number of cellular processes (Adjei, 2001; Rowinsky *et al.*, 1999) (Figure 4). Ras activation leads to enhanced signal transduction pathways that mediate the cell cycle, and plays a role in the proliferation of cells. It also leads to the activation of other types of signals ranging from upstream of receptor tyrosine kinases to a downstream cascade of protein kinases. These kinases control a wide variety of cellular processes including mitogenic signaling, cellular differentiation, nuclear localization, cytoskeleton rearrangement, and apoptosis (Adjei, 2001; Lowy and Willumsen, 1993).

Mutant oncogenic *ras* genes are frequent genetic aberrations found in 20% to 30% of all human tumors, although the incidences by tumor type vary greatly. The highest incidence of Ras mutations was detected in adenocarcinomas of the pancreas, the colon, and the lung (Reuter *et al.*, 2000). Mutated *ras* oncogenes were first identified by their ability to transform NIH 3T3 cells after DNA transfection. Subsequent analysis of a variety of tumor samples revealed that in each tumor, one of the three *ras* genes harbored a point mutation (Rowinsky *et al.*, 1999) (Table 2). Mutation of K-*ras* is most commonly found in human tumors, whereas N-*ras* mutations are encountered less often and H-*ras* mutations are encountered rarely (Oliff, 1999). Mutations of the K-*ras* gene are found exclusively in colon, lung, and pancreatic cancers. In cancers of the kidney, bladder, and

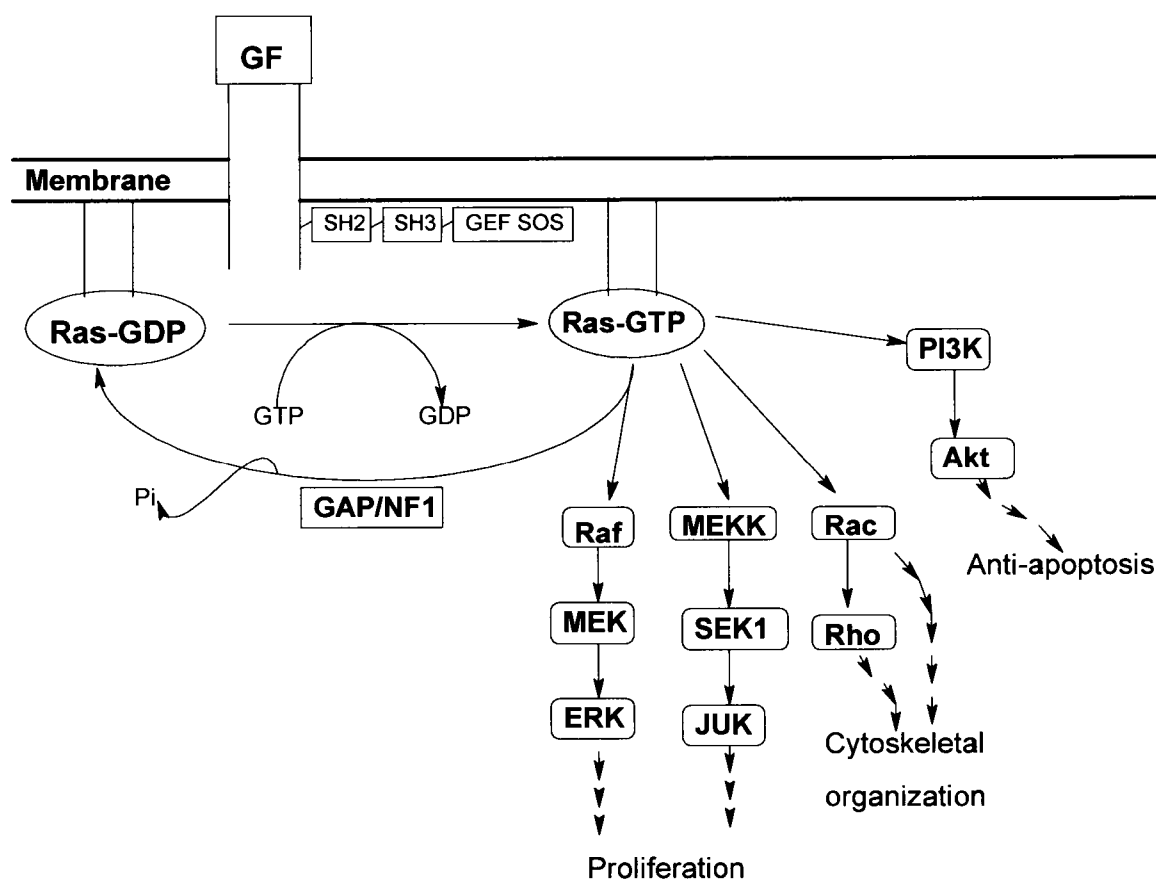


Figure 4 General downstream effectors of Ras protein and their functions. (GF, growth factor; GEF, guanine-nucleotide-exchange factor; SOS, son-of-sevenless; GAP, GTPase activator; NF1, neurofibromin; MEK, mitogen-activated protein (MAP) kinase kinase; ERK, extracellular signal-regulated kinase; MEKK, MAP kinase kinase kinase; JUK, Jun amino-terminal kinase; PI3K, phosphatidylinositol-3'-kinase; Akt, protein kinase B) (Rowinsky *et al.*, 1999).

Table 2 *Ras* mutation in human tumors

Tumor Type	Ras Mutation (%)	Predominant <i>ras</i> Mutation
Acute myelogenous leukemia	23-30	N
Bladder	10	H
Breast	2	H, K
Cholangiocarcinoma	> 50	N
Colon		
Adenocarcinoma	36	K
Adenoma	24-50	K
Colorectal	44	K
Endometrial carcinoma	18-40	K
Liver	10-30	N
Lung		
Large-cell carcinoma	21-33	K
Adenocarcinoma	22-50	K
Kidney	10	H
Melanoma	5-15	N
Ovarian carcinoma	23	K
Pancreatic carcinoma	> 80	K
Seminoma	43	K, N
Skin: keratoacanthoma	26	H
Thyroid		
Follicular adenoma	28	H, K, N
Follicular carcinoma	53	H, K, N
Undifferentiated carcinoma	60	H, K, N

(H, H-*ras*; K, K-*ras*; N, N-*ras*) Data adapted from (Adjei, 2001; Beaupre and Kurzrock, 1999; Rowinsky *et al.*, 1999).

skin, mutations are primarily in the H-*ras* gene, while mutations in the N-*ras* gene are consistently found in cases of leukemia (Adjei, 2001; Beaupre and Kurzrock, 1999). Thyroid carcinomas are unique in having mutations in one of the three *ras* genes. Most mutationally activated forms of *ras* genes identified in tumors result in disrupted guanine nucleotide regulation and constitutive activation of Ras (Lowy and Willumsen, 1993; Rowinsky *et al.*, 1999).

To inhibit constitutive activation of Ras in tumors, researchers have focused on the inhibition of FTase. Initial approaches to FTase inhibition involved the use of general inhibitors of isoprenylation. Synthesis of farnesyl groups can be blocked by HMG-CoA reductase inhibitors and the mevalonate pyrophosphate decarboxylase inhibitor (Adjei, 2001). Currently, a new class of antitumor drug has been described that targets the posttranslational modification of the Ras protein crucial for its activity (Gibbs *et al.*, 1994). Several different FTIs (FTase inhibitors) have been identified that competitively inhibit FTase by distinct biochemical mechanisms and can be placed into three major categories. The first of the general approaches has used design and synthesis of FPP analogs that compete with the substrate FPP for FTase (Tamanai *et al.*, 1993). The second class of FTIs is designed to mimic the Ras C terminus. The CAAX portion of Ras, starting at cysteine, contains all the critical determinants necessary for interaction with the enzyme FTase (Moore *et al.*, 1991). The final class of drugs combines the properties of the peptidomimetics and the farnesyl pyrophosphate analogs, and has therefore been termed bisubstrate inhibitor analogs (Manne *et al.*, 1995).

Ras protein is one of the primary target proteins of chemotherapy. It ordinarily behaves as a relay switch within the signal pathway that tells the cell to divide. It does this in response to external stimuli and results in activation of the signaling pathway. Activation of the *ras* protooncogene plays a role in the formation of cancers in the pancreas, the lung, and the colon (Bos, 1989). Even though there is some evidence to suggest that this oncogene influences the differentiation and proliferation of normal cells, as well as apoptotic processes, its precise role in malignancy is still unclear (Darley *et al.*, 1997). Ras has already been implicated in cell-signaling pathways of apoptosis, induced by various stimuli such as TNF- α (tumor necrosis factor- α), FAS, viral infection, and mechanical stress. Activation of Ras depends on both the inducer and the cell type; it will either inhibit or promote apoptosis (Billadeau *et al.*, 1995; Gulbins *et al.*, 1996; Trent *et al.*, 1996).

2. ARGLABIN-DMA

Sesquiterpenes are the largest class of terpenoids, and their structures are more diverse than any other class of terpenes. They are a widespread group of substances occurring in various plant species. Many sesquiterpene lactones have already been identified from the family Compositae, in particular Genera *Ixeris*, *Lactuca*, *Artemisia*, and *Cicholium* (Chung, 2001). The therapeutic properties of the *Artemisia* species, belonging to the Asteraceae family, are well known in the traditional pharmacopoeia of Europe, North America, and Asia (Klayman *et al.*, 1984; Norvaretti and Lemordant, 1990).

Sesquiterpene lactones inhibit a large number of enzymes involved in key biological processes such as DNA and RNA synthesis, purine synthesis, glycolysis, the citric acid cycle and the mitochondrial electron transport chain (Beekman *et al.*, 1997; Page *et al.*, 1987). They have been extensively used due to their wide variety of biological applications, which include: treatment of migraines and inflammation; cytotoxic and/or antitumor activities; shared the antibiotic property to act as phytotoxins, mammalian toxins, and insect-feeding deterrents; and also their ability to cause allergic contact dermatitis (Wen *et al.*, 2002). The cytotoxic and antitumor effects of sesquiterpene lactones have not been well studied due to their low potency. Recently it has been reported that some sesquiterpene lactones inhibit the *in vitro* growth of tumor cells in a cytostatic fashion, and it has been proposed that if their selectively cytotoxic or cytostatic actions against tumor cells can be established, sesquiterpene lactones may represent a new class of cancer chemotherapeutic drugs (Ross *et al.*, 1999).

Arglabin [1(R), 10(S)-epoxy-5(S), 6(S), 7(S)-guaia-3(4), 11(13)-dien-6,12-olide] (Figure 5), a sesquiterpene γ -lactone, was isolated in the early 1980s from the aerial part of the plant, *Artemisia glabella*, a species of wormwood endemic to the Karaganda region of Kazakhstan (Shaikenov *et al.*, 1998; Shaikenov *et al.*, 2001). The monoterpene limonene blocks cellular protein prenylation and causes the differentiation of tumor cells (Crowell *et al.*, 1991, Haag *et al.*, 1992). Arteminolode, a sesquiterpene lactone from *Artemisia vulvatica*, showed selective inhibitory activity against rat FTase (Lee *et al.*, 1998). Therefore, the structural similarity with cellular isoprenoids makes natural terpenoids attractive for development of inhibitors of protein prenylation, in particular,

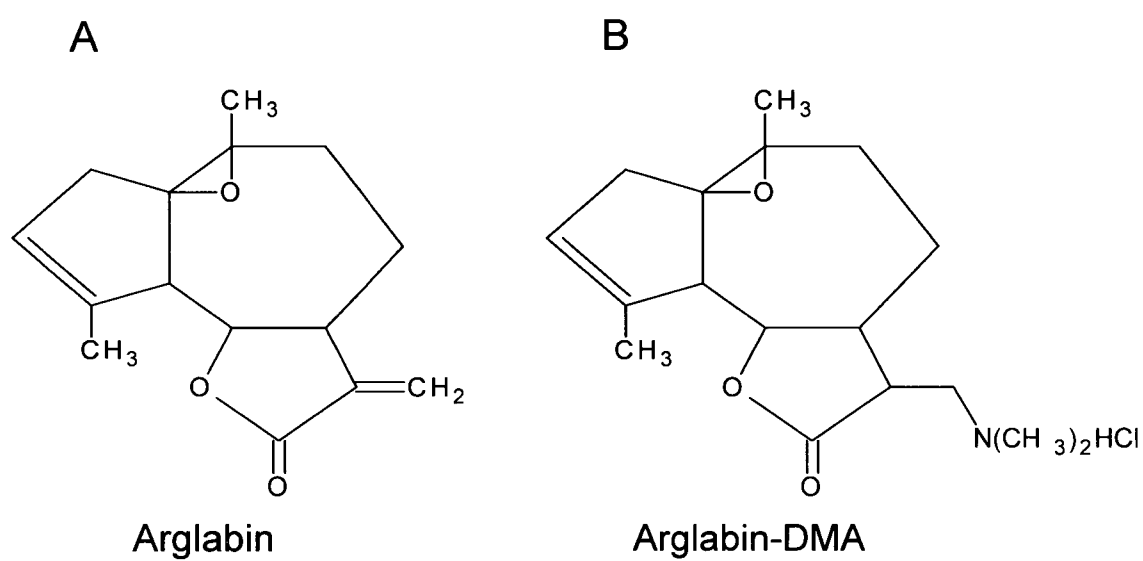


Figure 5 Structure of Arglabin and Arglabin-DMA. The sesquiterpene γ -lactone Arglabin, which is 1(R), 10(S)-epoxy-5(S), 6(S), 7(S)-guaia-3(4), 11(13)-dien-6, 12-olide (A), and water soluble dimethylaminohydrochloride derivative of Arglabin (Arglabin-DMA) (B) (Shaikenov *et al.*, 2001).

inhibition of FTase and the oncogenic activation of the Ras protein (Shaikenov *et al.*, 1999). Arglabin is a derivative of farnesyl pyrophosphate, which is a substrate for FTase, and has structural similarity with cellular polyprenols (Epstein *et al.*, 1991). Initial investigations focused on the ability of Arglabin to inhibit polyprenylation of cellular proteins because sesquiterpenes are made from farnesyl pyrophosphate (Shaikenov *et al.*, 1998).

The natural plant compound Arglabin is transformed by phosphorylation within cells, and this phosphorylated derivative can prevent farnesylation of Ras proteins, arresting cell proliferation. The mechanism of action of this new antitumor compound, Arglabin, suggests that some of the plant sesquiterpenes may be useful for anticancer therapy. Arglabin inhibits farnesylation without influencing the geranylgeranylation of cellular proteins. The mechanism of inhibition of the FTase reaction may be due to competition between farnesyl pyrophosphate and a phosphorylated derivative of Arglabin, affecting farnesyl at the C-terminal cysteine residue of the Ras proteins (Shaikenov *et al.*, 1998).

Arglabin is registered and approved for usage in clinics in the Republic of Kazakhstan as an antitumor medicine. The results of laboratory and preclinical studies have supported this usage by showing its high antitumor activity against malignant tumors. Importantly, the results of clinical trials found the preparation is non-toxic for the liver and the kidneys. Also, as a result of treatment, it has been reported that there was a

definitive change in the cancerous activities of hepatocellular cancer and tumors of the lungs, breast and ovaries. The patients with liver cancer exhibited rapid reduction in the size of tumors. There was general overall improvement of patient's condition and biochemical indices, blood tests, and immune status all improved. The tumors were slightly reduced in size and then stopped growing. The ovarian cancer patients reported no accumulation of ascetic fluid, and tumors were reduced by 30-40% of their original size prior to treatment. The preparation had no suppressive effect on hematopoiesis, no side effects, and appeared to normalize the functions of the immune system. Also, this drug did not affect the arterial pressure or other important functions of cardiovascular system (Institute of phytochemistry catalog, 2000).

Arglabin is a colorless crystalline compound isolated from *A. glabella*, chromatographically purified, and then recrystallized. In general, this compound is not water soluble and does not exhibit potent cytotoxicity. To overcome these limitations, the compound has been modified to render it water-soluble through the addition of a dimethylaminohydrochloride group to the C₁₃ carbohydrate moiety, yielding Arglabin-DMA (Figure 5). This renders it more effective against tumors at lower concentration. Arglabin-DMA is phosphorylated intracellularly. This complex inhibits the farnesylation of Ras protein by competing with farnesyl diphosphate and inhibits cell proliferation of a variety of tumor types (Shaikenov *et al.*, 2001).

The mutation of *ras* genes may affect alternative mechanisms of activation in tumor cells. The screening of natural compounds and the design of chemical molecules

therefore target the posttranslational modification of the Ras protein. Some of these compounds inhibit the enzyme FTase which is required for Ras to associate with the plasma membrane (Ouintero *et al.*, 1999). Arglabin, as a biosynthetic derivative of the farnesyl pyrophosphate, has a similar chemical structure to the farnesyl moiety of Ras proteins. Arglabin inhibition of cell growth may be mediated by its phosphorylated metabolites in the cells. The action mechanism of the new antitumor compound, Arglabin, suggests that some of the plant sesquiterpenes may be useful for anticancer therapy (Shaikenov, 1997; Shaikenov *et al.*, 1998).

3. APOPTOSIS

Birth and death are the two extremes of life. A balance between birth and death regulates cell numbers, relating proliferation, growth arrest, and cell death. A single ovum after fertilization (zygote) is capable of dividing and giving rise to a fully grown individual whose normal somatic cells are programmed to die after aging (i.e. programmed cell death (PCD) or apoptosis). Normal somatic cells are programmed to die (or undergo apoptosis); on the other hand, cancer cells program themselves to survive (Michaelson, 1991; Vermes and Haanen, 1994).

Cancer cells can be considered as a disturbance in the relative rates of cell proliferation and cell death. Cancer cells, whose origins are from a group of normal somatic cells, have many characteristics in common with those cells. Somatic cells

undergo aging and die, however, cancer cells also undergo cellular aging but are able to survive, sometimes achieving immortality (Michaelson, 1991). Some mutated genes responsible for production of growth factors and their receptors, misdirected signal transduction pathways, and many cell-cycle regulatory genes have been implicated in this process of cancerous transformation (Hanahan and Weinberg, 2000; Lundberg and Weinberg, 1999; Nowell, 1990).

Traditionally, more attention has been paid to aspects of cell proliferation rather than to cell death. There are two major way in which cells die: necrosis and apoptosis. Necrosis is a pathological death (an uncontrolled cell death), characterized by cell swelling, mitochondrial damage, a breakdown of homeostatic control, cell membrane lysis, aspecific DNA degradation, and leakage of cell content, etc. Necrotic cells cause inflammatory reactions in adjacent tissues. However, apoptosis, physiological death, is a genetically encoded cell elimination program that controls cell numbers and organ size (Figure 6). Although apoptosis as a form of cell death has been recognized for a long time, its relevance to cancer has been appreciated only in recent years. Indeed, cells with DNA damage, which escape the DNA repair mechanisms, are committed to suicide. This mechanism attempts to prevent the inheritance of DNA mutations in the potential progeny of sublethally damaged cells. Cells from a wide variety of malignancies show a decreased ability to undergo apoptosis in response to physiological stimuli (Nowell, 1990).

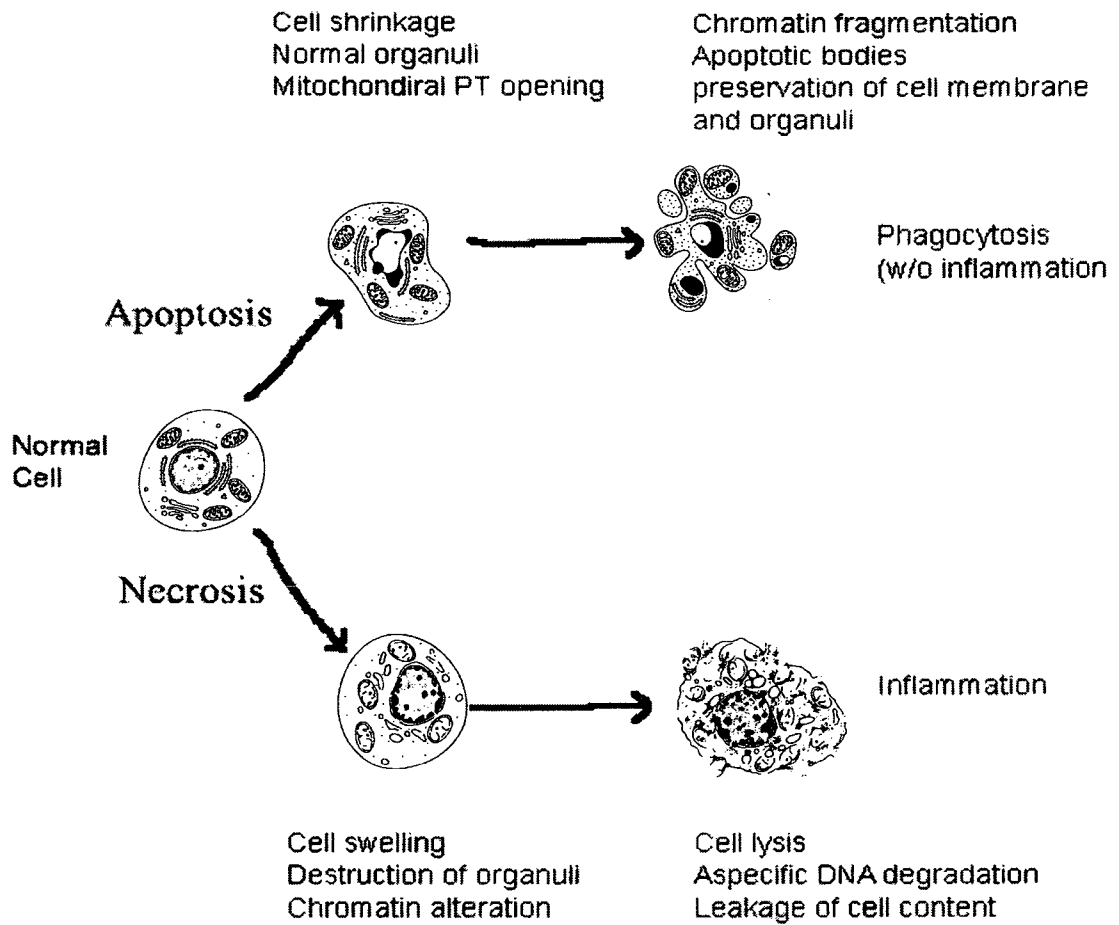


Figure 6 Main differences between apoptosis and necrosis (Michaelson, 1991; Tomei and Cope, 1991).

In all self-renewing tissues and cells, reproduction through mitosis is counterbalanced by cell removal through apoptosis. Cells dying by apoptosis usually undergo characteristic morphological and biochemical changes. Apoptosis, an orderly and genetically controlled form of cell death, concludes with phagocytosis of cellular remnants without causing damage such as inflammatory reactions to adjacent tissues. Apoptotic cells undergo separation from their neighbors by morphological, biological, and molecular changes. The types of morphological change consist of membrane blebbing, chromatin condensation, nuclear breakdown into micronuclei, and cytolysis into condensed apoptotic bodies. The types of biological and molecular changes include BCL-2/BAX interactions, loss of mitochondrial membrane potentials, phosphatidylserine externalization, DNA denaturation and cleavage (50-300kb) among others. Apoptosis is an important feature that allows the organism to eliminate DNA-damaged cells, and prevents survival of suboptimal cells in tissues which may otherwise lead to the propagation of damaged DNA to daughter cells (Hanada *et al.*, 1993; Rechavi *et al.*, 1989; Vermes and Haanen, 1994).

One of the first organisms in which suicide genes were identified was *Caenorhabditis elegans*, a nematode. Two death genes, *ced-3* and *ced-4*, and an anti-death gene, *ced-9*, have been identified in *C. elegans*. In mammalian cells, a cancer-causing oncogene, *bcl-2*, which is an anti-suicide gene similar to *ced-9* of *C. elegans*, is 23% identical in sequence. Bcl-2 was shown to prevent lymphocytes from committing suicide and to block apoptosis. Recently, the *ced-3* gene of roundworm, which plays a role in apoptosis, was shown to have sequence homology with a mammalian gene, "ICE".

This gene encodes a cysteine protease involved in the processing of interleukin-1b and acts as an interleukin-1b-converting enzyme (ICE) (Stewart, 1994; White, 1993).

Therefore, the *C. elegans* model has been valuable in the identification of the proteins that control apoptosis in human cells (Wickremasighe and Hoffbrand, 1999).

The process of apoptosis is dependent on the activities of a family of proteases known as caspases (Alnemri *et al.*, 1996). Caspases exist as inactive zymogens in all animal cells (Nagata, 1997). The activities of these proteases are governed directly or indirectly by a variety of other proteins that either promote or inhibit apoptosis. The final step of apoptosis induction, termed 'execution', involves proteases of the ICE family, which directly causes destruction of the cell (Martin and Green, 1995). Members of a family of proteases, the cysteine proteases, have an active site with a conserved amino acid sequence allowing them to cleave proteins following aspartate residues. These proteases play a direct role in the hydrolytic digestion of the cell. They have been dubbed caspases (cysteine-dependent aspartate cleaving protease) due to their shared structural and functional features. Caspase-1 was the first member to be identified as a protease involved in mammalian apoptosis, due to its homology with CED-3 (White, 1993; Yuan *et al.*, 1993). To date, ten members of this family have been identified by structure and substrate specificity in mammalian models. It is now generally accepted that the proteins involved in the execution of apoptosis belong to the caspase family. The caspases can be activated to induce apoptosis under a variety of conditions which include receptor-ligand coupled signal transduction, DNA damage, lack of growth factors, oxidative stress, and breakage of cell-cell and cell-matrix interactions.

When cells undergo apoptosis, they generally follow one of two major pathways. Though they share some mechanisms, these two pathways can be separated into an extrinsic pathway, and an intrinsic pathway. In some cases, extrinsic signals such as death receptor ligands (cytokines) are the apoptotic stimuli. This is also called the death receptor pathway. Such a mechanism is best illustrated by the tumor necrosis factor receptor (TNFR) family members, such as Fas (CD95, specific for the Fas ligand) and TNFR1 (which binds TNF). Upon its activation, Fas recruits FADD (Fas-associated death domain) by interaction of their respective death domains (DDs). Similarly, TRADD (TNFR1-associated death domain protein) binds to TNFR1 and connects it to FADD via interaction between these domains. Subsequently, FADD activates FLICE (FADD-like ICE, caspase), inducing the apoptotic execution which is the final stage of apoptosis. The procaspase recruited by death domain is immediately cleaved to produce the initiation caspase (caspase 8) (Green, 1998; Nagata, 1997). In the second type of death pathway, intrinsic signals such as damage caused by radiations and chemicals induce apoptosis in p53-dependent manner (Bennett, 1999). This is also called the intrinsic pathway or drug-induced pathway of apoptosis. The mitochondria and caspase apoptotic pathways are intimately connected (Sellers and Fisher, 1999). One of the proapoptotic factors released from mitochondria during apoptosis is cytochrome *c*. In the cytosol, cytochrome *c* binds the adapter protein Apaf-1 and, in the presence of ATP/dATP, it promotes the assembly of a multiprotein complex called the apoptosome which, in turn, binds and activates procaspase-9 (Ferraro *et al.*, 2003). The apoptosome is the executioner of the intrinsic pathway.

The second apoptotic pathway is likely regulated by the Bcl-2 family of proteins. The Bcl-2 family consists of proapoptotic factors such as Bax, Bak, Bcl-X_S, Bid, and Bad, and antiapoptotic factors such as Bcl-2, Bcl-X_L, and Bcl-W. Proapoptotic Bcl-2 family members translocate to and alter the permeability of the mitochondrial membrane. The proapoptotic factors activate to release cytochrome *c*. Bcl-2 and Bcl-X_L however act to prevent cytochrome *c* release and thus interfere with this caspase pathway (Green, 1998). The caspases are influenced by association of two different factors in Bcl-2 family such as Bax-Bax homodimer functions in active apoptosis. Also, the process can be opposed by heterodimerization of Bax with Bcl-2 (Bax-Bcl-2). This association blocks caspase activity and therefore allows for cell survival. It has been suggested that the ratio of Bcl-2 to Bax determines survival or death following an apoptotic stimulus (Hoffman and Liebermann, 1994; Oltvai *et al.*, 1993; Yang and Korsmeyer, 1996).

Recently, Bid, one of the Bcl-2 family, was found to be a very important mechanism for cross-talk between the apoptotic pathways. Inefficient activation of caspase-8 under the extrinsic pathway cleaves Bid, producing a C-terminal fragment. Activated Bid binds to mitochondria and induces release of cytochrome *c*, causing the formation of the apoptosome in the intrinsic pathway. The initiation caspases (caspase-8, -9) activate execution caspases such as caspase-3, -6, and -7. These execution caspases cleave relevant substrates that induce the biochemical and morphologic characteristics of apoptotic cells (Pruschy *et al.*, 2001) (Figure 7).

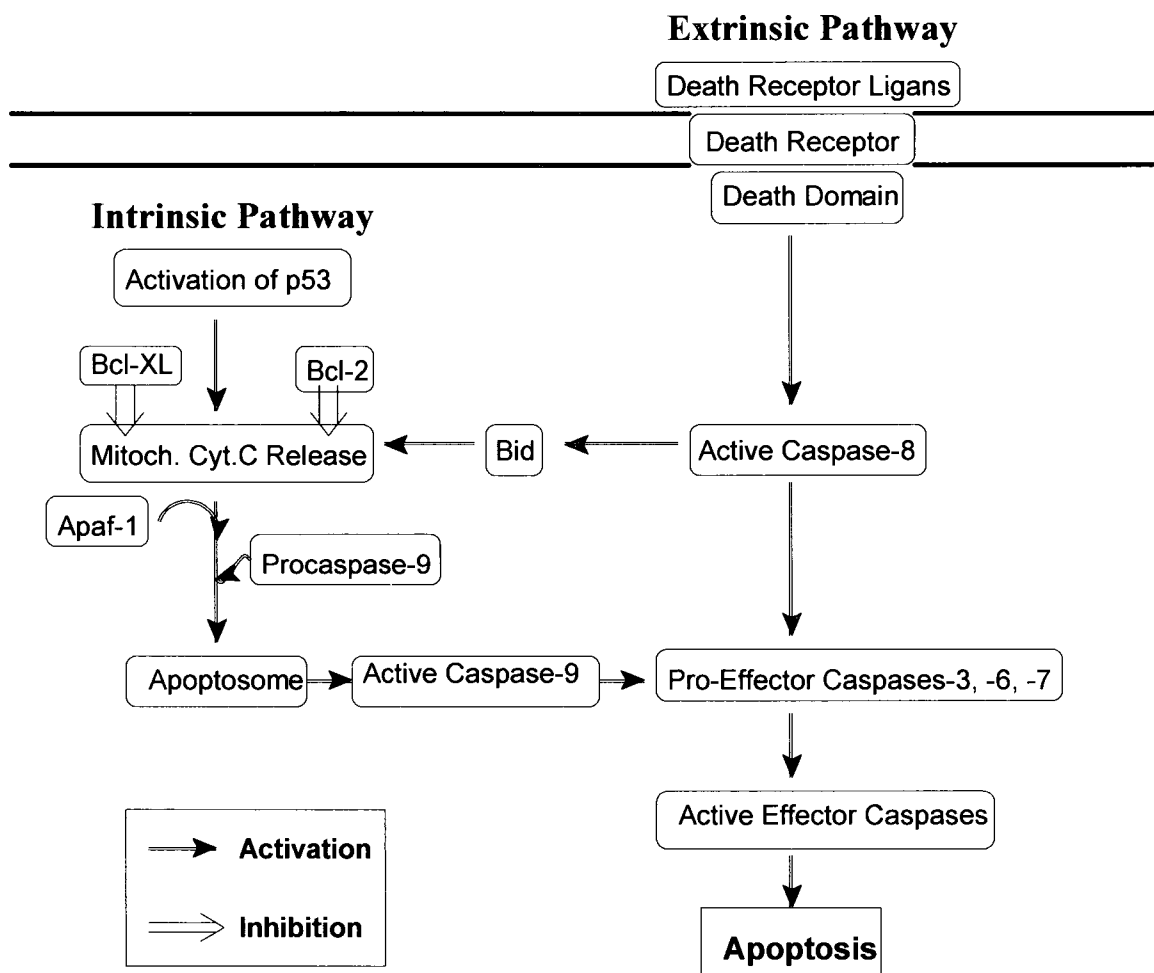


Figure 7 Three major apoptosis pathways. The first pathway, the intrinsic pathway, is represented by the death receptor. The second, the extrinsic pathway, is represented by the radiation and chemicals. The third pathway is a mechanism for cross-talk between the apoptosis pathways; in a weak caspase 8 of the extrinsic pathway, activates Bid mediate to release cytochrome c thus entering the intrinsic pathway. (Pruschy *et al.*, 2001; Schuler and Green, 2001).

Oncogenes and tumor suppressor genes control the balance between apoptotic death and antiapoptotic survival signals, which determine whether a cell proliferates or dies. Changes in products of oncogene or tumor suppressor genes can be responsible for initiation of apoptosis (Leblanc *et al.*, 1999). The *c-myc* oncogene, which is known to be important for control of cell proliferation, plays a part in the regulation of apoptosis. Ras activation of PI3K suppresses c-Myc-induced apoptosis through the activation of PKB/Akt (Kauffmann-Zef *et al.*, 1997). Removal of activated Ras in tumor cells initiates apoptosis that is dependent on c-Myc activity. Apoptosis is also initiated by an inhibitor of MEK (MAPK/ERK kinase), a kinase downstream of Ras (Tsuneoka and Mekada, 2000). Ras-mediated signals, that inhibit apoptotic cell death, were shown to act through PI3-kinase and AKT pathways, both leading to a block of cytochrome c release from the mitochondrial outer membrane (Kennedy *et al.*, 1999). The tumor suppressor gene, *p53*, has been shown to have dramatic effects on apoptosis. Its gene product, p53, can delay cell cycle progression before initiation of DNA replication by arresting this cycle following DNA damage (Slichenmyer *et al.*, 1993). The putative oncogene *bcl-2* is considered to be an apoptosis suppressor gene. Multiple genetic events are associated with tumor development. Among those events are the activation of *bcl-2*, and the inactivation of apoptotic inducers such as p53 and bax (Chiou *et al.*, 1994).

Cell death can also be induced by hormonal signals (prostaglandin E, adenosine) to the membrane. cAkinase (cAMP-dependent kinase) has been implicated in the nuclear response to hormonal signals for cell proliferation, differentiation, and apoptosis. Apoptosis is induced by agents that increase cAMP (eg. cholera toxin, theophylline,

Isobutylmethylxanthine) and cAkinase agonists (eg. cAMP analogues). Blocking gene expression and/or protein synthesis can prevent cAMP-induced apoptosis. ICER (inducible cAMP early repressor) represses cAMP-induced cell death. This indicates that cell death was induced by transcriptional activation of a 'killer' gene regulated by a CRE-containing promoter. ICER and Bcl-2 both act as upstream and downstream switches respectively, and may decide the fate of the cell (Raghoebier *et al.*, 1991; Rechavi *et al.*, 1989).

Many chemotherapeutic agents of diverse origin and different primary target molecules may induce apoptotic cell death in cancer cells via a convergence of intracellular pathways. Thus, the identification of impediments to this pathway, whether of genetic origin (e.g. *bcl-2*) or by interacting cytokines or factors as yet unknown, are important in identifying potential causes of drug resistance in malignant cells (Allen *et al.*, 1993).

Recent data show that in some cellular models, chemotherapeutic agents induce apoptosis, an irreversible process which leads to a rapid and complete elimination of tumor cells. However, further studies also demonstrated that apoptosis, induced by anticancer agents, is highly regulated by multiple signaling pathways which are themselves influenced by oncogenes, protein kinase/phosphatase activities, external stimuli, and the oxidative balance. Often, they share the same signaling pathways with cell proliferation and differentiation (Ruchaud and Lanotte, 1997). Therefore, it appears that cell death commitment is controlled by both external and internal factors which

interfere with the downstream of drug-target interactions. The characterization of these factors may provide novel targets for modulating drug-activated intracellular signaling pathways in order to promote apoptosis in drug-resistant cells (Allen *et al.*, 1993; Sellers and Fisher, 1999).

Two major cellular effects of FTI are cell cycle effects and induction of apoptosis. The FTI-induced apoptosis involves changes in mitochondrial membrane potential and the release of cytochrome c. The released cytochrome c activates caspase-9 and caspase-3, leading to DNA fragmentation. Detection of FTI-induced apoptosis requires special conditions, such as exposing cells to low serum conditions to inhibit cells from attaching to extracellular matrix (Edamatsu *et al.*, 2000; Lebowitz *et al.*, 1997; Suzuki *et al.*, 1998).

4. CELL CYCLE

The cell cycle consists of four sequential stages. G_1 is the first gap phase in which cells prepare for DNA replication. In this phase, cells monitor both their internal and external environments to determine whether to divide or to remain in a state of quiescence, known as G_0 . In G_0 , basic cellular metabolism is depressed in general, including transcription and translation. Once initiated, the cell progresses from the G_1 to S phase, the period of DNA synthesis for the reproduction of the whole genome. Then, the cell progresses to the G_2 phase, the second gap phase in which cells prepare for mitosis, before entering into M phase. M phase, or mitosis, is the period of cell division in which two identical daughter cells are produced during prophase, prometaphase, metaphase,

anaphase, and telophase. Cells can exit the cell cycle to settle into the G_0 phase in response to the deprivation of nutrients and growth factor stimulation (Brooks and La Thangue, 1999; Owa *et al.*, 2001).

As cells enter the cycle from G_0/G_1 , one or more D-type cyclins (D1, D2, and D3) are expressed as part of the delayed early response to mitogen stimulation. Progression through the G_1 phase initially depends on holoenzymes composed of one or more of the D-type cyclins (D1, D2, and/or D3) in association with cyclin-dependent kinases (CDKs), CDK 4 or CDK 6. The CDKs are protein complexes that are composed of a regulatory cyclin subunit and a catalytic partner. This is followed by activation of cyclin E-CDK 2 complex as cells approach the G_1/S transition. Together, cyclin E and CDK 2 act to hyperphosphorylate retinoblastoma protein (pRb), which then releases the elongation factor E2F that activates DNA polymerase. This step, termed the restriction point, represents the point of no return; cell commitment to undergo DNA synthesis (S phase) and mitosis is inevitable. In the S/ G_2 transition and G_2 phase, cyclin A binds CDK 2 and CDC 2, giving two distinct cyclin A kinase activities, one appearing in S phase, the other in G_2 (Pagano *et al.*, 1992). The G_2/M transition is mediated by activation of the cyclin B/CDC 2 kinase complex (Figure 8). At each phase of cell cycle, the scheduled activity of the CDKs is controlled by their association with cyclins and the CDK inhibitors (CKIs). CKI molecules are categorized into two separate families: 1) INK 4 family (p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}) specifically inhibit the catalytic subunits of CDK 4 and CDK 6; and 2) Cip/Kip family (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) inhibit the activities of cyclin D-, E-, and A-dependent kinases (Ammit and Panettieri, 2001; Sherr

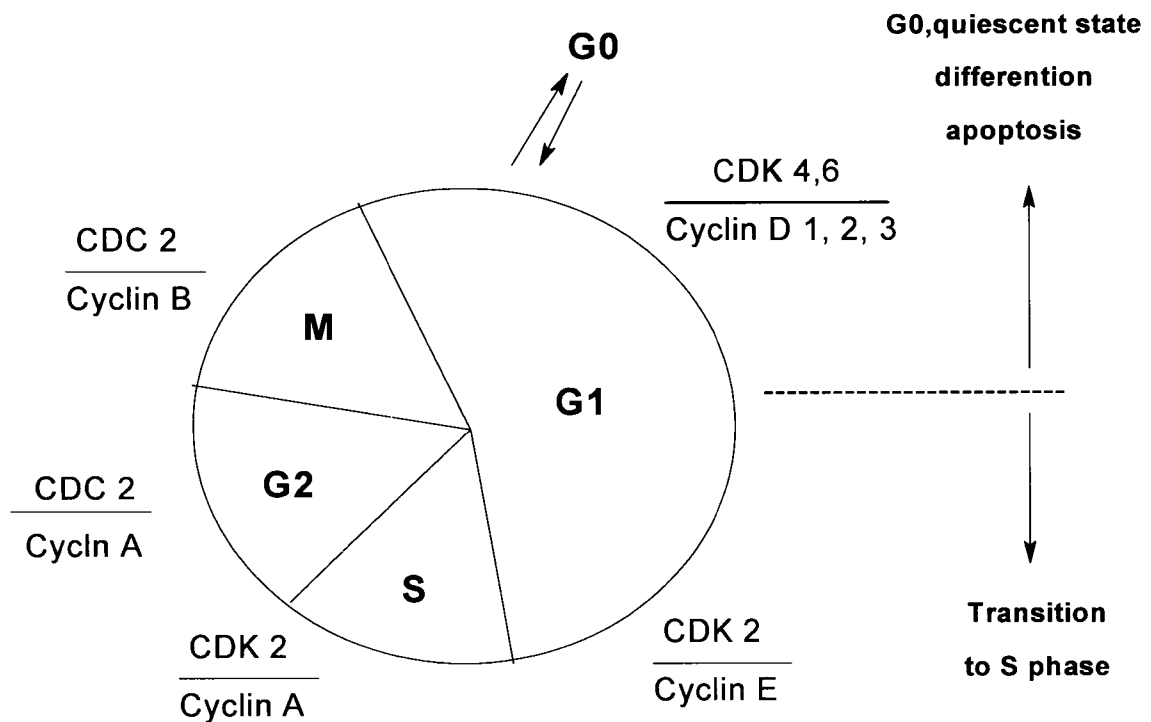


Figure 8 The cell cycle. The normal cell division cycle is sub-divided into four distinct phases: two gap phase (G1 and G2), S phase and M phase. CDK; cycling-dependent kinase, CDC 2; cell-division cycle, one of the CDKs (Brooks and La Thangue, 1999).

and Roberts, 1999). Each phase of the cycle is under the control of specific positive and negative regulators that either promote or arrest cell-cycle progression depending on their relative levels of expression (Brooks and La Thangue, 1999; Shapiro and Harper, 1999).

Proliferative disorders such as cancers are recognized as diseases of the cell cycle. The disorders are found in genetic alterations affecting proteins that control cell cycle progression. The mechanisms that normally function to restrain cell division are defective, while those that promote division become more active. The genes responsible for these changes in growth potential are generally named tumor suppressors and oncogenes, respectively (Brooks and La Thangue, 1999; Wang *et al.*, 1996).

Ras proteins are encoded from the proto-oncogene *ras*, and are involved in cell signaling and regulation of cell proliferation. Activated Ras interacts with a diverse spectrum of effectors, initiating a multitude of cytoplasmic signaling cascades. Recent studies have begun to establish the links between Ras signaling pathways and cell cycle regulatory proteins. The mechanism by which aberrant Ras and Rho GTPase activation promotes oncogenesis clearly involves a deregulation of cell cycle progression. Much is now known regarding how Ras and Rho signaling can control both positive and negative regulators to facilitate an exit from G₀, progression through G₁, and initiation of DNA synthesis (Bar-Sagi and Hall, 2000). Raf signal, activated by Ras, can induce cyclin D1 expression, allowing cyclin D1 to complex with Cdk4 and Cdk6 and phosphorylate Rb to affect the G1 to S transition. Rho activates CDK 2 and also inhibits p21^{Cip1} and p27^{Kip1} to induce cyclin D1 and stimulate the G1 to S transition. Rho represses p21^{Cip1} expression to

block p21^{Cip1} induction by Ras and to allow Ras induced progression from G1 to S (Liberto *et al.*, 2002). Also, Rac, one of the Rho family members appears to induce cyclin D1 expression and induce the G1 to S transition primarily through activation of NF- κ B to activate the cyclin D1 promoter. The cooperative action of Ras, Rac and Rho to induce cyclin D1 expression is a key component of oncogenic transformation (Chang *et al.*, 2003; Pruitt and Der, 2001).

Recent excitement in cancer research concerns the development of a new generation of antitumor drugs. These drugs are designed to target molecular events specific to cancer cells, including FTIs (farnesyl transferase inhibitors). FTIs have been developed in an attempt to inhibit membrane association of Ras. While it is demonstrated that FTIs are effective in reverting phenotypes due to H-ras activation, a number of reports suggested that there are targets other than Ras that are critical for the FTI effects. Studies on protein farnesylation define the effects of FTIs on cancer cells with the aim of gaining insight into the involvement of farnesylated proteins other than Ras on FTIs. These include members of the Ras superfamily G-proteins (Foster *et al.*, 1996; Lebowitz *et al.*, 1997; Murphy *et al.*, 1996). One of two major effects of FTI is the ability to cause changes in cell cycle progression (Jiang *et al.*, 2001; Tamanoi *et al.*, 2001). The cell cycle effect involves enrichment of either G₀/G₁ phase cells or G₂/M phase. Characterization of the FTI-induced G₀/G₁ enrichment led to the findings that FTI affects the expression and modification of a number of cell cycle related proteins. With some cell lines, G₂/M enrichment was detected after the treatment with FTI (Jiang *et al.*, 2001).

5. PURPOSE OF THIS STUDY

Many chemotherapeutic agents induce cell death in tumor cells via a convergence of intracellular pathways, which lead to apoptosis. After chemotherapy, the balance between the relative rates of cell proliferation and cell death is related to the regulation of apoptosis or the cell cycle. In previous studies, the major cellular effects of FTase inhibitors (FTIs) were cell cycle effects or induction of apoptosis. Arglabin-DMA has been shown to be a potent inhibitor of tumor growth. It may inhibit FTase as the chemotherapeutic target. However, the mechanisms by which Arglabin-DMA inhibits tumor growth *in vivo* and *in vitro* are still relatively unknown. To elucidate this mechanism, we hypothesize that Arglabin-DMA has major cellular effects similar to those of other FTIs to inhibit tumor growth.

The present study addresses the effects of Arglabin-DMA *in vitro*, using a cultured cell model to confirm or refute its proposed role as a Ras-dependent FTI. To determine the mechanism by which this drug inhibits tumor growth, the effects of Arglabin-DMA are examined in human colon tumor cells. Experimentation is divided into three specific aims: In the first specific aim, we re-examine the ability of Arglabin-DMA to inhibit Ras activity *in vitro*. The second examines whether Arglabin-DMA inhibits cancer growth in an apoptosis-dependent manner. Finally, we determine that Arglabin-DMA induces cell cycle arrest in human colon tumor cell lines.

5.1. Reexamination of the ability of Arglabin-DMA to inhibit farnesylation on the Ras protein *in vitro*

Viability assays are principally used to enumerate the proportion of living and dead cells in a population. Trypan blue is generally used for the viability assay. The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not enter the cell unless the membrane is damaged. This stain is actively extruded from viable cells, but readily enters and stains dead cells. Using viability assays, the viable population percentage between groups, which have been exposed for varying times or concentrations of the drug, is compared. These comparisons are used to determine the effectiveness of the drug action.

In today's pharmaceutical industry, most of the new anticancer drug candidates fail clinical trials due to their toxicity, even though they significantly inhibit tumor cell growth rate. These high failure rates may be reduced if potential drugs are screened for toxicity earlier in the drug discovery process. Evaluation of toxic effects of potential drugs is cost effective in an *in vitro* system using cultured cell lines or primary cells. Also, cytotoxicity studies are used to predict drug metabolism pathways in humans. To compare the effects of Arglabin-DMA in a normal human cell line and colon tumor cell lines for toxicity, several positive controls (5-Fluorouracil, Lovastatin, or Taxol) were used.

Previous studies showed that Arglabin-DMA inhibits polyprenylation of the cellular proteins, presumably because plant sesquiterpenes function as polyprenols of

cellular proteins. Arglabin can inhibit farnesylation without influencing geranylgeranylation (Shaikenov *et al.*, 1998). We attempted to reconfirm that Arglabin-DMA inhibits polyprenylation and the formation of isoprenoids (farnesyl pyrophosphate and geranylgeranyl pyrophosphate) which are byproducts of cholesterol synthesis.

5.2. Test whether Arglabin-DMA inhibits cancer cell growth in an apoptosis-dependent manner

We examine a broad and chronologically characteristic pattern of morphological, biochemical and molecular changes of apoptosis to determine whether Arglabin-DMA activates apoptosis in human colon tumor cells or not. At the biochemical level of apoptosis, double-strand cleavages of genomic DNA are found by conventional agarose gel electrophoresis and are also detected by the TUNEL assay in our experiments. Differences in the permeability of the cell membranes of living, dead, and apoptotic cells, are used to identify apoptotic cells using two DNA dyes, acridine orange (AO) and ethidium bromide (EB). The fine architecture of the cell membrane changes in the initial phase of apoptosis. This shift in the architecture of the plasma membrane involves the redistribution of the various phospholipid species between the two leaflets of the membrane. We can detect membrane conversion in apoptotic cells through the use of Annexin V. Finally, alterations of the plasma membrane in apoptotic cells lead to the accumulation within the cell of a detectable dye.

5.3. Testing whether Arglablin-DMA induces cell cycle arrest in human colon tumor cell lines

In the analysis of the mechanism of Arglablin-DMA using flow cytometry, the relationship of Arglablin-DMA and the cell cycle is studied in colon tumor cells. Once cells are stained with propidium iodide (PI) as the DNA fluorochromes, they are analyzed by flow cytometry. Where the relative content of DNA indicates the distribution of a population of cells throughout the cell cycle.

The aim of this last study is to compare the changes of the cell cycle after exposure to Arglablin-DMA. Studies using increased concentrations of Arglablin-DMA, followed by examination of cells using flow cytometry, are capable of showing the drug's concentration correlation with the inhibition of cell progression. Also, DNA content frequency histograms may reveal fractional DNA content, represented by the sub-diploid ($<2n$: sub- G_1) peak. In late apoptotic cells, DNA is degraded to such an extent that it becomes extracted during the routine procedure of cell staining. This approach is currently the most frequently used in flow cytometry to identify and quantify apoptotic cells (Nicoletti *et al.*, 1991).

MATERIALS AND METHODS

1. HUMAN CELL LINES

We investigated the effects of Arglabin-DMA on two human colon cancer cell lines, each having different Ras and p53 status (HCT 116 and HT-29). HCT 116, derived from a colon carcinoma, contains a mutation in codon 13 of the *K-ras* protooncogene and wild-type *p53*. HT-29, derived from a colon adenocarcinoma, has a mutation in codon 237 of the *p53* and a wild-type *ras*. The normal cell line (Hs 27), used as a control for examining cytotoxicity, is a fibroblast. All cell lines were obtained from American Tissue Culture Collection (ATCC, Rockville, MD. USA).

2. DRUG EXPOSURE

Arglabin, like other sesquiterpenes, is biosynthetically derived from farnesyl diphosphate by cyclization, rearrangements and oxidation. The water soluble form of Arglabin is Arglabin-dimethylaminohydrochloride (Arglabin-DMA; Figure 2). Powdered Arglabin-DMA was obtained from the Institute of Phytochemistry, Republic of Kazakhstan. It was dissolved with distilled water and presented at various final concentrations (15.25, 30.50, and/or 45.75 μM).

5-Fluorouracil, Lovastatin, Mynumycin A, or Taxol were used as positive controls in several experiments. They were obtained from Sigma (St. Louis, MO. USA). 5-Fluorouracil is a potent antitumor agent that affects pyrimidine synthesis by inhibiting thymidylate synthetase. It was dissolved in DMSO and administrated at 19.22 μM (Nita *et al.*, 1998). Lovastatin (Mevinolin) is a potent inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Lovastatin was dissolved in methanol and administrated at 10 μM (Agarwal *et al.*, 1999; Agarwal *et al.*, 2002). Manumycin A is a FTI (farnesyltransferase inhibitor) in that it is FPP (farnesyl pyrophosphate) analog. It was dissolved in methanol and administrated at 20 μM (Di Paolo *et al.*, 2000). Taxol (Paclitaxel) is an anti-tumor agent which binds to the N-terminal region of β -tubulin. It was dissolved in distilled water and administrated at 58 nM (Banerjee *et al.*, 1997). All drugs were stored at over -20°C and solutions were prepared fresh for each experiment.

3. INITIATION AND MAINTENANCE OF HUMAN CELL LINES

Two colon tumor cell lines and one fibroblast line were stored at -155°C , according to the instructions of ATCC for use. Complete media for HCT 116 and HT-29 was prepared using McCoy 5A medium (Sigma, St. Louis, MO. USA). Complete media for Hs27 was prepared with Dubecco's modified Eagle's medium (Sigma, St. Louis, MO. USA), each containing 10% fetal bovine serum (GIBCO BRL, Life Technologies, Gaithersburg, MD. USA), 200 mM Glutamine, 50 units/ml penicillin, and 50 mg ml^{-1} streptomycin (Sigma, St. Louis, MO. USA). Cells were thawed and placed in 25 cm^2 culture flasks (T-25) (Corning, Corning, NY. USA) in volumes of 5ml of complete media.

Cells were allowed to adhere to flasks, and the flasks were placed in a 37°C incubator with 95% air, 5% CO₂ humidified atmosphere. Cells were fed every 2 days with complete media after old media were discarded. The primary cultures were allowed to grow to subconfluence, trypsinized, and passed into 75 cm² culture flasks (T-75) (Corning, Corning, NY. USA). Depending on the cell density, cells in a given flask were subcultured into two or three flasks before reaching confluence.

Under these conditions, the population doubling time of HCT 116 was about 24 hours, that of HT-29 was about 30 hours, and that of Hs27 was about 48 hours. Cultures were regularly examined under the microscope for contamination and/or confluence; if the culture was contaminated, we added 1 to 2 ml of bleach and disposed of cultures in an appropriate biohazard container. If the cells grew to confluence, they were split. Cultures were found to be free of Mycoplasma contamination procedure. All experiments involved cells that were passaged more than 30 times. All procedures were performed under a sterile hood utilizing sterile techniques.

4. TRYPSINIZATION AND CELL PASSAGE

Cultures were passaged and harvested using 0.05% trypsin-EDTA (Sigma, St. Louis, MO. USA) to detach the cells. The cells grown to confluence were prepared to be split. The media from flasks was removed and discarded in bleach. The flasks were rinsed with 3 ml of trypsin-EDTA to remove residual media. Two ml of trypsin-EDTA in T-25 flasks, or 4 ml of trypsin-EDTA in T-75 flasks were added. The flasks were placed in the

incubator at 37°C. Trypsinization was periodically observed under the light microscope, as various cell types detach at different rates (trypsinization is typically complete within 3 to 10 minutes). When most of the cells had become “rounded” (i.e., when the trypsin had disrupted the cells’ protein connection to the flask surface), the flasks were tapped gently on each side to dislodge the attached cells into suspension. Each flask was neutralized with complete media, using the same as the amount of trypsin-EDTA. The cells and media were triturated to obtain a homogenous suspension. The cell suspension of colon tumor cell lines was aliquoted in 1 ml volumes into new sterile T-25 flasks or aliquoted in 3 ml volumes into new sterile T-75 flasks. The cell suspension of Hs 27 (normal cell line) was aliquoted in 2 ml volumes into new sterile T-25 flasks (5 ml total volume) or aliquoted 6 ml volumes in to new sterile T-75 flasks (15 ml final volume).

5. CELL COUNTING AND CELL VIABILITY

Cells were plated in T-25 at a density of 2×10^4 cells per cm^2 . After incubation for 24 hours, old media were discarded. This step was done to insure that more precise data would be obtained, due to the fact that Arglabin-DMA induces cell rounding and detachment. Complete media containing Arglabin-DMA were added to the flasks at various final concentrations (0, 15.25, 30.50, and 45.75 μM). 5-Fluorouracil (19.22 μM); Lovastatin (10 μM); or Taxol (58 nM) was used, separately as positive controls, and compared to the growth inhibition rate of Arglabin-DMA. Detached and attached cells in control and drug exposure samples were collected by trypsinization at different times (24, 48, and 72 hours) of incubation. As in the previously described trypsinization, the cells

and media were triturated to a homogenous suspension. 300 μ l of the suspension was diluted 1:1 with pipette into 300 μ l of 0.4% trypan blue solution (Sigma, St. Louis, MO, USA) into a disposable culture tube. The diluted suspension was mixed to the disposable culture tube containing the cell suspension. One drop from the disposable culture tube was pipetted into the hemacytometer and allowed to stand for 5 minutes.

The viable cells appear clear and round with no blue-colored dye; the blue-dyed, non-viable cells were counted in the four corner square chambers of the grid of a hemacytometer. Use of a hemacytometer is critical for obtaining an accurate count of cells. The cell count was calculated using the equation: $\text{cells/ml} = (n) \times 10^4$, where: n = the average cell count per square of the four corner square chamber counted. We determined the total number of cells in the total suspension volume. The percent viability of cells in culture was calculated. The cellular morphologies of human cell lines in various assays were examined under Olympus phase light microscope (Tokyo, Japan) at $100 \times$ magnification. Light photomicrographs (ISO 200 and 400) were taken with an Olympus camera (Tokyo, Japan).

6. ADD-BACK EXPERIMENTS

To evaluate the cellular mechanisms involved in Arglabin-DMA growth inhibition, add-back experiments were performed, adding intermediates (farnesyl pyrophosphate (FPP), or geranylgeranyl pyrophosphate (GGPP)) in cholesterol biosynthesis which belong to a class of molecules called isoprenoids (Holstein et al.,

2002). Isoprenoids are attached to several cellular proteins, including G proteins, by a posttranslational modification termed isoprenylation (Agarwal *et al.*, 1999). Lovastatin, which served as a positive control, induces morphological changes (cell-rounding and detachment) similar to Arglabin-DMA-induced cell-rounding and detachment. It is an HMG-CoA reductase inhibitor. Inhibition of HMG-CoA reductase inhibits the formation of isoprenoids and thereby impairs the post-translational processing of Ras and Ras-related proteins (Rap1a, RhoA, and RhoB). Isoprenylation of target proteins, like the GTP-binding protein Ras, is essential for their membrane localization (Agarwal *et al.*, 2002). Manumycin A, a second positive control, is a natural product derived from the bacterium *Streptomyces*, and is a FPP analog similar to Arglabin-DMA. It acts by the specific inhibition of the FTase and exerts antiproliferative effects on human tumor cells which harbor a mutated *K-ras* gene (Di Paolo *et al.*, 2000).

HCT 116 or HT-29 cells were seeded at 2×10^4 cells per cm^2 on T-25 flask, permitted to attach and grow for 24 hours. We discarded the old media and divided the cells into two groups after adding 30 μM Lovastatin, 61 μM Arglabin-DMA, or 20 μM Manumycin A, dissolved in fresh, complete media. 100 μM Farnesyl pyrophosphate (Sigma, St. Louis, MO. USA), or 10 μM geranylgeranyl pyrophosphate (Sigma, St. Louis, MO. USA) was then added. The samples were incubated for 3 days and photographs were taken after 1 and 3 days incubation for comparing the morphological appearances of the samples. 1×10^6 cells of each sample were collected. We followed the experimental procedures for cell viability for counting cells and also checked the change in the number of the floating cells, related to apoptotic cells. Higher doses of Lovastatin (30 μM),

Manumycin A (20 μ M), and Arglabin-DMA (61 μ M) were used in these experiments to clarify the inhibiting potential effect of these drugs.

7. ANNEXIN V ASSAY

HCT 116 or HT-29 cells were seeded at 2×10^4 cells per cm^2 in T-25 flask and incubated for 24 hours. We discarded the old media and added three final concentrations of Arglabin-DMA (0, 15.25, 30.50 and 45.75 μ M) dissolved in fresh, complete media. After three days of incubation, each cell line was split into three separate groups (control, attached in Arglabin-DMA treatment, and floating in Arglabin-DMA treatment). The following steps were followed using the ApoAlert Annexin V protocol (Clontech, Palo Alto, CA, USA). At each time point, 1×10^6 cells of each group were collected. The cells were rinsed and resuspended with $1 \times$ Binding Buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM CaCl_2). 10 μ l PI and 5 μ l EGFP-labeled recombinant human Annexin V was added. For analyzing cells, the cell suspensions were incubated on a glass slide at room temperature for 5-15 minutes in the dark. Slides were covered with a glass coverslip and observed under an Olympus fluorescence microscope (Tokyo, Japan), at a magnification of $200 \times$, using a filter set for acridine orange. Light photomicrographs (ISO 100) were taken with a Nikon camera (Tokyo, Japan) using 640T Tungsten chrome color film of Imation (Oakdale, MN, USA).

8. APOPTOSIS DYE-UPTAKE ASSAY

A second technique to detect alterations of the plasma membrane is the Apopercantage assay (Biocolor, Newtownabbey, Northern Ireland). During apoptosis, cells undergo the onset of the 'flip-flop' alteration to the cell membrane, causing translocation of phosphatidylserine to the exterior surface of the cell membrane. The Apopercantage Dye enters the cell following this event, and dye uptake continues until 'blebbing' is induced. At this stage, no further dye can enter the cell. Once in cells, Apopercantage does not escape (Wilson and Elliott, 2001).

Cells of each cell line were seeded at 2×10^4 cells per cm^2 in T-25 flask and allowed to attach overnight. The medium was replaced with fresh media or fresh media containing $30.50 \mu\text{M}$ Arglablin. After exposure of these cells to Arglablin-DMA, two cell lines of different *ras* status, HCT 116 and HT-29, underwent the morphological changes causing them to round up and become detached from the substrate. In determining cell viability, 80-95% floating cells were live cells that did not stain with trypan-blue. For examining floating cells that were morphologically affected by Arglablin-DMA, we collected and examined cells divided into two groups (floating or attached groups) in each of the colon cell lines after three days incubation. 2×10^5 attached cells or floating cells were collected and counted. Harvested cells were given two washes with PBS. $100 \mu\text{l}$ aliquots of complete culture medium containing Apopercantage Dye Label were added and samples were incubated for 1 hour at 37°C . Cells in each group were collected and resuspended in 1 ml of serum-free medium. The cellular morphologies in the

Apoptotic assay were examined. Normal cells, which appear clear, are round and exclude the dye; the apoptotic cells stained purple-red were counted in a hemacytometer under an Olympus phase light microscope (Tokyo, Japan) at a magnification of $100\times$. Light photomicrographs (ISO 200 and 400) were taken with an Olympus camera (Tokyo, Japan). Total apoptotic and normal cell numbers and the percent apoptotic cells were calculated.

9. IDENTIFICATION OF DYING CELLS BY APOPTOTIC NUCLEAR MORPHOLOGY

Detection of apoptotic cells in this experiment is based on differences in the permeability of the cell membranes of living, dead, and apoptotic cells. Two DNA dyes, acridine orange (AO; Sigma, St. Louis, MO. USA) and ethidium bromide (EB; Sigma, St. Louis, MO. USA), were used to find apoptotic cells.

HCT 116 or HT-29 cells were inoculated into 25 ml flasks with a viable cell density of 2×10^4 cells/cm². After incubation for 24 hours, old medium was discarded, and fresh complete media with or without 30.50 μ M Arglablin-DMA was added. After samples were incubated for three days, cells were divided to in three groups (control, attaching in Arglablin-DMA treatment, and floating in Arglablin-DMA treatment) and trypsinized for different times for each group. Cell counts were made to determine cell viability and cytotoxicity.

After counting, cells were given two washes with $1 \times$ phosphate buffered saline (PBS) (0.14 M NaCl; 3 mM KCl; 10 mM Na_2HPO_4 ; 2 mM KH_2PO_4 , pH 7.2). Twenty-five microliters of the cell suspension were mixed with 1 μl of the dye mixture containing 100 $\mu\text{g/ml}$ AO and 100 $\mu\text{g/ml}$ EB in PBS (pH 7.2). EB, as a fluorescent DNA-binding dye, is taken up by dying cells only. Metachromatic dye AO is taken up by both live and dead cells. But apoptotic cells can be distinguished by their different morphologies (Reed, 2000). After staining, cells were mounted on a hemacytometer. Cells were observed under an Olympus fluorescence microscope (Tokyo, Japan), at a magnification of $200 \times$, using a filter set for AO and 640T Tungsten chrome color slide film of Imation (Oakdale, MN USA). Light photomicrographs (ISO 100) were taken with a Nikon camera (Tokyo, Japan).

The status of cells was identified by counting the number of cells exhibiting one of five staining patterns: Stage one is bright green chromatin with organized cell structure-viable cells with normal nuclei. Stage two is bright green chromatin that is highly condensed-viable cells with apoptotic nuclei in an early phase of apoptosis. Stage three is bright orange chromatin that is highly condensed-nonviable cells with apoptotic nuclei in a late phase of apoptosis. Stage four is bright orange chromatin that is fragmented-nonviable cells with apoptotic nuclei in the late phase of apoptosis. Stage five is red-dead cells (Reed, 2000).

10. TUNEL ASSAY

The TUNEL (terminal deoxynucleotidyl transferase-(TdT)-mediated dUTP nick end labeling) assay was carried out using the Apoptosis Detection Kits protocol (R&D Systems, Minneapolis, MN. USA). The assay detects biotinylated nucleotides incorporated onto the free 3'-hydroxyl residues of DNA fragments, added by Terminal deoxynucleotidyl transferase (TdT). Also, it inserts a deoxynucleotide-triphosphate, dNTP, that has a chromophore attached. The use of this assay results in intense brown staining of the nuclei of apoptotic cells. Since necrotic cells may also contain DNA fragments that can incorporate label, the identification of apoptotic cells can be visualized on a histological section by light microscopy (Reed, 2000). In this assay, attached cells (HCT 116 and HT-29) were exposed to Arglabin-DMA, and then monitored by the TUNEL assay to test the occurrence of apoptotic events.

Sample preparation: Microscope slides and glass coverslips were sterilized by autoclaving for 20-30 minutes with or without pretreatment with poly-L-lysine or collagen to assist in cell adhesion. The sterile slides were transferred into a tissue culture dish. 2×10^4 cells/cm² of HCT 116 or HT-29 were seeded in the tissue culture dish and allowed to attach overnight. The media were replaced with fresh media or fresh media containing 30.50 μ M Arglabin. After 72 hours of incubation, the media were removed from the dish and the slides with adherent cells were carefully rinsed with 1 \times PBS (0.14 M NaCl; 3 mM KCl; 10 mM Na₂HPO₄; 2 mM KH₂PO₄, pH 7.2). The rinsed cells were

fixed by adding 3.7 % formaldehyde solution (1 × PBS with 37% Formaldehyde) for 10 minutes at 18-24 °C and then washed with 1 × PBS.

Labeling and detection: Each sample was permeabilized by incubation with 50 µl Proteinase K Working Solution (1 µl Proteinase K; 50 µl sterile water). The slides were incubated in a humidity chamber for 30 min at 18-24 °C and washed twice. To prepare the Nuclease-generated Positive Control using TACS-Nuclease, 50 µl of TACS-Nuclease Working Solution (1 µl TACS-Nuclease with 50 µl TACS-Nuclease Buffer) was pipetted onto a no drug sample. A coverslip was placed, and the slide incubated for 10 minutes in a humidity chamber. The other samples were covered with TACS Nuclease buffer during preparation of the Positive Control. The samples were immersed in Quenching Solution (30% H₂O₂ with 45 ml Methanol) for 5 minutes, quenching potential high levels of endogenous peroxidase, and then the samples were washed for 1 minute to remove H₂O₂ solution. The samples were immersed in 1 × TdT (Terminal deoxynucleotidyl Transferase) Labeling Buffer (10 × Labeling Buffer) for 5 minutes. The entire specimen was covered with 50 µl of Labeling Reaction Mix (1 µl TdT dNTP Mix; 1 µl 50 × Mn²⁺; 1 µl TdT enzyme; 50 µl 1 × TdT Labeling Buffer). To prepare an unlabeled Sample Control, one sample was treated with Labeling Reaction Mix from which the TdT enzyme had been omitted. All of the samples were placed in a humidity chamber in a 37°C incubator for 1 hour. The reaction was stopped by immersing the samples in 1 × TdT Stop Buffer for 5 minutes. The samples were washed twice with 1 × PBS for 5 minutes per wash and dried. 50 µl of Streptavidin-HRP Working Solution (1 µl

Streptavidin-HRP; 50 μ l of 1 \times PBS) were pipetted onto each sample to cover the entire specimen. The samples were incubated for 10 minutes and washed twice, 2 minutes per wash with 1 \times PBS. The samples were immersed in DAB Working Solution (30% H₂O₂; 250 μ l diaminobenzidine; 50 μ l 1 \times PBS) as background staining may occur. The samples were washed twice in tap water for 2 minutes each wash.

Counterstaining: The samples were immersed in 1% Methyl Green Counterstain for 1 minute and washed sequentially by dipping in each of the following solutions: deionized water (2 changes, ten times each); 95% ethanol (2 changes, ten times each); 100 % ethanol (2 changes, ten times each); xylenes (2 changes, ten times each). Each sample was pipetted with 50 μ l of mounting medium (Permount, Williston, VT, USA) and grown on glass coverslips. The cellular morphologies of human cell lines in various assays were examined under Olympus phase light microscope (Tokyo, Japan) at 100 \times or 200 \times magnification. Light photomicrographs (ISO 200 and 400) were taken with an Olympus camera (Tokyo, Japan).

11. ANALYSIS OF DNA FRAGMENTATION

In apoptotic cells, DNA fragmentation is regular, in contrast to the random DNA degradation that occurs in necrotic cells, in that it involves the generation of large DNA fragments. Apoptotic cells reflect double-strand cleavage of linker DNA between nucleosomes. Large DNA fragments were found by conventional agarose gel

electrophoresis, followed by the appearance of a ladder of low molecular weight fragments that are multiphases of ~180bp (Vermes and Haanen, 1994; Reed, 2000).

In previous studies of cell viability, exposure of cells to Arglabin-DMA caused them to round up and become detached from the substrate, suggesting that this compound may induce cells to undergo apoptosis. Therefore, three groups (control, attached in Arglabin-DMA treatment, and floating in Arglabin-DMA treatment) were separately collected. In order to detect the induction of apoptosis, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was detected with DNA samples extracted from the Arglabin-DMA treated cells at one or three days.

2×10^4 cells/cm² per cell line (HCT 116 or HT-29) were seeded in T-75 flasks and allowed to attach overnight. The media were replaced with fresh media, or fresh media containing 30.50 μ M Arglabin-DMA, 10 μ M Lovastatin or 58 nM Taxol. After 1 or 3 days of incubation, cells were collected in three groups (control, attached in Arglabin-DMA treatment, and unattached in Arglabin-DMA treatment), and counted. Harvested cells (over 3×10^6 cells) were lysed and transferred to centrifuge tubes. After centrifuging at 600g for 5 minutes, the supernatant was incubated with 18 μ g/ml proteinase K overnight at 50°C. The samples were centrifuged at 2000g for 5 minutes, and 100 μ l of sodium perchlorate was added. The upper layer was extracted in 150 μ l of Nucleon resin (Amersham Life Science, Buckinghamshire, UK). DNA was precipitated with 2 volumes of cold absolute ethanol. Precipitates were washed twice with 1 ml cold 70% ethanol and re-centrifuged at top speed (minimum 4000g). The pellet was air dried

for 10 min. The DNA was dissolved in 100 μ l TE buffer (10 mM Tris HCl; 1 mM EDTA, pH 8.0). After treatment with 2 μ g/ml RNase solution for 30 minutes at 37°C, the DNA sample was diluted, 2 μ l into 1000 μ l distilled water, and the DNA samples were placed in a quartz cuvette in order to read the optical density (OD). The concentration of DNA was read by measuring the absorbance of a sample at 260nm (A_{260}) on a UV-visible recording spectrophotometer (Shimadaza, Kyoto, Japan). A 1.8% agarose gel was made with 0.6 g agarose containing ethidium bromide ($0.5 \mu\text{g ml}^{-1}$) in 40 ml TBE buffer (89 mM Tris base; 89 mM Boric acid; 2 mM EDTA, pH 8.2). 10 μ g DNA samples in 1/5 DNA volume of 6 \times loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol; 30% glycerol) were loaded on the agarose gel and electrophoresed at 100 volts until the dye marker had migrated almost to be bottom of the gel. 1 kb plus DNA ladder (Life Technologies, Rockville, MD, USA) was used as the molecular weight DNA marker. After electrophoresis, the gel was visualized under UV light, and photographed using a Kodak DC 120 zoom digital camera (Eastman Kodak Company, Rochester, NY. USA).

12. FLOW CYTOMETRY ANALYSIS OF CELL CYCLE

The method is based on elution of low-molecular weight DNA from the ethanol-fixed cells, which is followed by cell staining with propidium iodide (PI; Sigma, St. Louis, MO. USA) as the DNA fluorochrome. PI binds to the major groove of double-stranded DNA after permeabilization of cells, which produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centered around 600 nm. Since PI can also bind to double-stranded RNA, it is necessary to treat the cells with RNase for

optimal DNA resolution (Banerjee *et al.*, 1997; Krishan, 1975). The relative content of DNA indicates the distribution of a population of cells throughout the cell cycle. For cycling cells, cells in the G₀/G₁ phases of the cell cycle have a diploid DNA content (2n). Cells within the G₂/M phases have a DNA content of 4n, while S phase has a DNA content greater than 2n and less than 4n (DNA synthetic) during which the DNA content has doubled (Ormerod, 1999).

The experimental groups consisted of cells treated with three concentrations of Arglabin-DMA (15.25 and 30.50 μM). Also, 19.5 μM 5-Fluorouracil was used for a positive control to demonstrate correctly stained cells and cell cycle effects. 5-Fluorouracil is a potent antitumor agent that affects pyrimidine synthesis by inhibiting thymidylate synthetase. Treatment of cells with 5-FU leads to an accumulation of cells in S-phase (Takeda *et al.*, 1999). Cells which were not subjected to any treatment with the drug were used as a negative control. HCT 116 and HT-29 cells were seeded at a density of 2×10^4 cells/cm². After 24 hours incubation in a CO₂ incubator, the media in the flasks were replaced with fresh media or with media containing Arglabin-DMA (15.25 and 30.50 μM) or 5-Fluorouracil (19.5 μM). The cells were incubated for a further 24 hours.

For each time interval, a cell pellet was obtained by centrifugation of floating cells and attached cells. The pellet was then washed once with 1 × PBS (0.14 M NaCl; 3 mM KCl; 10 mM Na₂HPO₄; 2 mM KH₂PO₄, pH 7.2). It was fixed in 70% ethanol and stored at 4 °C. At the time of processing, a pellet was obtained by centrifugation of the

samples at 2000 r.p.m. for 5 minutes. The resulting pellet was washed once with $1 \times$ PBS and then resuspended in 3 ml of $1 \times$ PBS.

100 μ l of 0.5 mg ml^{-1} RNase was added to each sample. The samples were incubated at 37°C for 30 minutes. The samples were then stained with 40 μ l of $0.5 \text{ }\mu\text{g ml}^{-1}$ propidium iodide. All samples were filtered through a $40 \text{ }\mu\text{M}$ pore mesh to protect the nozzle of the flow chamber in cytometer. The samples were analyzed using two different flow cytometers: a FACSCalibur flow cytometer (Becton-Dickinson Biosciences: Immunocytometry Systems, San Jose, CA, USA) at Eastern Virginia Medical School or the Coulter EPCICS C clinical flow cytometer (Beckman Coulter, Miami, FL, USA) at Old Dominion University.

13. STATISTICAL ANALYSIS

We used three different final inoculation cell densities (4×10^3 , 2×10^4 , or 4×10^4 cells/cm²). Cell growth in HCT 116 or HT-29 was tested with the addition of three different concentrations (15.25, 30.50, or 45.75 μM) of Arglabin-DMA for three different durations (1, 2, or 3 days) of incubation. Data under the same conditions were collected and analyzed. Samples chosen from data of untreated HCT 116 or HT-29 cells were compared as control groups with data from cells treated with Arglabin-DMA in HCT 116 or HT-29. The data files were analyzed using Adventures in Statistics 7.0 (SPSS, Syracuse, NY, USA). Statistical analysis shows that the critical t-value (t_{cal}) is greater than the observed t-values (t table), with the probability value (P-value).

First, differences in all test results between decreased cell numbers in HCT 116 or HT-29 after treatment with Arglablin-DMA were analyzed by a 2-tailed Student's *t* test. We hypothesize that the decrease in total cell growth numbers after treatment with Arglablin-DMA in HCT 116 or HT-29 is not significantly different. The student *t* test was used to compare mean values, and statistical significance was achieved when $P < 0.05$.

Second, a Paired Student's *t* test was performed to compare both the percentage of decreased cell numbers between HCT 116 and HT-29. The null hypothesis is that there are no difference in growth inhibition between HCT 116 (ras-; K-*ras* mutant) and HT-29 (ras +; wide type ras) after exposure of Arglablin-DMA. The cut-off level for statistical significance of differences was predetermined as $P < 0.05$ for all comparisons.

RESULTS

1. REEXAMINATION OF THE ABILITY OF ARGLABIN-DMA TO INHIBIT FARNESYLATION OF RAS PROTEIN *IN VITRO*.

1.1. Cell Viability

In our studies, three concentrations (15.25, 30.50, and 45.75 μM) of Arglabin-DMA were used. Two cell lines of different *ras* status, HCT 116 and HT-29, underwent similar cell rounding and detachment after treatment with Arglabin-DMA (Figure 9 and 10). Distribution of floating cells in HCT 116 and HT-29 was calculated from sixteen cell culture data sets. The range of floating cells per ml for HCT 116 after exposure of three different concentrations of Arglabin-DMA was $1.76 \sim 6.76 \times 10^4$ and for HT-29 it was $2.50 \sim 5.66 \times 10^4$ in a dose-dependent manner. The percentages of floating cells to total cell number was 8~39%, and 9~32%, respectively (Table 3).

About 4×10^4 cells/cm² were examined after 24hrs, because the population doubling times of both HCT 116 and HT-29 after exposure with Arglabin-DMA are about 24 hours. Cells were cultured for three more days. After incubation with no drug (control) and three different concentrations of Arglabin-DMA, total cell numbers in HCT 116 or HT-29 decreased in direct relation to the concentration of Arglabin-DMA (Figure 11), and three different concentrations (15.25, 30.50 or 45.75 μM) of Arglabin-DMA led to a reduction in cell numbers of 24.5%, 58.3%, and 66.2% in HCT 116, and by 11.8%,

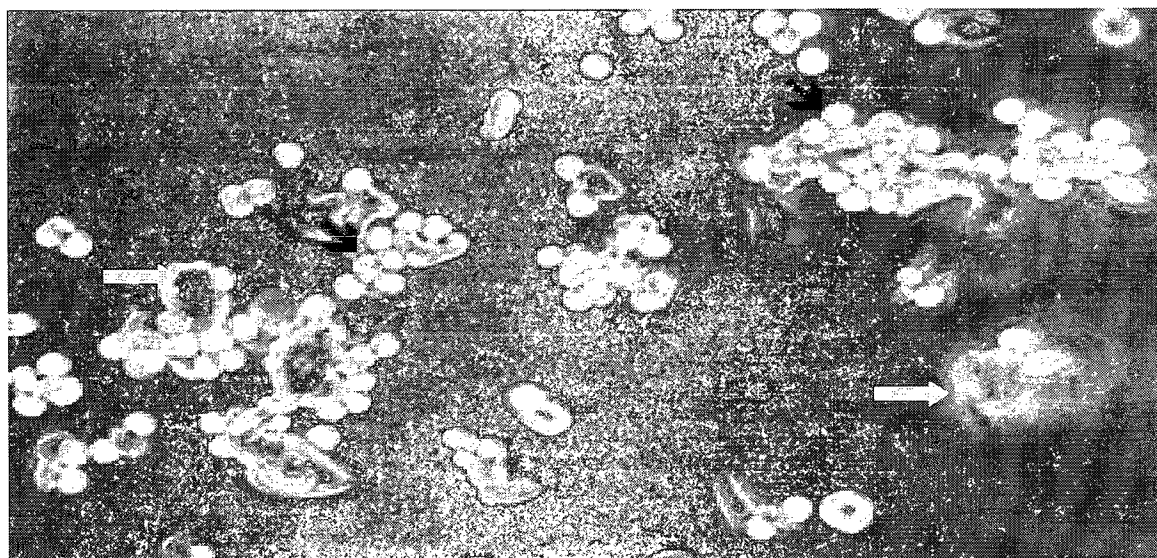


Figure 9 Morphology of floating cells and adherent cells in HCT 116. Adherent cells show anchorage-dependent growth, whereas floating cells show rounding and detachment after treatment with Arglabin-DMA (black arrow (→) indicates floating cell; gray arrow (→) indicates attached cells).

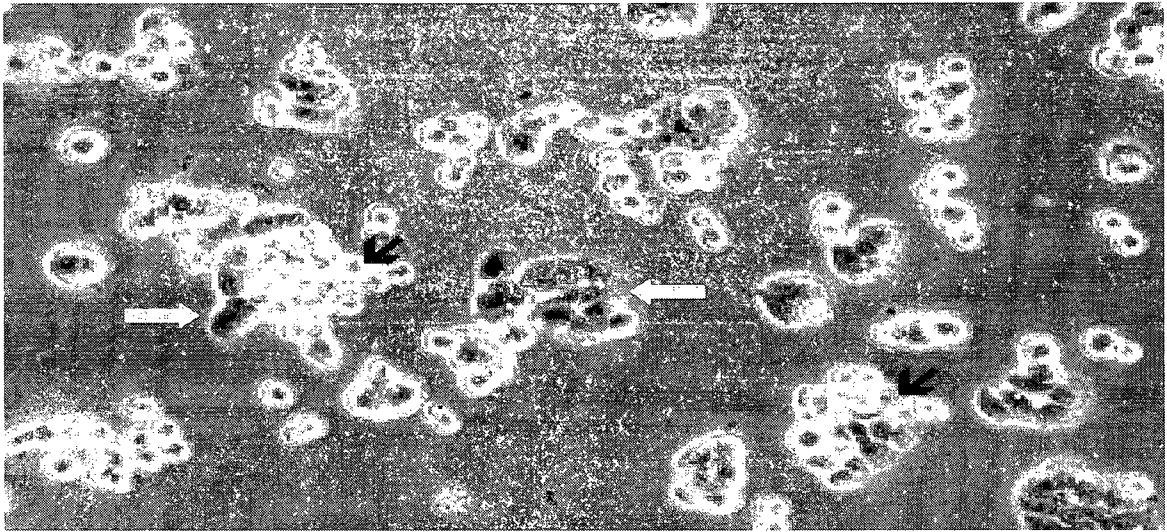


Figure 10 Morphology of floating cells and adherent cells in HT-29. Adherent cells show anchorage-dependent growth, whereas floating cells show rounding and detachment after treatment with Arglab-DMA (black arrow (→) indicates floating cell; gray arrow (←) indicates attached cells).

Table 3 Distribution of floating cells in HCT 116 and HT-29 after three different concentrations (15, 30 or 45 μM) of Arglabin-DMA (Floating cells: rounding and detached cells)

Floating cells	Mean (Cells/ml)	Range (Cells/ml)
HCT 116	3.54×10^4	$1.76 \sim 6.76 \times 10^4$ (8~39%)
HT-29	3.74×10^4	$2.50 \sim 5.66 \times 10^4$ (9~32%)

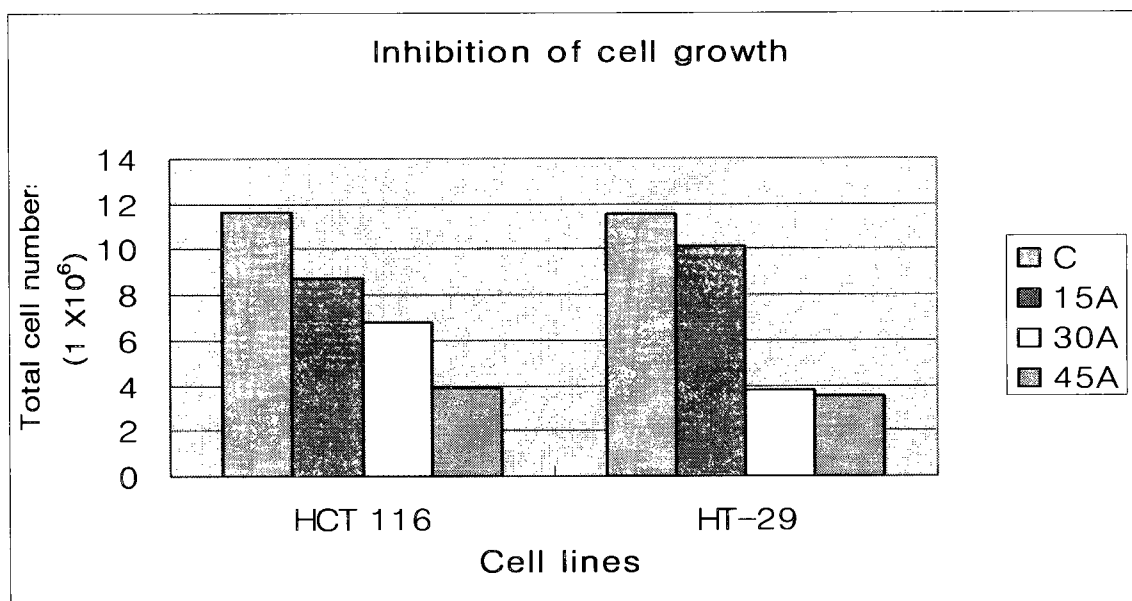


Figure 11 Inhibition of cell growth after exposure to Arglabin-DMA in HCT 116 or HT-29 cells. (C: untreated drug (Control), 15A: 15.25 μ M Arglabin-DMA, 30A: 30.50 μ M Arglabin-DMA, 45A: 45.75 μ M Arglabin-DMA).

67.4%, and 69.1% in HT-29 relative to the control values (Figure 12). Arglabin-DMA thus inhibits the proliferation of both cultured human colon tumor cells (HCT 116 and HT-29), having different ras status *in vitro*. It is probable that reduction in cell number after treatment with Arglabin-DMA is not affected by ras status.

Changing the cell number in the inoculation produced a significantly different result in regards to cell growth. Three different inoculation cell sizes (4×10^3 , 2×10^4 , and 4×10^4 cells/cm²) were used. Cell growth in HCT 116 or HT-29 was tested with the addition of three different concentrations of Arglabin-DMA (Figure 13 and Table 4). Also, the percentage of inhibition of cell growth in each group of the various concentrations of Arglabin-DMA was calculated by comparing the untreated group and the Arglabin-DMA treated group (Figure 14 and Table 5). It was determined that inhibition of cell growth was directly related to the concentration of Arglabin-DMA, and inversely related to the total cell number of inoculation in both HCT 116 and HT-29. Arglabin-DMA inhibits tumor cell growth in a dose-dependent manner. Also, no difference is seen between cell lines based on Ras status.

Although many anticancer drugs have been developed that have proven to inhibit the growth of cancer cells *in vitro*, many of these treatments involve chemicals that may also injure normal cells in cancer patients, resulting in impaired cell functioning. Serious harm to normal cells by anticancer drugs is an important problem in the development of drugs. After 72 hours of incubation with several positive controls (30.50 μ M Arglabin-

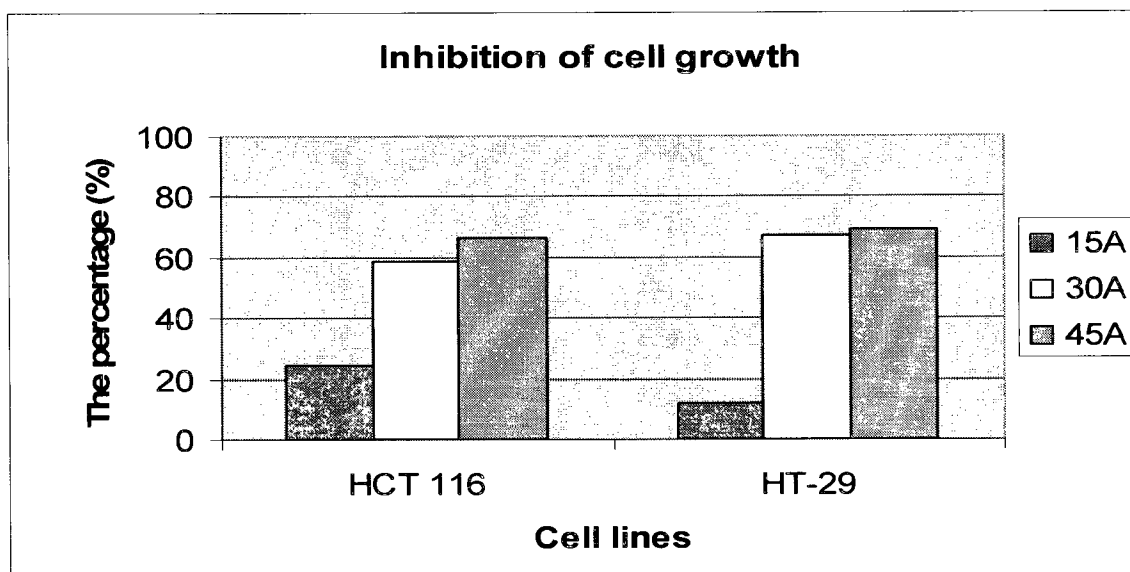


Figure 12 Percentage of cell growth inhibition after exposure of Arglabin-DMA in HCT 116 and HT-29 cells. (15A: 15.25 μ M Arglabin-DMA, 30A: 30.50 μ M Arglabin-DMA, 45A: 45.75 μ M Arglabin-DMA).

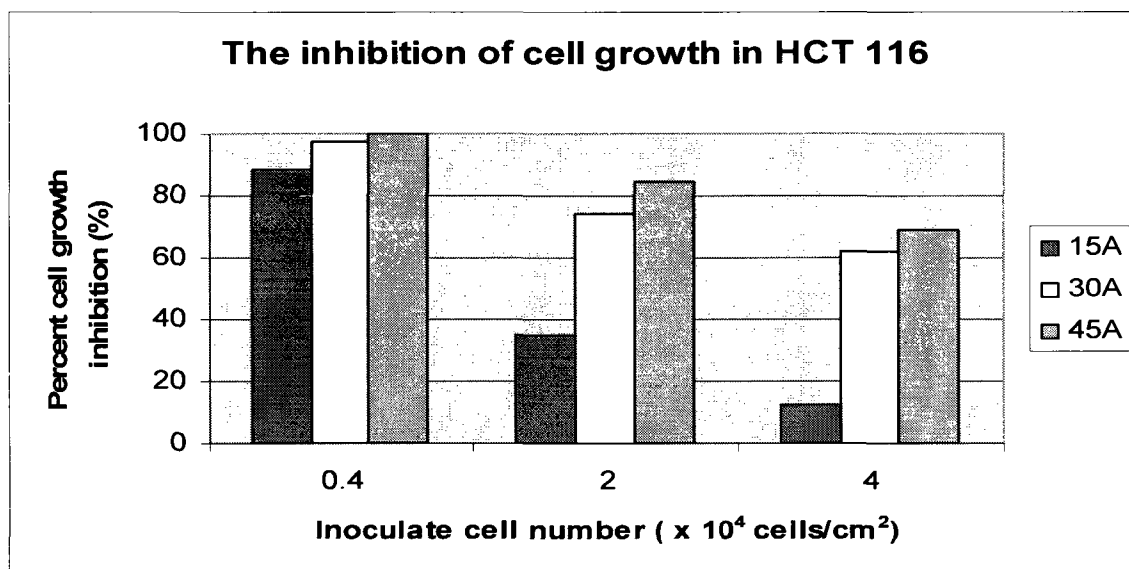


Figure 13 Percent inhibition of cell growth between inoculation numbers and concentrations of Arglabin-DMA in the HCT 116 cell line. (15A: 15.25 μ M Arglabin-DMA, 30A: 30.50 μ M Arglabin-DMA, 45A: 45.75 μ M Arglabin-DMA).

Table 4 Percent inhibition of cell growth between inoculation numbers and concentrations of Arglabin-DMA in the HCT 116 cell line

HCT 116 (cells/cm ²)	15A	30A	45A
4×10^3	88.58%	97.40%	100%
2×10^4	35.02%	74.31%	84.39%
4×10^4	12.15%	62.13%	69.10%

(15A: 15.25 μ M Arglabin-DMA, 30A: 30.50 μ M Arglabin-DMA, 45A: 45.75 μ M Arglabin-DMA).

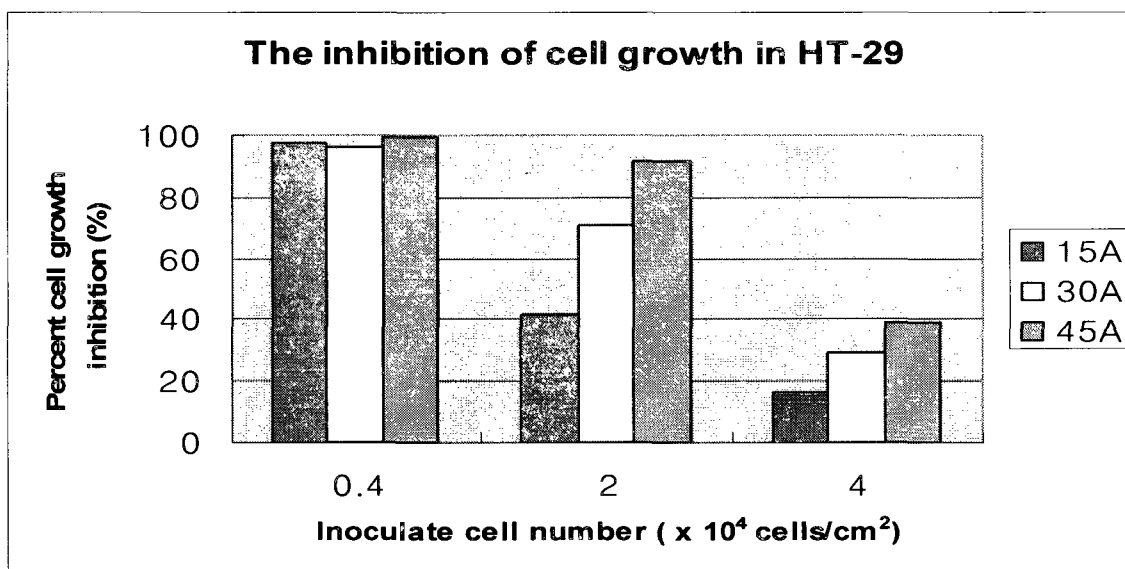


Figure 14 Percent inhibition of cell growth between inoculation numbers and the concentrations of Arglabin-DMA in the HT-29 cell line. (15A: 15.25 μ M Arglabin-DMA, 30A: 30.50 μ M Arglabin-DMA, 45A: 45.75 μ M Arglabin-DMA).

Table 5 Percent inhibition of cell growth between inoculation numbers and the concentrations of Arglabin-DMA in the HT-29 cell line

HT-29 (cells/cm ²)	15A	30A	45A
4×10^3	97.09%	95.81%	99.53%
2×10^4	41.60%	70.94%	91.74%
4×10^4	16.04%	29.18%	69.10%

(15A: 15.25 μ M Arglabin-DMA, 30A: 30.50 μ M Arglabin-DMA, 45A: 45.75 μ M Arglabin-DMA).

DMA, 19.5 μ M 5-Fluorouracil, 10 μ M Lovastatin, or 58 nM Taxol), Arglabin-DMA was examined to investigate relative toxic effects in normal cells (Hs27, fibroblast) in comparison with these other drugs.

Cell growth after exposure to Arglabin-DMA in HCT 116 or HT-29 cell lines was significantly inhibited: 79.13% or 77.06% decrease in total cell numbers, respectively (Figure 15, 16). Taxol in HCT 116 and HT-29 inhibits 84.20% and 78.40% of cell growth. 5-Fluorouracil in HCT 116 and HT-29 inhibited 64.57% and 69.31% cell growth. Arglabin-DMA, 5-FU, and Taxol thus significantly inhibit cell growth in both human tumor cell lines. In contrast, HCT 116 cells treated with Lovastatin decrease 52.39%, while HT-29 had only a slight decrease (19.01%) in cell growth. Our study showed that Lovastatin is thus highly effective in mutant *K-ras* HCT 116 cells, but not in normal *ras* HT-29 cells. The inhibition of cell growth due to treatment with Arglabin-DMA is similar to that of Taxol, and slightly greater than 5-FU and Lovastatin. Statistical analysis of cell growth inhibition between each drug compared with Arglabin-DMA in HCT 116 or HT-29 cell lines showed that Arglabin-DMA affects cell growth inhibition as that of Taxol or 5-FU and is more effect than Lovasatin (Figure 15, 16).

In a normal cell line (Hs27), cells treated with Lovastatin or Taxol decreased cell survival by 68.71% and 57.82% after three days of incubation. The 5-FU treated group was reduced 43.30%. Arglabin-DMA inhibited 41.58% cell growth in Hs27. Statistical analysis showed that the probability value of both Taxol and Lovastatin compared with

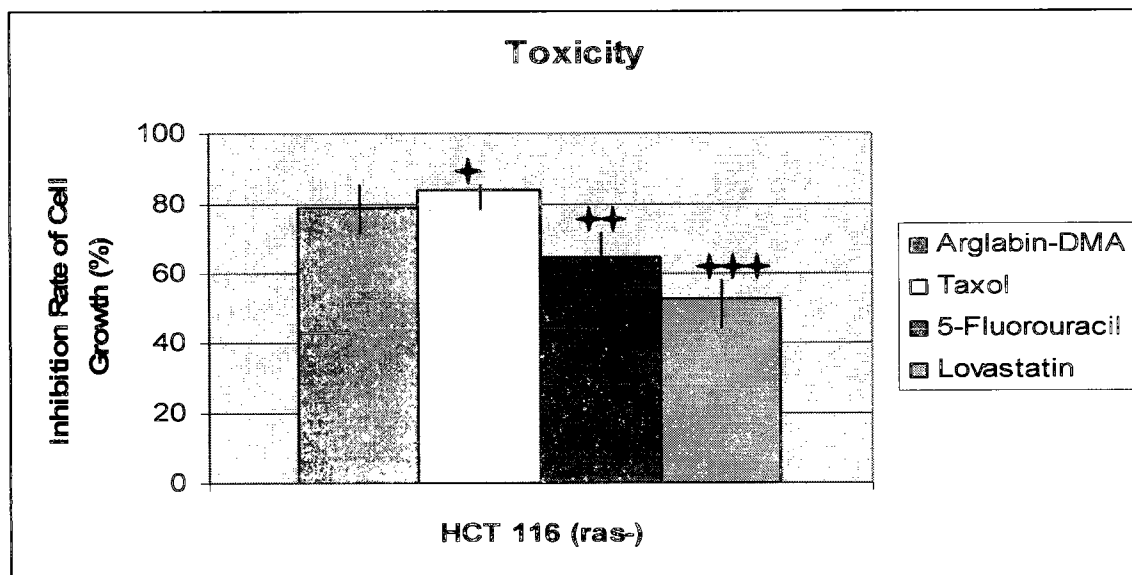


Figure 15 Arglabin-DMA cytotoxicity in HCT 116 after three days incubation. (30.50 μ M Arglabin-DMA, 19.5 μ M 5-Fluorouracil, 10 μ M Lovastatin, 58 nM Taxol).

◆ : P= 0.202, Taxol vs. Arglabin-DMA.

◆◆ : P= 0.120, 5-Fluorouracil vs. Arglabin-DMA.

◆◆◆ : P= 0.001, Lovastatin vs. Arglabin-DMA.

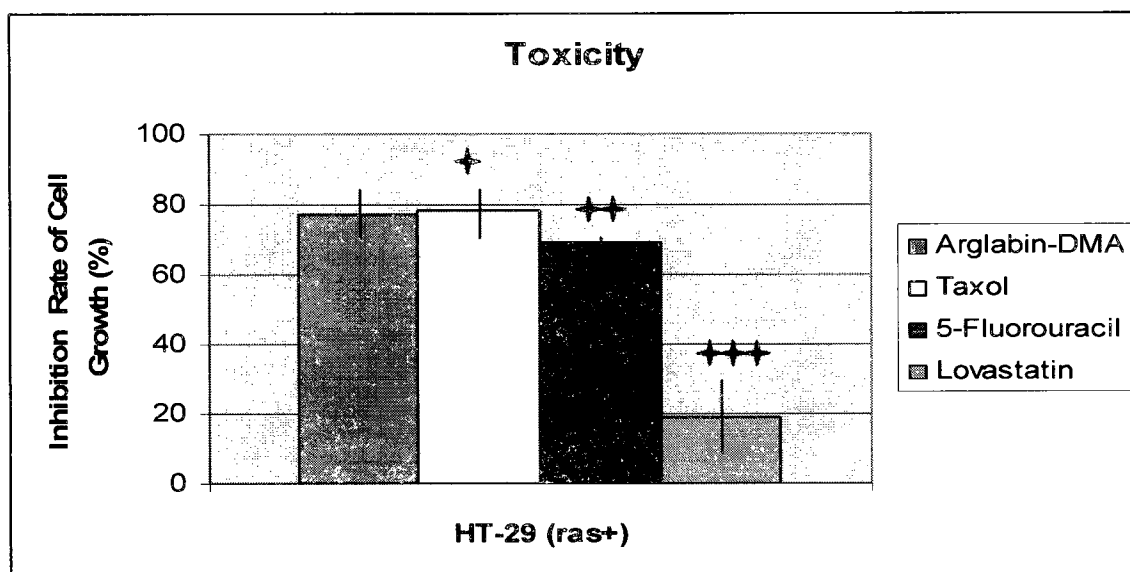


Figure 16 Arglabin-DMA cytotoxicity in HT-29 cell line after three days incubation. (30.50 μ M Arglabin-DMA, 19.5 μ M 5-Fluorouracil, 10 μ M Lovastatin, 58 nM Taxol).

- ◆ : P= 0.833, Taxol vs. Arglabin-DMA.
- ◆◆ : P= 0.268, 5-Fluorouracil vs. Arglabin-DMA.
- ◆◆◆ : P= 0.010, Lovastatin vs. Arglabin-DMA.

Arglabin-DMA in HCT 116 or HT-29 cell lines are smaller than 0.05; in contrast, the probability value, compared with 5-Fluorouracil, showed greater than 0.05 (Figure 17). Therefore cell growth inhibition of Arglabin-DMA in normal Hs27 is less than that of Lovastatin or Taxol, and is similar to that of 5-FU. After three or eight days incubation with Arglabin-DMA or Taxol, it was found that the viability of Hs27 cells treated with Arglabin-DMA had increased from 41.38% to 53.01%. The groups treated with Taxol had a more dramatic decrease in total cell growth (68.97% to 85.79%) (Figure 18 and Table 6). This signifies that normal cells (Hs27) are less susceptible to Arglabin-DMA-induced toxicity, and the drug has less potential for inhibiting normal cell growth, particularly after longer exposures.

1.2. Statistical analysis

Changing the cell number of the inoculation, the concentration of drug, or the incubation time in both human colon tumor cell lines (HCT 116 or HT-29) produced significantly different results in regards to cell growth and viability.

To examine whether Arglabin-DMA inhibits specifically mutant *K-ras* tumor colon cells or not, statistical analysis was executed to analyze cell culture data obtained from each experiment. The results of the *t* test for both HCT 116 and HT-29 were significant when cell lines treated with Arglabin-DMA were compared to the untreated control (Table 7). Therefore, cell growth inhibition by Arglabin-DMA in HCT 116 or HT-29 has a significant effect. Also, we compared toxicity in HCT 116 and HT-29 after

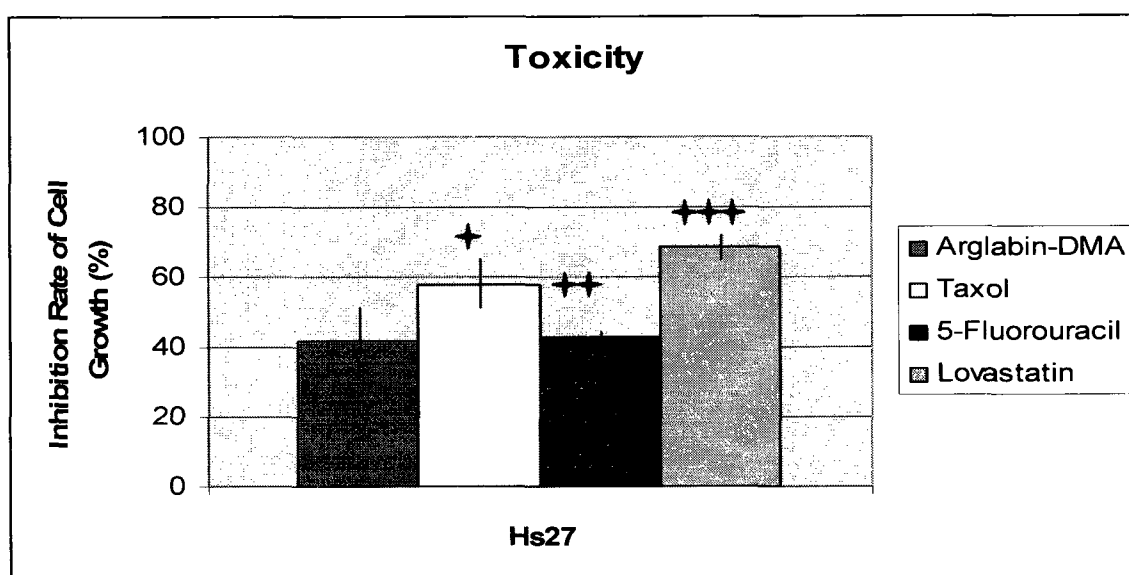


Figure 17 Cytotoxicity in Hs27 after three days incubation with four anticancer drugs. (30.50 μ M Arglabin-DMA, 19.5 μ M 5-Fluorouracil, 10 μ M Lovastatin, 58 nM Taxol).

- ◆ : P= 0.028, Taxol vs. Arglabin-DMA.
- ◆◆ : P= 0.073, 5-Fluorouracil vs. Arglabin-DMA.
- ◆◆◆ : P= 0.007, Lovastatin vs. Arglabin-DMA.

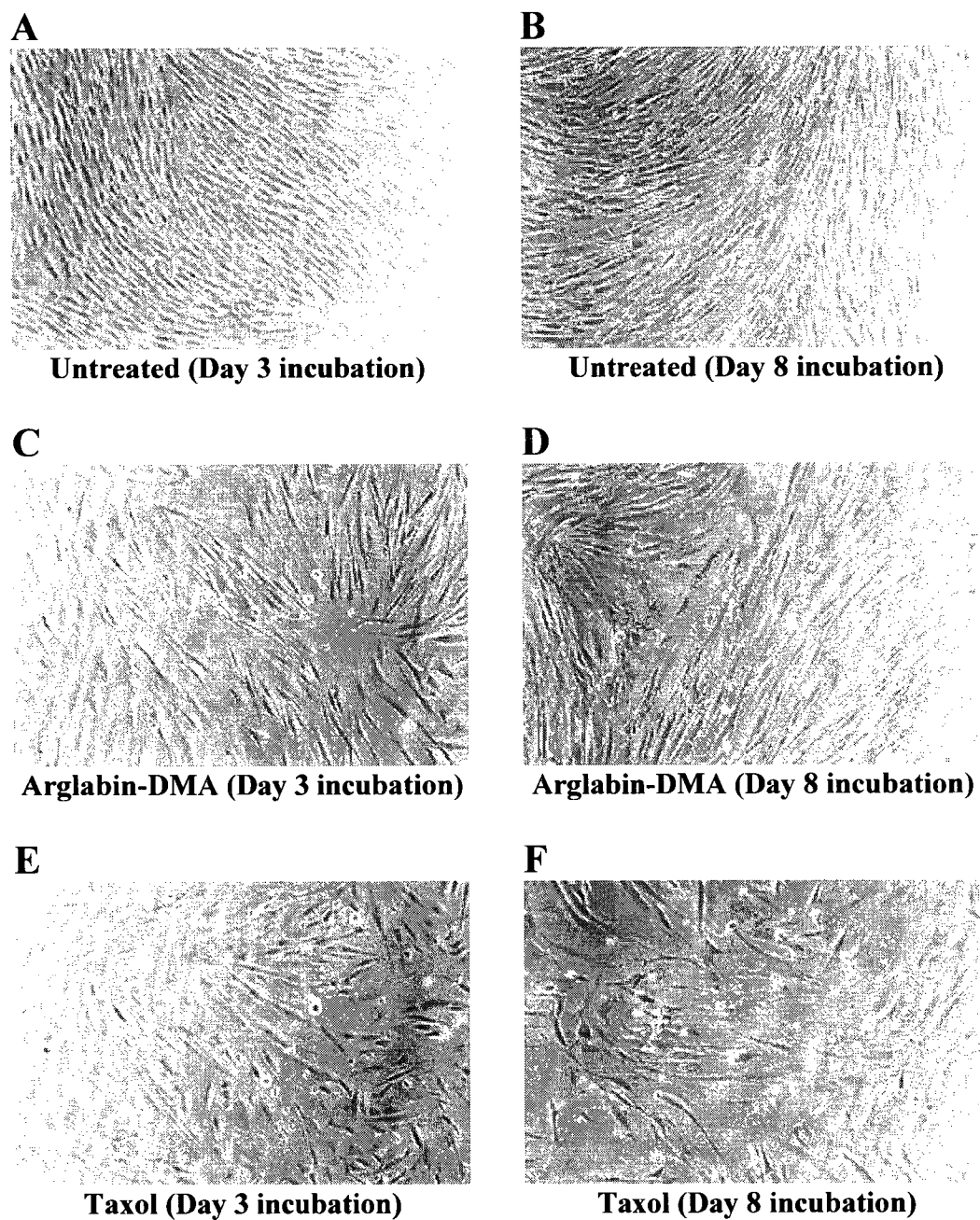


Figure 18 The morphological cell changes in Hs27 after two different incubation periods (three or eight days) after treatment with Arglabin-DMA or Taxol. (A and B: Untreated-control group, C and D: 30 μ M Arglabin-DMA, E and F: 58 nM Taxol).

Table 6 Percentage of cell growth inhibition in Hs27 after two different incubation periods (three or eight days) after treatment with Arglabin-DMA or Taxol

Hs27	Day 3 (cells/cm²)	Day 8 (cells/cm²)
Control	5.24 x 10 ⁴	1.10 x 10 ⁵
30 μM Arglabin-DMA	3.06 x 10 ⁴ (41.38%)	5.16 x 10 ⁴ (53.01%)
58 nM Taxol	1.62 x 10 ⁴ (68.97%)	1.56 x 10 ⁴ (85.79%)

Table 7 Statistical analysis of decline of the total cell number between control group and Arglablin-DMA group

A

HCT 116		t-test for Equality of Means		
Two sample test	<i>t</i> cal.	<i>t</i> table	P-value	Ho: $\bar{U}_i = \bar{U}_j$
Control - Drug	3.488	2.0322	0.001	Reject Ho

B

HT 29		t-test for Equality of Means		
Two sample test	<i>t</i> cal.	<i>t</i> table	P-value	Ho: $\bar{U}_i = \bar{U}_j$
Control - Drug	2.105	2.0322	0.043	Reject Ho

A: HCT 116 cell line, B: HT-29 cell line.

Ho: $\bar{U}_i = \bar{U}_j$, (\bar{U}_j = Hypothetical value)

$t_{table} = t_{0.05(2), df}$, (df = n-1, n=sample number)

| *t* cal. | < *t* table (P>0.05) then do not reject Ho

| *t* cal. | ≥ *t* table (P<0.05) then reject Ho

exposure to Arglabin-DMA (Table 8) and found that there was no significant difference in toxicity between the two cell lines (HCT 116 or HT-29). Therefore, statistical analysis indicated that Arglabin-DMA equally affects cell growth inhibition in both wild-type *ras* and mutant *K-ras* tumor cells.

1.3. Add-Back experiments

Higher doses of Lovastatin, Manumycin A, and Arglabin-DMA at 90% inhibitory concentrations for HCT 116 were used in these experiments to clarify the inhibiting potential of these drugs. Total cell numbers of HCT 116 (*ras*-) after Lovastatin treatment were decreased by 79.89%, but only 32.92% in HT-29 (*ras*+). In the group treated by both farnesyl pyrophosphate and Lovastatin in HCT 116 and HT-29, the number of viable cells decreased in comparison to the only Lovastatin treated group. In contrast, the group treated by both geranylgeranyl pyrophosphate and Lovastatin in HCT 116 significantly increased cell growth (85.99%) compared to the data of Lovastatin treated group (20.11%) (Figure 19). Also, detached HCT cells induced by Lovastatin were reattached in the group treated by both geranylgeranyl pyrophosphate and Lovastatin in HCT 116 cell (data not shown). In HT-29, cell growth rate in between the group treated by Lovastatin and the group treated by geranylgeranyl pyrophosphate and Lovastatin changed 65% to 82% (Figure 19). The geranylgeranyl pyrophosphate slightly prevented the cell growth inhibition by Lovastatin. These data imply that geranylgeranyl pyrophosphate prevented Lovastatin-induced cell rounding and detachment, suggesting that Lovastatin may act by inhibiting geranylgeranylation and not farnesylation of target proteins. Manumycin A

Table 8 Statistical analysis of the percent of decrease in cell number between HCT 116 and HT-29 cell lines after treatment with Arglablin-DMA

Cell line	t-test for Equality of Means				
	Paired test	<i>t</i> cal.	<i>t</i> table	Sig.(2-tail)	Ho: $\bar{U}_i = 0$
HCT 116 – HT-29		1.562	2.1098	0.137	Accept Ho

Ho: $\bar{U}_i = 0$

$t_{table} = t_{0.05(2), df}$, (df = n-1, n=sample number)

| $t_{cal.}$ | < t_{table} (P>0.05) then do not reject Ho

| $t_{cal.}$ | ≥ t_{table} (P<0.05) then reject Ho

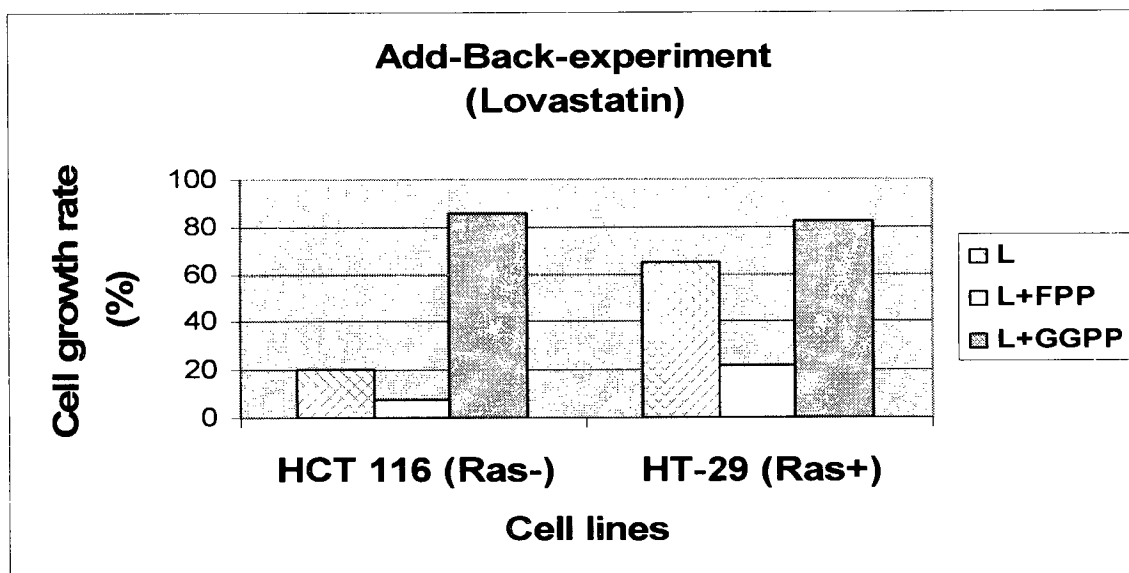


Figure 19 Add-back experiment after treatment with Lovastatin. Lovastatin in both HCT 116 and HT-29 after three days incubation inhibits polyprenylation and the formation of isoprenoids. (farnesyl pyrophosphate and geranylgeranyl pyrophosphate). (L: 30 μ M Lovastatin, M: FPP: 100 μ M Farnesyl pyrophosphate, GGPP: 10 μ M geranylgeranyl pyrophosphate).

was chosen because FPP is a substrate not only for FTase but also for other enzymes catalyzing the synthesis of squalene and other prenylation reactions and is expected to affect the isoprenoid biosynthetic pathway. The result is that Manumycin A did not affect the groups with added isoprenoid (Figure 20). Significantly, in spite of higher concentration of Manumycin A, it slightly affected HT-29 cells (ras⁻) (24.25%) compared to cell growth inhibition (5.34%) of HCT 116 cells (ras⁺). Arglabin-DMA, in contrast significantly inhibited cell growth in both colon tumor cell lines. Addition of isoprenoid (farnesyl pyrophosphate or geranylgeranyl pyrophosphate) did not relieve inhibition in either cell line (Figure 21). Therefore, Arglabin-DMA had no effect on the isoprenylated proteins, and the data with Arglabin-DMA is similar to that of Manumycin A. In contrast, inhibition of geranylgeranylation is the probable mechanism of action of Lovastatin. These results cast doubt on the suggested FTI-based mechanism of inhibition for Arglabin-DMA.

2. TEST OF WHETHER ARGLABIN-DMA INHIBITS CANCER CELL GROWTH IN AN APOPTOSIS-DEPENDENT MANNER.

2.1. Annexin V assay

We have observed that colon tumor cells lose their ability to maintain an asymmetric lipid bilayer after exposure to Arglabin-DMA. The most pronounced feature of this normal asymmetry is the significant absence of phosphatidylserine (PS) in the

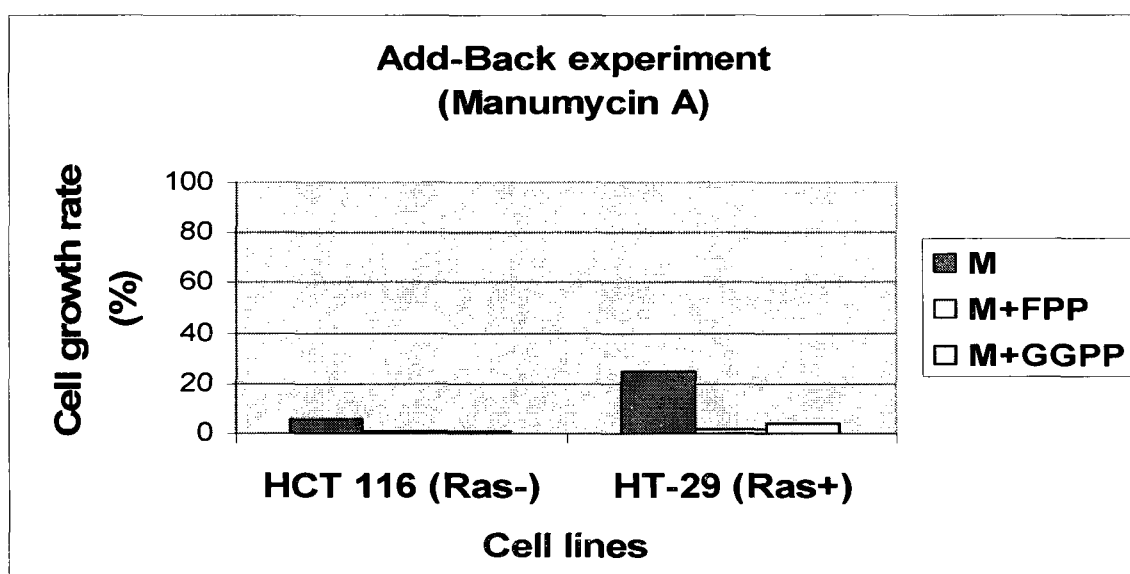


Figure 20 Add-back experiment after treatment with Manumycin A. Manumycin A in both HCT 116 and HT-29 after three days incubation inhibits polyprenylation and the formation of isoprenoids. (farnesyl pyrophosphate and geranylgeranyl pyrophosphate). (M: 20 μ M Manumycin A, FPP: 100 μ M Farnesyl pyrophosphate, G: 10 μ M geranylgeranyl pyrophosphate).

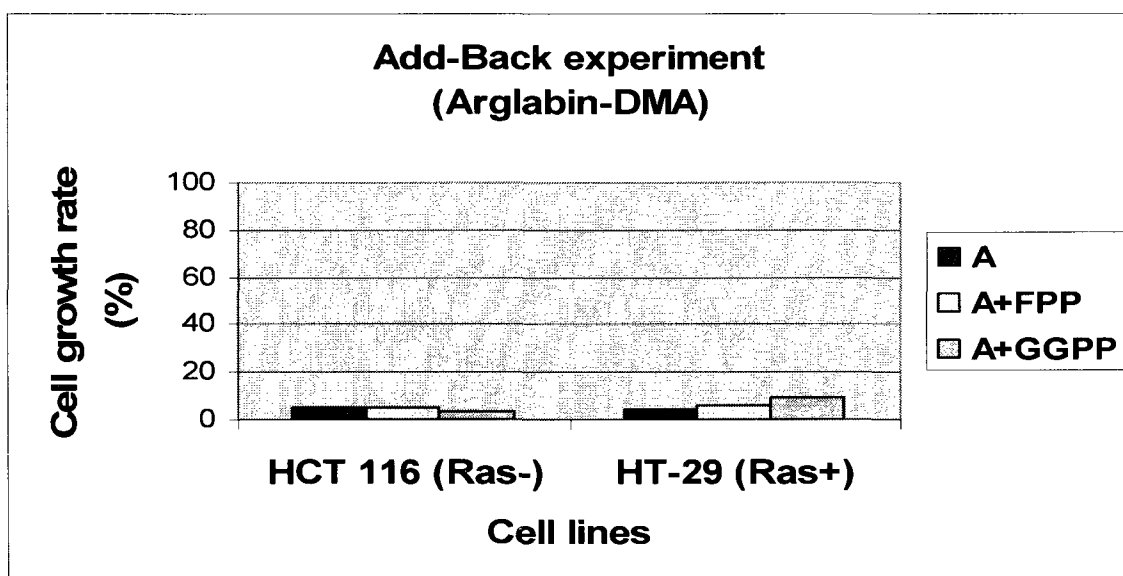


Figure 21 Add-back experiment after treatment with Arglabin-DMA. Arglabin-DMA in both HCT 116 and HT-29 after three days incubation inhibits polyprenylation and the formation of isoprenoids. (farnesyl pyrophosphate and geranylgeranyl pyrophosphate). (A: 61 μ M Arglabin-DMA, FPP: 100 μ M Farnesyl pyrophosphate, GGPP: 10 μ M geranylgeranyl pyrophosphate).

outer leaflet of the plasma membrane. During the initial phase of apoptosis, phospholipid redistribution caused by membrane alterations is detected. When PS is exposed, Annexin V binds specifically to the phospholipid membrane in the presence of Ca^{2+} ions (Vermes and Haanen, 1994). Apoptotic cells are distinguished by using Annexin V as an EGFP (enhanced green fluorescent protein) conjugate in combination with propidium iodide (PI). Annexin V-EGFP is the brightest green fluorescent reagent available for Annexin V-based detection of apoptosis (Koopman *et al.*, 1994).

After being treated with 30 μM Arglabin-DMA in HCT 116 and HT-29, early apoptotic cells with intact cell membranes stained green (Annexin V positive/PI negative) were detected through photography at three days in floating cells (Figures 22 and 23). Also, dimly stained green cells that did not have enough intensity to be detected by photography were present in both attached and floating groups. Arglabin-DMA thus induces apoptosis in floating cells that form phospholipid redistribution based on membrane alterations. It is possible that apoptosis induced by Arglabin-DMA occurs in attached cells, perhaps representing an early stages of apoptosis that will be followed later by detachment.

2.2. Apoptosis dye-uptake assay

Cell uptake of a purple-red dye happens only in the apoptotic process (Figure 24). Colon tumor cells (HCT 116 and HT-29) not treated with Arglabin-DMA did not exhibit dye uptake. Small numbers of cells in the attached HCT 116 and HT-29 cells undergo

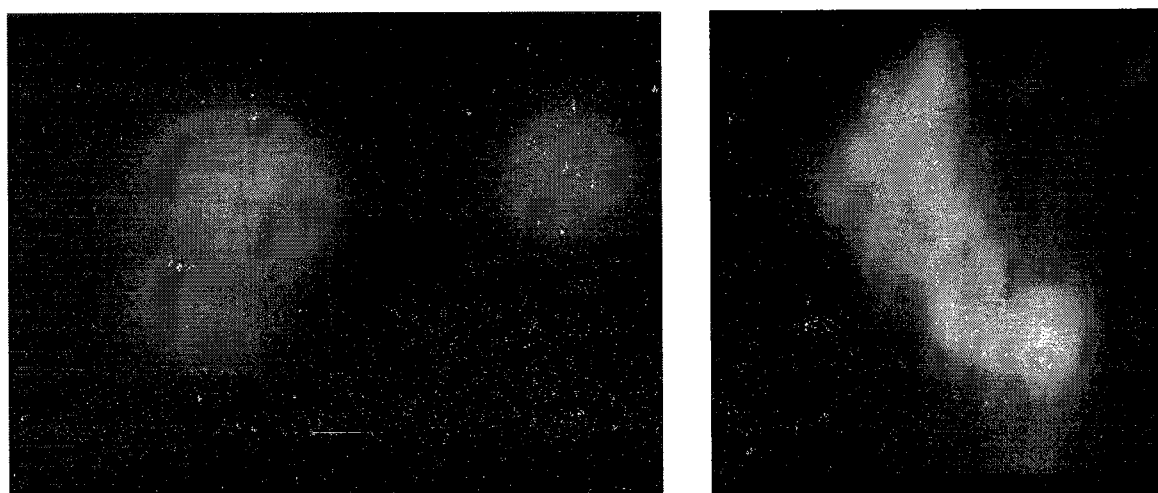


Figure 22 Annexin V assay in HCT 116. Early apoptotic cells in floating cells, after treatment with 30.5 μ M Arglabin-DMA for three days incubation.

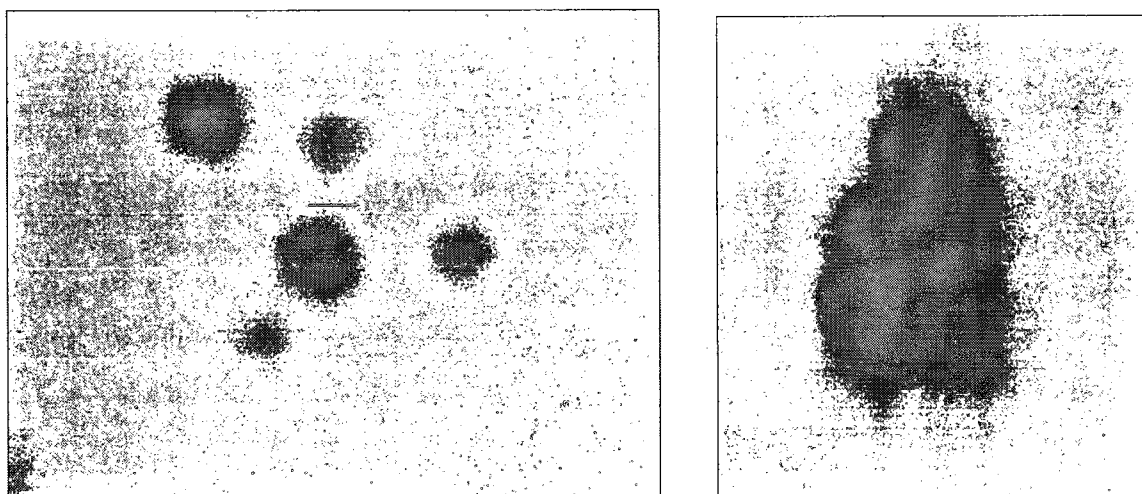


Figure 23 Annexin V assay in HT-29. Early apoptosis in floating cells treated with 30.5 μM Arglabin-DMA for three days incubation.

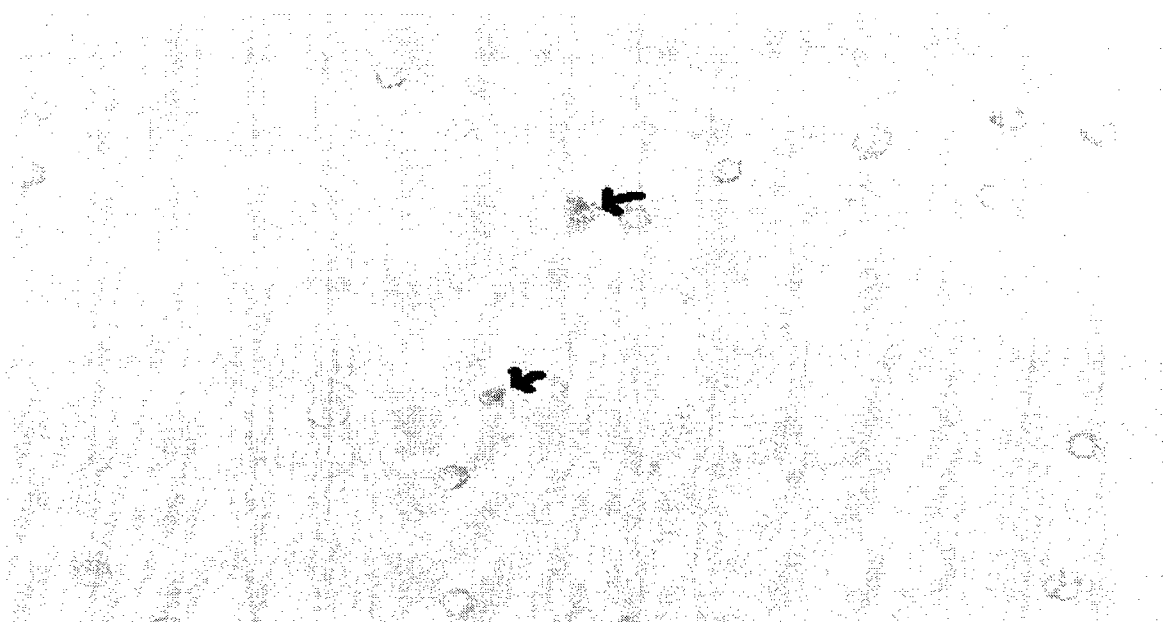


Figure 24 Image of apoptotic cells in Apoppercentage assay. HCT 116 and HT-29 cells undergoing apoptosis have become visible by purple-pink dye. Black arrow (→) indicates apoptotic cell.

apoptosis after exposure to Arglabin-DMA. After one day incubation with Arglabin-DMA, 13.39% of floating HCT 116 cells were apoptotic. Under the same conditions, 29.43% of floating group in HT-29 were apoptotic. At three days, over 26% of floating HCT 116 and HT-29 cells were apoptotic (Figures 25 and 26; Tables 9 and 10). Thus, early phase apoptotic cells, stained by Apoppercentage dye, were increased in Arglabin-DMA induced floating cells on HCT 116 and HT-29.

2.3. Identification of dying cells by apoptotic nuclear morphology

Cells dying by apoptosis usually undergo characteristic morphological and biochemical changes. The types of morphological change consist of membrane blebbing, chromatin condensation, nuclear breakdown, and cytolysis into condensed apoptotic bodies. Dramatic changes occur within the nucleus during apoptotic death (Vermees and haanen, 1994). Cells undergoing apoptosis display increased chromatin condensation and nuclear fragmentation. These morphological changes are triggered by the activation of members of the caspase family including caspase activated DNase, and several novel proteins including AIF (apoptosis-inducing factor) and CIDE (Cell death-Inducing DFF-like Effector) (Kroemer and Zamzami, 1999). An apoptotic chromatin condensation inducer in the nucleus, Acinus, induces apoptotic chromatin condensation. Acinus is cleaved by caspase-3 and an additional unknown protease, generating a small active peptide p17, which causes chromatin condensation *in vitro* (Sahara *et al*, 1999).

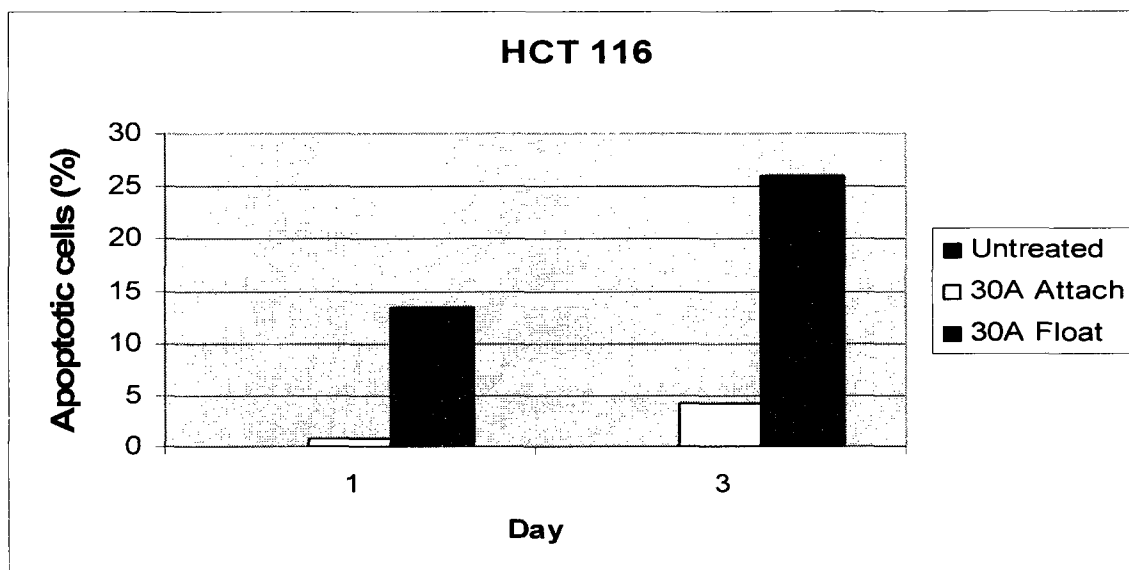


Figure 25 Apoptosis dye-uptake assay in HCT 116. Percentage apoptotic cells after one and three days incubation following treatment with 30.5 μ M Arglabin-DMA.

Table 9 Apoptosis dye-uptake assay in HCT 116

Incubation day	Cell type	Untreated Group	30A Attached Group	30A Floating Group
1 day	Normal cells	1.23×10^6	5.33×10^5	1.27×10^5
	Apoptotic cells	0 (0%)	4.24×10^3 (0.80%)	1.70×10^4 (13.39%)
3 days	Normal cells	8.87×10^6	1.06×10^6	1.98×10^5
	Apoptotic cells	0 (0%)	4.35×10^4 (4.10%)	5.16×10^4 (26.06%)

The number of apoptotic HCT 116 cells.

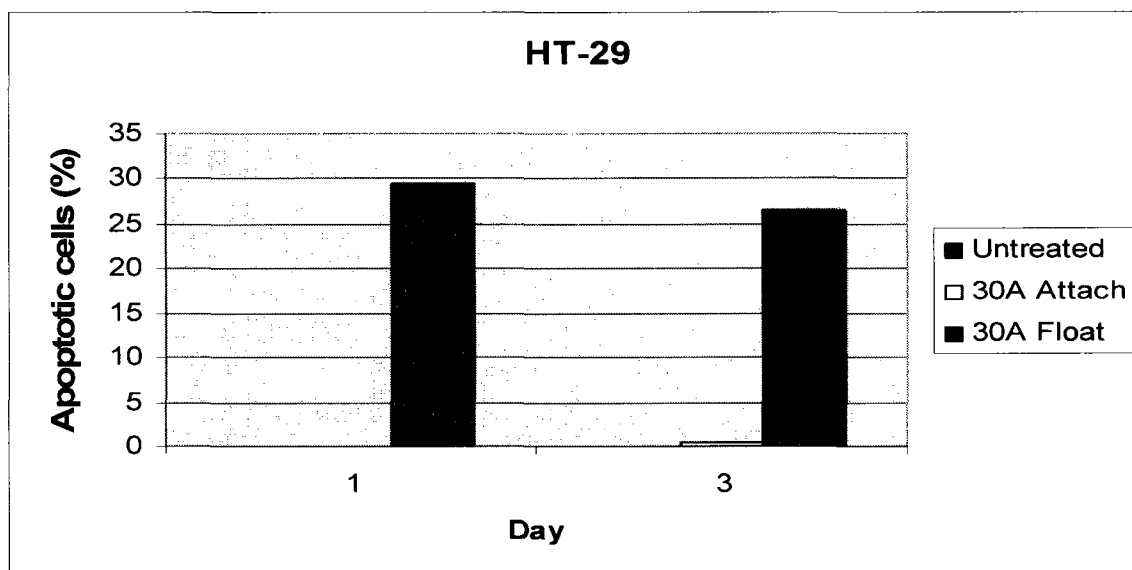


Figure 26 Apoptosis dye-uptake assay in HT-29. Percentage apoptotic cells after one and three days incubation following treatment with 30 μ M Arglabin-DMA.

Table 10 Apoptosis dye-uptake assay in HT-29

Incubation day	Cell type	Untreated Group	30A Attaching Group	30A Floating Group
1 day	Normal cells	1.45×10^6	4.74×10^5	2.27×10^5
	Apoptotic cells	0 (0%)	0 (0%)	6.68×10^4 (29.43%)
3 days	Normal cells	7.47×10^6	1.40×10^6	2.83×10^5
	Apoptotic cells	0 (0%)	4.69×10^3 (0.34%)	7.53×10^4 (26.61%)

The number of apoptotic HT-29 cells.

Fluorescent DNA-binding dyes, such as ethidium bromide (EB), are taken up only by dying cells. EB fluoresces a red-orange color under ultraviolet light and with increased fluorescence when bound to double-stranded DNA. Metachromatic dye acridine orange (AO) can be taken up by both live and dead cells, which can be distinguished by their different morphologies. AO intercalates into DNA (green) and also binds to RNA (red-orange). The difference in the uptake of these two dyes (AO/EB) allows the identification of viable versus non-viable cells by fluorescence microscopy. An assay of cell viability based on simultaneous cell staining with AO and EB allows one to discriminate between live vs. apoptotic vs. necrotic cells (Reed, 2000).

In Tables 11 and 12, over 95% HCT 116 or HT-29 cells not exposed to Arglabind-DMA are live cells (Stage 1; Figure 27; A). After exposure to Arglabind-DMA, many attached cells in HCT 116 or HT-29 cell line were live in Stage 1, while over 80% floating cells in HCT 116 or HT-29 were in Stages 2 and 3 (Figures 27; B and C). However, fewer than 8% of attached cells in HCT 116 and HT-29 showed morphologies classified as Stage 2 (Figure 27; B). Floating cells in HCT 116 and HT-29 after treatment with Arglabind-DMA were identified as apoptotic cells in Stage 4, which includes bright orange chromatin that is fragmented, or apoptotic bodies in the late phase of apoptosis (Figure 27; D). Red color cells, Stage 5, are dead cells, (Figure 27, C and E). The increase of dead cells in the untreated group is attributed to nutritional deficiencies in the medium due to the increased growth of colon tumor cells.

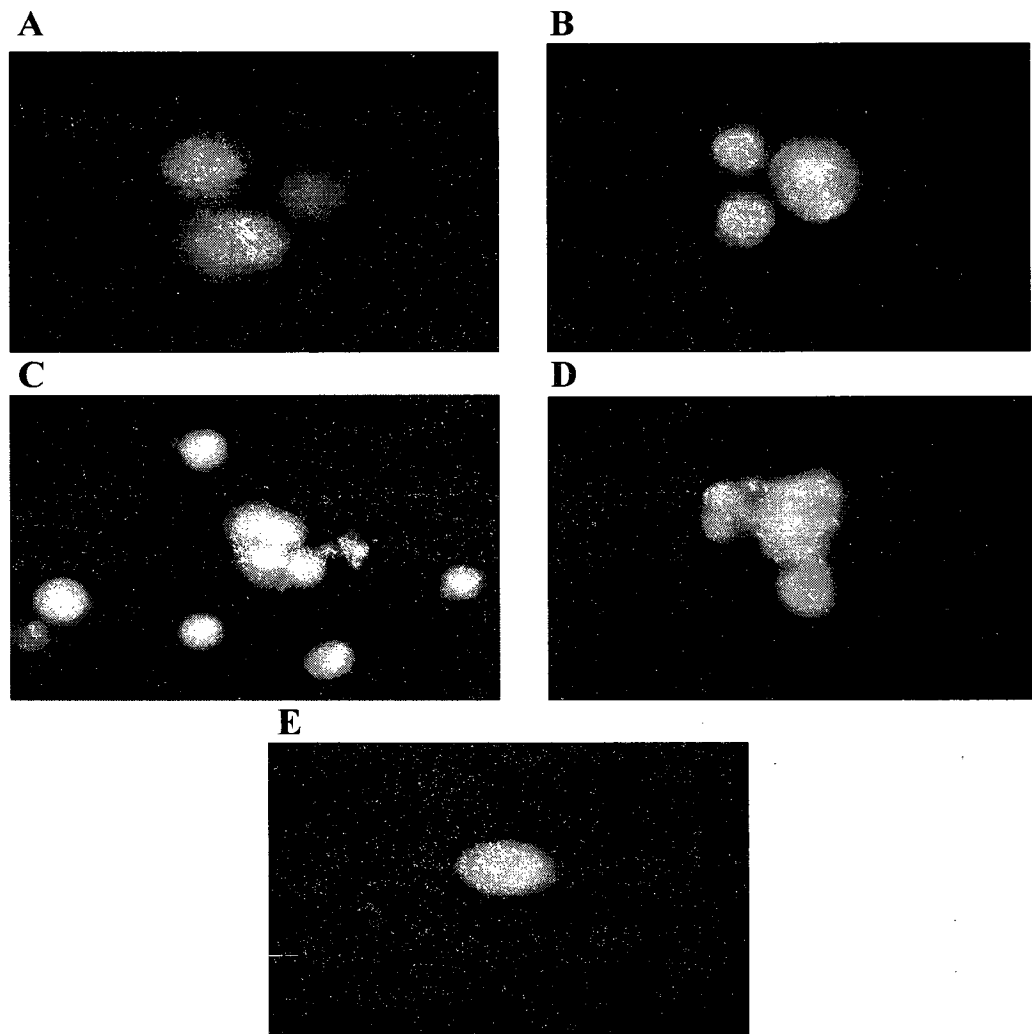


Figure 27 Identification of dying cells by apoptotic nuclear morphology. A: Stage one - bright green chromatin with organized cell structure: viable cells with normal nuclei (uniform distribution). B: Stage two - bright green chromatin that is highly condensed or fragmented: viable cells with apoptotic nuclei, early phase of apoptosis. C: Stage three or four - bright orange chromatin (uniform staining) that is highly condensed or fragmented: nonviable cells with apoptotic nuclei, late phase of apoptosis. D: Stage four - bright orange chromatin that is highly fragmented: nonviable cells with apoptotic nuclei, late phase of apoptosis. E: Stage five - red cell (lower left) that is a dead cell.

Table 11 Identification of dying cells by apoptotic nuclear morphology

HCT 116	TCN	Stage			
		1	2-3	4	5
Control Group	8.87 x 10 ⁶	96.08%	negligible	none	3.92%
Attached Group	1.06 x 10 ⁶	91.36%	7.41% (Stage 2)	none	1.24%
Floating group	1.98 x 10 ⁵	2.59%	86.2%	4.31%	6.90%

Morphological changes after treatment with 30.5 μ M Arglablin-DMA for three days incubation in HCT 116. (TCN: Total cell number).

Stage 1 = Bright green chromatin with organized cell structure – Normal cells.

Stage 2 = Bright green chromatin that is highly condensed – Early phase of apoptosis.

Stage 3 = Bright orange chromatin that is highly condensed - Late phase of apoptosis.

Stage 4 = Bright orange chromatin that is fragmented - Late phase of apoptosis.

Stage 5 = Red cells – Dead cells.

Table 12 Identification of dying cells by apoptotic nuclear morphology

HT-29	TCN	Stage			
		1	2-3	4	5
Control Group	7.47 x 10 ⁶	95.74%	negligible	none	4.26%
Attached Group	1.40 x 10 ⁶	56.60%	32.08% (Stage 2)	none	8.93%
Floating Group	2.83 x 10 ⁵	4.35%	81.16%	11.59%	2.90%

Morphological changes after treatment with 30.5 μ M Arglablin-DMA for three days incubation in HT-29. (TCN: Total cell number).

Stage 1 = Bright green chromatin with organized cell structure – Normal cells.

Stage 2 = Bright green chromatin that is highly condensed – Early phase of apoptosis.

Stage 3 = Bright orange chromatin that is highly condensed - Late phase of apoptosis.

Stage 4 = Bright orange chromatin that is fragmented - Late phase of apoptosis.

Stage 5 = Red cells – Dead cells.

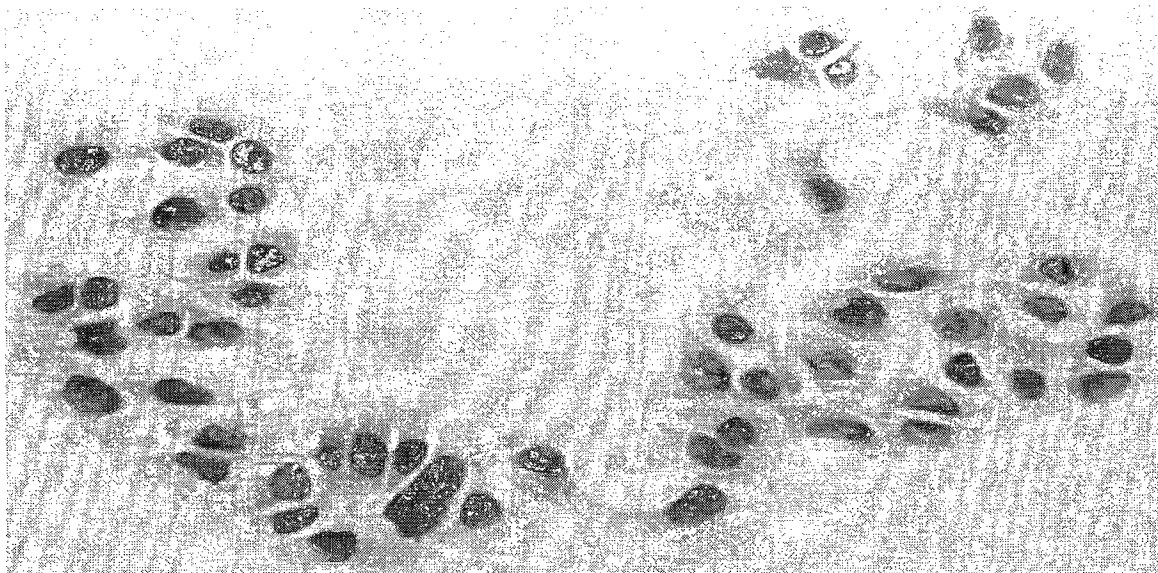
Typical early or late apoptotic morphological changes induced by Arglabin-DMA were observed in the floating cells of HCT 116 and HT-29 cell lines, including evident cytoplasmic and nuclear shrinkage, chromatin condensation, and membrane blebbing, which are all characteristic morphological alterations of apoptosis (Figure 27; B, C, and D). Also, early phase apoptotic cells, with bright green chromatin that is highly condensed, were observed in the attached cells treated with Arglabin-DMA. Therefore, Arglabin-DMA induced apoptosis in floating cells and to a lesser degree in attached cells.

2.4. TUNEL assay

The DNA strand-breaks of the untreated group generated by TACS-Nuclease in the positive control have uniform brown staining in HCT 116 or HT-29 (Figures 28). A negative control sample omitted terminal deoxynucleotidyl transferase (TdT) in the enzymatic reaction (not shown but very similar to Figure 29).

The attached group treated with Arglabin-DMA, in both colon tumor cell lines, was no different than the negative control sample (Figure 29). This indicates that the anchorage-dependent cells did not have the free 3-hydroxyl residues of DNA fragments, and apoptotic cells were not found in the attaching group after the exposure to Arglabin-DMA. No apoptotic cells were detected in the untreated sample or the attached cells of HCT 116 and HT-29 exposed to 30 μ M Arglabin-DMA. The 10-30% floating cells could not be monitored because anchorage-dependent cells were the only types examined. These floaters are likely the apoptotic population.

A



B

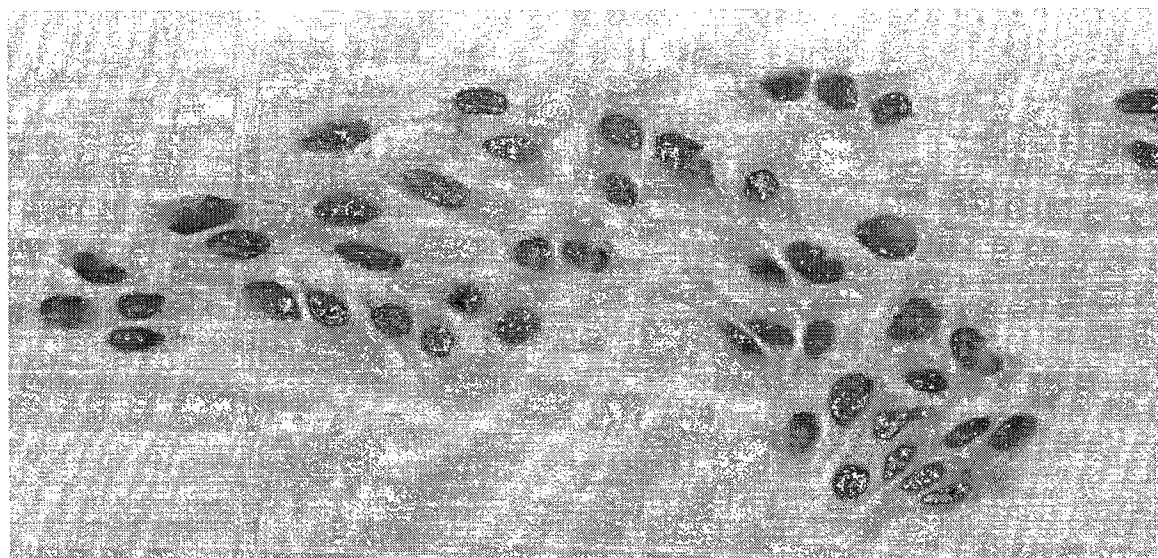


Figure 28 Positive control of TUNEL assay. DNA strand breaks generated by nuclease in essentially every untreated cell were stained as brown color (A: HCT 116 B: HT-29).

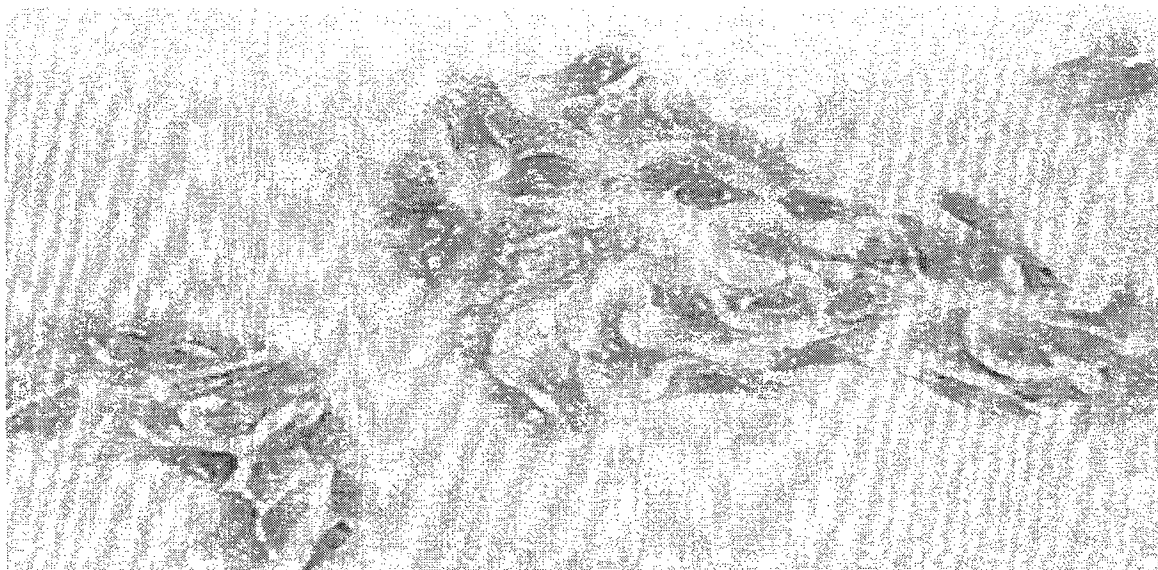
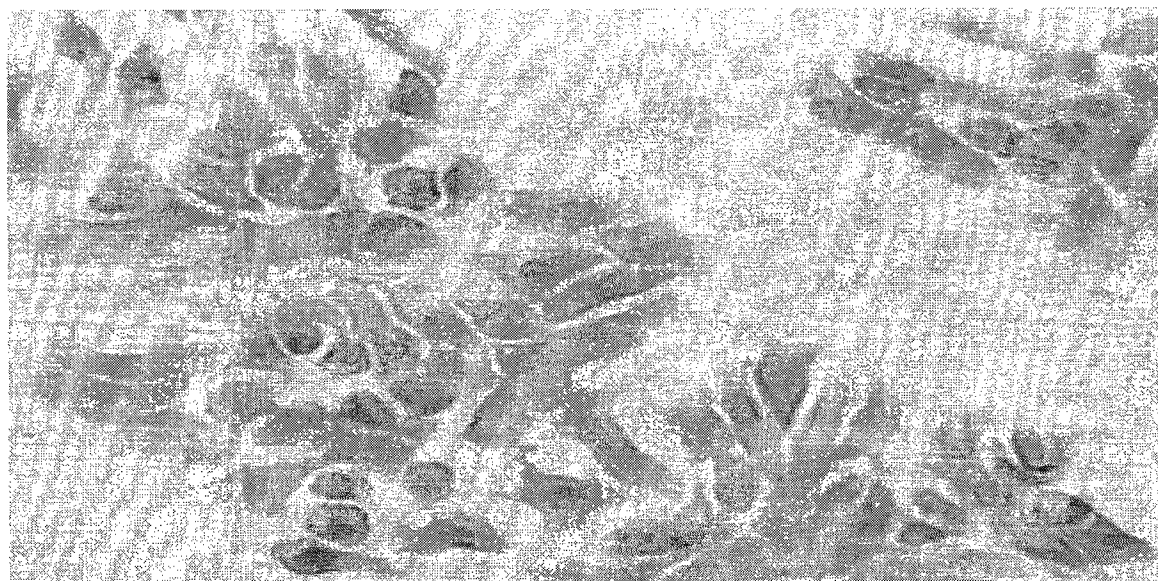
A**B**

Figure 29 TUNEL assay in attached cells after treatment with 30.5 μ M Arglabin-DMA for three days (A: HCT 116 B: HT-29).

2.5. Analysis of DNA fragmentation

As a positive control, we used Taxol since this drug induces morphological changes like Arglabin-DMA and has the ability to induce apoptosis. As seen in DNA fragmentation assays, in the floating cells in HCT 116 or HT-29, exposure to 58 nM Taxol results in DNA ladder formation within one day, whereas fragmentation was absent in the untreated sample or the attached sample exposed to 30 μ M Arglabin-DMA (Figure 30 and 32). However, after Arglabin-DMA or Taxol treatment, DNA fragmentation increased in the floating HCT 116 cells after three days of incubation (Figure 31). Similarly in the floating HT-29 cells, DNA ladder bands were detected similar to HCT 116 (Figure 33). Lovastatin induced morphological changes like Arglabin-DMA in HCT 116. The floating HCT 116 cells showed ladder bands after three days of incubation (Figure 34). DNA fragmentation was still not detectable in the untreated sample, or drug treatment (Arglabin-DMA or Taxol) in the attached cells of either cell line. Cell death induced by Taxol was more rapid than by Arglabin-DMA.

Data from the DNA fragmentation assay showed that floating cells had undergone apoptosis. In an additional experiment, we examined the relationship between p53 and apoptosis. Taxol and Lovastatin were used as positive controls for this experiment. In previous studies, Taxol was shown to display p53-independent apoptosis. (Lanni *et al.*, 1997; O'Connor *et al.*, 1997). Lovastatin has been shown to induce p53-dependent apoptosis in human colon tumor cells (Agarwal *et al.*, 1999). After exposure to Arglabin-DMA, Lovastatin, or Taxol in each of the cell lines, HCT 116, HT-29 and Hs27, we



Figure 30 Analysis of DNA fragmentation in HCT 116 after 24 hours incubation with 30.5 μ M Arglabin-DMA or 58 nM Taxol. (M: Molecular Marker; 1 kb plus DNA ladder, 1: Control-untreated cells, 2: Attached cells treated with Arglabin-DMA, 3: Floating cells treated with Arglabin-DMA, 4: Attached cells treated with Taxol, 5: Floating cells treated with Taxol).

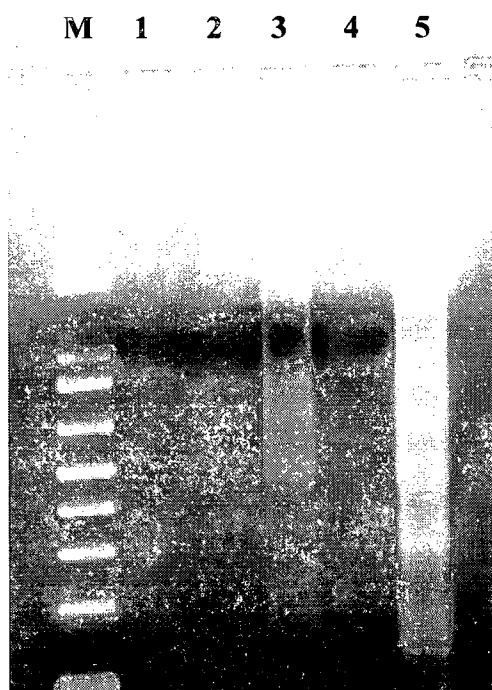


Figure 31 Analysis of DNA fragmentation in HCT 116 after three days incubation with 30.5 μ M Arglabin-DMA or 58 nM Taxol. (M: Molecular Marker; 1 kb plus DNA ladder, 1: Control-untreated cells, 2: Attached cells treated with Arglabin-DMA, 3: Floating cells treated with Arglabin-DMA, 4: Attached cells treated with Taxol, 5: Floating cells treated with Taxol).

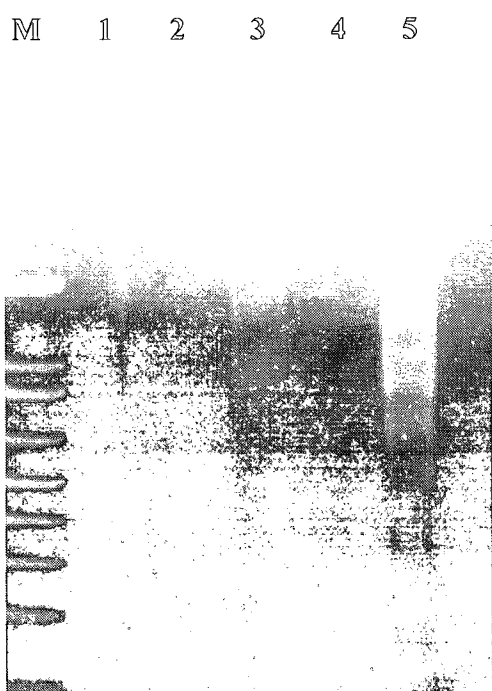


Figure 32 Analysis of DNA fragmentation in HT-29 after one day incubation with 30.5 μ M Arglabin-DMA or 58 nM Taxol. (M: Molecular Marker; 1 kb plus DNA ladder, 1: Control-untreated cells, 2: Attached cells treated with Arglabin-DMA, 3: Floating cells treated with Arglabin-DMA, 4: Attached cells treated with Taxol, 5: Floating cells treated with Taxol).



Figure 33 Analysis of DNA fragmentation in HT-29 after three days incubation with 30.5 μ M Arglabin-DMA or 58 nM Taxol. (M: Molecular Marker; 1 kb plus DNA ladder, 1: Control-untreated cells, 2: Attached cells treated with Arglabin-DMA, 3: Floating cells treated with Arglabin-DMA, 4: Attached cells treated with Taxol, 5: Floating cells treated with Taxol).

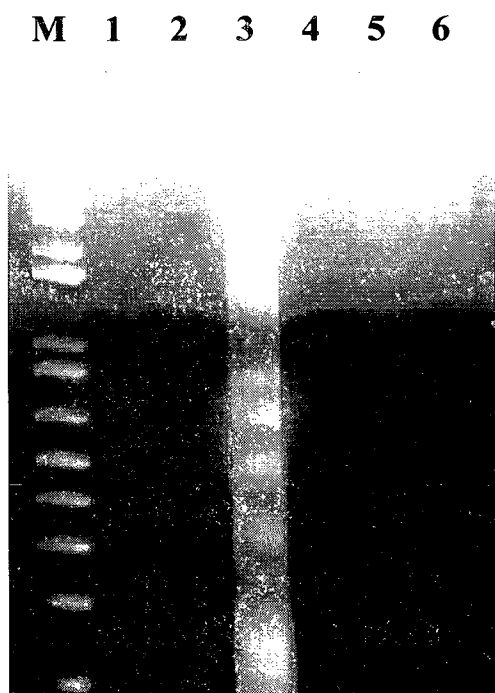


Figure 34 Analysis of DNA fragmentation in two human colon tumor cell lines after three days incubation with 10 μ M Lovastatin. (M: Molecular Marker; 1 kb plus DNA ladder, 1: Control-untreated cell of HCT 116, 2: Attached cell treated with Lovastatin in HCT 116, 3: Floating cell treated with Lovastatin in HCT 116, 4: Control-untreated cells of HT-29 5: Attached cells treated with Lovastatin in HT-29, 6. Floating cells treated with Lovastatin in HT-29).

counted the number of floating cells in each drug group. Both HCT 116 and Hs27 cells, having wild-type p53, showed increased numbers of floating cells after exposure to Lovastatin. The number of floating cells decreased significantly within the HT-29 cell line (p53 mutant) after treatment with Lovastatin in comparison with HCT 116 or Hs27 cell lines. Taxol, however, proved to increase the number of floating cells in all three cell lines. After exposure to Arglabin-DMA, the number of floating cells that resulted was shown to be similar to the number of floating cells after treatment with Taxol in all three cell lines (Table 13). The number of floating cells with Lovastatin in HT-29, having mutant p53, decreased abruptly. This indicates that Lovastatin may induce p53-dependent apoptosis. The numbers of floating cells of the three cell lines treated with Arglabin-DMA were similar to that of floating cells exposed to Taxol, which induces p53-independent apoptosis. This implies that Arglabin-DMA may induce p53-independent apoptosis.

3. TESTING WHETHER ARGLABIN-DMA INDUCES CELL CYCLE ARREST IN HUMAN COLON TUMOR CELL LINES.

3.1. Flow cytometry analysis of the cell cycle

In analysis of the cell-cycle profile, each phase of the cell cycle was displayed with respect to DNA content. Thus, flow cytometry analysis of DNA content revealed whether Arglabin-DMA treated cells arrest in G₂/M.

Table 13 The relation between p53 and floating cells undergoing apoptosis

Cell types	Hs27 (Ras+, p53+)	HCT 116 (Ras-, p53+)	HT-29 (Ras+, p53-)
Arglabin-DMA	5 x 10 ⁴ (6.53%)	2.08 x 10 ⁵ (11.35%)	2.51 x 10 ⁵ (9.46%)
Lovastatin	1.98 x 10 ⁵ (27.12%)	7.63 x 10 ⁵ (18.26%)	1.19 x 10 ⁴ (1.27%)
Taxol	6.7 x 10 ⁴ (13.99%)	3.07 x 10 ⁵ (22.13%)	4.07 x 10 ⁵ (16.23%)

Three cell lines (HCT 116, HT-29 and Hs27), either wild-type or mutated p53, were grown to subconfluence, exposed to the respective drugs (30.5 μ M Arglabin-DMA, 10 μ M Lovastatin or 58 nM Taxol) for 3 days and the number of floating cells was counted.

As shown in Figure 35 and Table 14 using a FACSCalibur (Becton-Dickinson flow cytometer) at Eastern Virginia Medical School, untreated HCT 116 cells exhibited a typical cell cycle distribution (72.31% in G₁, 20.26% in S phase, and 7.43% in G₂/M phase). When those were exposed to 19.5 μM 5-FU as a positive control, we observed 48.58% in G₁, 48.70% in S phase, and 2.72% in G₂/M phase. Therefore, 5-FU reduces the number of cells entering the G₂/M phase of the cell cycle as compared to the untreated sample. 15. 25 μM or 30.5 μM Arglabin-DMA treatment altered the cell cycle of the HCT 116 cells, arresting them in G₂/M phase. In data from the Becton-Dickinson flow cytometer, the Arglabin-DMA treated group again showed an increased yellow peak (tetraploid: 4n) in the G₂/M phase (Figure 35).

After exposure 15.25 μM Arglabin-DMA in the HCT 116 cell line, using the Coulter flow cytometer, the data showed that the result is an increase in cells blocked at G₂/M phase (Figure 36 and Table 15). The results that HCT 116 cells treated with Arglabin-DMA showed the arrest of G₂/M phase at 24 hours is similar to the results of Becton-Dickinson flow cytometer. In Coulter flow cytometric analysis in HT-29 cells, cells with two different concentrations of Arglabin-DMA accumulated in G₂/M by 24 hours and decreased G₁ and S phase population (Figure 37 and Table 16).

In summary, analysis with two different instruments showed flow cytometry profiles of nuclear DNA content that revealed that Arglabin-DMA altered the DNA content and induced a G₂/M phase arrest of cell cycle in HCT 116 and HT-29 cells.

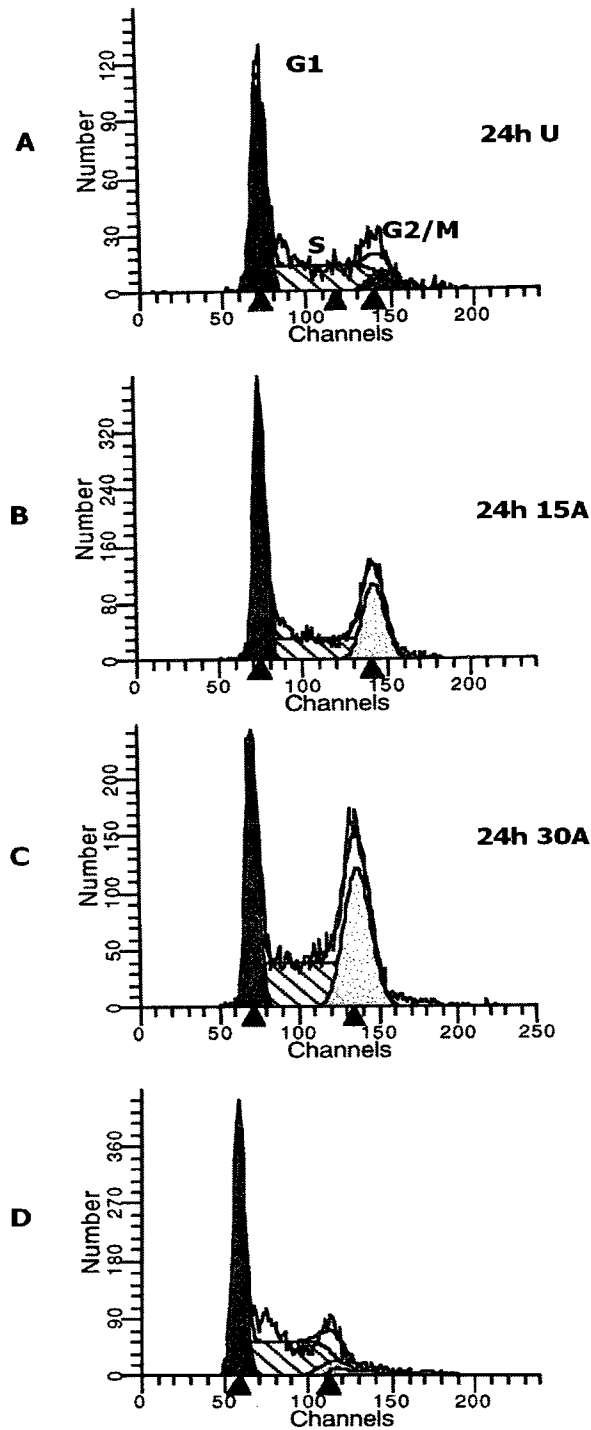


Figure 35 DNA content frequency histograms of HCT 116 from a FACSCalibur (Becton-Dickinson flow cytometer). A: Untreated group, B: 15.25 μ M Arglabin-DMA, 24 hrs, C: 30.5 μ M Arglabin-DMA, 24 hrs, D: 19.5 μ M 5-FU. The red peak on the G₁ or the G₂/M phase represents diploid cells. The yellow peak (tetraploid: 4n) is showed on the G₂/M phase of B and C graphes in Arglabin-DMA treatment group.

Table 14 Flow cytometry analysis of cell cycle in HCT 116 from a FACSCalibur (Becton-Dickinson flow cytometer)

HCT 116	G₁	S	G₂/M
A. Untreated (Control)	72.31 %	20.26 %	7.43 %
B. 15.25 μ M Arglabin-DMA	42.38 %	32.72 %	24.90 %
C. 30.5 μ M Arglabin-DMA	28.57 %	37.78 %	33.65 %
D. 19.5 μ M 5-Fluorouracil	48.58 %	48.70 %	2.72%

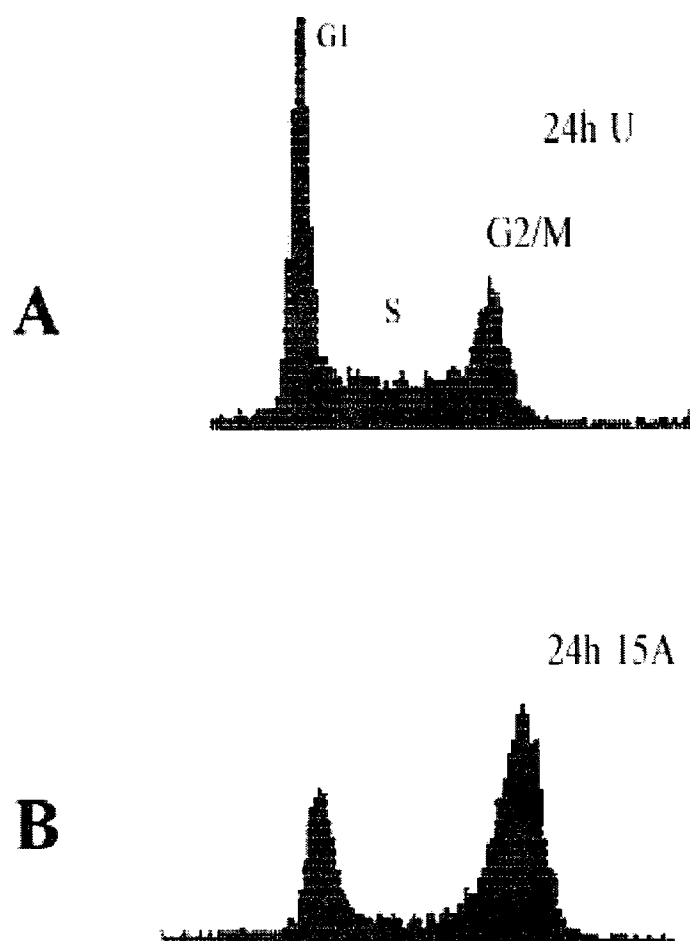


Figure 36 DNA content frequency histograms of HCT 116 from the Coulter EPCICS C clinical flow cytometer. A: Untreated group, B: 15.25 μ M Arglabin-DMA, 24 hrs.

Table 15 Flow cytometry analysis of cell cycle in HCT 116 from the Coulter EPCICS C clinical flow cytometer

HCT 116	G₁	S	G₂/M
A. Untreated (Control)	46.70 %	25.85 %	27.45 %
B. 15.25 μ M Arglabin-DMA	26.54 %	15.54 %	57.92 %

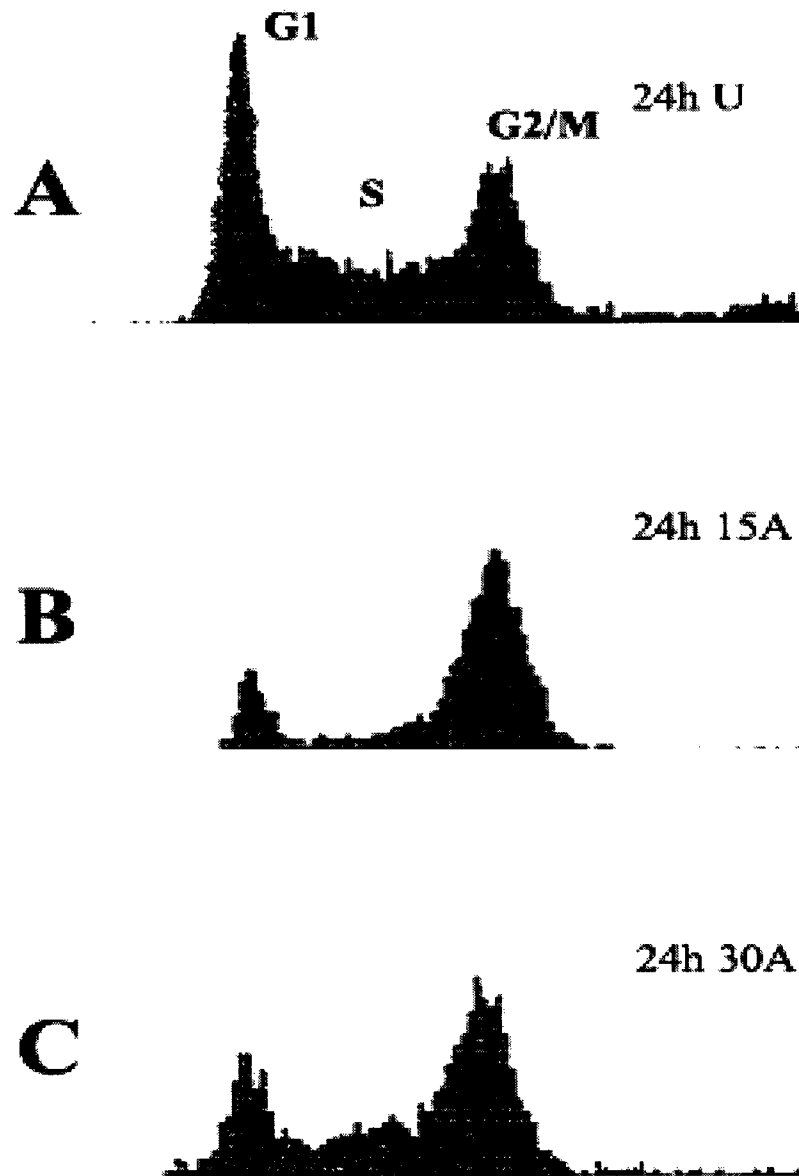


Figure 37 DNA content frequency histograms of HT-29 from the Coulter EPCICS C clinical flow cytometer. A: Untreated group, B: 15.25 μ M Arglabin-DMA, 24 hrs, C: 30.5 μ M Arglabin-DMA, 24 hrs.

Table 16 Flow cytometry analysis of cell cycle in HT-29 from the Coulter EPCICS C clinical flow cytometer

HT 29	G₁	S	G₂/M
A. Untreated (Control)	45.58 %	27.79 %	26.63 %
B. 15 μ M Arglabin-DMA	15.45 %	15.28 %	69.27 %
C. 30 μ M Arglabin-DMA	21.85 %	23.61 %	54.54 %

DISCUSSION

A previous study suggested that Arglabin-DMA, modified to render it water-soluble, is a FTI that inhibits the binding of farnesyl diphosphate (FDP) to FTase, thus inhibiting farnesylation of Ras protein, and that Arglabin-DMA inhibits anchorage-independent growth of mouse neuroblastoma cells as well as KNRK cells (a normal rat kidney [NRK] cell line transformed by Kirsten murine sarcoma virus) (Shaikenov *et al.*, 2001). Our results showed that Arglabin-DMA has a marked effect on the morphology and the growth of both mutant K-*ras* and wild-type *ras* human colon tumor cell lines (HCT 116 and HT-29), and these effects occur in a concentration-dependent manner. Cytotoxicity results, compared between the two colon tumor cell lines, suggested that those tumors that were sensitive to Arglabin-DMA were not dependent on Ras function activated by mutated K-*ras*. This means that Arglabin-DMA as an FTI does not specifically affect the activated Ras.

Preclinical studies on the response of cultured human tumor cells to FTI treatment showed that several cell types with wild-type *ras* are sensitive to FTIs, both *in vivo* and *in vitro* (Cox, 2001). In other studies, about 50% of K-*ras* transformed cell lines were observed to be as resistant as nontransformed cell lines, although it was very easy and straightforward to inhibit both processing and transformation as a result of oncogenic H-Ras (Rowell *et al.*, 1997). Proteins ending with a serine, such as H-Ras, bind more weakly to FPTase than proteins having a methionine, such as Ki4B-Ras and lamin B

(Reiss *et al.*, 1991). The differences in affinity imply that proteins in cells have different sensitivities to an FTI (Gibbs and Oliff, 1997). The basis for the resistance of K-Ras to FTIs is that its affinity for the FTase enzyme is high (Rowell *et al.*, 1997). It has been reported that K-Ras processing could escape FTI inhibition; nevertheless, tumors containing mutated *K-ras* could be inhibited by FTIs (Sepp-Lorenzino *et al.*, 1995).

Another possible mechanism based on the cytotoxic data that Arglabin-DMA affects the growth of HCT 116 and HT-29 cell lines is that it affects the farnesylation of one or more non-Ras proteins. It means that Arglabin-DMA may affect inhibition of the processing of other farnesylated proteins. Farnesylated proteins clearly have important roles to play in normal cells. Among the known farnesylated CAAX proteins other than Ras, the G-proteins (for example, rap-1, rab, and rho), lamin A and B, and inositol triphosphate 5-phosphatase type I may have relevance to intracellular signaling and apoptosis (Yeung *et al.*, 2000). The nuclear lamins essential for nuclear structural integrity (Dalton *et al.*, 1995), proteins of the retinal visual signal transduction system (transducin γ subunit, cGMP phosphodiesterase α , and rhodopsin kinase) (Gibbs *et al.*, 1994), the human homologue of the yeast molecular chaperone YDJ1 (Caplan *et al.*, 1992), the skeletal muscle phosphorylase kinase, as well as other proteins yet to be identified or whose precise function is unknown (for example Pxf, RhoE, and Rap2) (Foster *et al.*, 1996) are all thought to be farnesylated (Gibbs and Oliff, 1997).

In previous studies, researchers found that FTIs inhibit the farnesylation of one or more non-Ras proteins such as Rho B to exert their anti-tumor effects (Cox, 2001; Rowell *et al.*, 1997; Whyte *et al.*, 1997). Protein prenylation is catalyzed by three different enzymes that recognize both the isoprenoid and the acceptor protein: FTase (farnesyl transferase) and GGTase (geranylgeranyl transferase) I and II (Casey and Seabra, 1996). However, the specificity of these enzymes for their substrates is not absolute. Thus, it appears that proteins usually modified by geranylgeranyl pyrophosphate (GGPP) may be prenylated by farnesyl pyrophosphate (FPP) and vice versa, given the appropriate circumstances (Guijarro *et al.*, 1998). Previous investigations into the biologic mechanism of the growth inhibition of Ras-transformed cells have shown that farnesylation of K-Ras and N-Ras is more resistant to FTase inhibitors than farnesylation of H-Ras (James *et al.*, 1995; Nagasu *et al.*, 1995; Sun *et al.*, 1998). In the absence of FTase inhibitors, all Ras proteins are present only in the farnesylated form. However, K-Ras and N-Ras (but not H-Ras) become geranylgeranylated by GGTase I *in vivo* in a dose-dependent manner when intracellular farnesylation is inhibited by an FTase inhibitor (Rowell *et al.*, 1997; Whyte *et al.*, 1997). Subsequently, both FTase and GGTase I inhibitors are required for inhibition of K-Ras processing (Lemer *et al.*, 1997; Sun *et al.*, 1998). Like K-rasB and N-ras, the low-molecular-weight protein Rho B also can be either farnesylated or geranylgeranylated (Armstrong *et al.*, 1995; Casey and Seabra, 1996). Recently, it has been suggested that the antitransforming effects of FTIs are mediated at least in part by alteration of farnesylated Rho proteins, including RhoB (Adjei, 2001; Chen *et al.*, 2000; Lebowitz and Prendergast, 1998). In contrast to Ras proteins, RhoB exists normally *in vivo* in a farnesylated (RhoB-FF) and a

geranylgeranylated version (RhoB-GG). Treatment with FTIs results in a loss of RhoB-GG and a gain of RhoB (Du *et al.*, 1999).

Arglabin-DMA also affected the growth of normal cells. Our studies show that this drug blocks the growth of tumor cells, irrespective of ras genotype, with limited toxicity toward normal cells (Hs27). It has been reported in the literature that FTIs can be less growth inhibiting in normal cells in vitro and have fewer side effects in animal and human studies (Kohl *et al.*, 1993; Rowinsky *et al.*, 1999). FTI treatment inhibits and reverses malignant transformation without severely affecting normal cells (Du and Prendergast, 1999). Interestingly, tumor cell lines with wild-type Ras were also sensitive to FTI. In contrast, nontransformed epithelial cell lines were far less sensitive. It has been suggested that nontransformed cells may produce a form of Ras that is isoprenylated even in the presence of FTase inhibitors (Nagasu *et al.*, 1995; Emanuel *et al.*, 2000). Additionally, this phenomenon may be due to functional redundancy within the Ras family (Reuter *et al.*, 2000). The genetic ablation of FPTase does not cause the yeast cells to die. These observations provided evidence that inhibition of FPTase, even though it is important to the function of a number of proteins other than Ras, would not be toxic to normal cells (Gibbs and Oliff, 1997).

Preclinical studies showed that Arglabin-DMA, a sesquiterpene γ -lactone, competitively inhibits the binding of farnesyl diphosphate to FTase and may inhibit the function of other farnesylated cell proteins such as rho and related cytoskeletal proteins

(Shaikenov *et al.*, 2001). Our studies with add-back experiments examined the possible explanation that Arglabin-DMA, as a sesquiterpene γ -lactone, may affect the synthesis of cholesterol and suppress protein prenylation, particularly inhibition of FTase and the oncogenic activated *ras* protein. Geranyl pyrophosphate and farnesyl pyrophosphate, respectively, are intermediates in the synthesis of monoterpene and sesquiterpenes (Elson, 1995). It is been reported that the monoterpene, limonene, and the sesquiterpene, β -ionone, suppress HMG-CoA reductase activity and lower the level of low-density lipoprotein (LDL) cholesterol (Crowell *et al.*, 1991), causing differentiation of tumor cells (Haag *et al.*, 1992). The secondary action of assorted isoprenoids is the direct inhibition of the protein isoprenyl transferase activities. These isoprenoids markedly suppress the growth of tumors in vitro and in vivo; the growth of the host animal is not impaired (Elson, 1995). Arteminolide, a sesquiterpene lactone from *Artemisia vulvatica*, shows selective inhibitory activity against rat FTase (Lee *et al.*, 1998).

Our data from add-back experiments proved that Lovastatin acts by inhibiting geranylgeranylation and not farnesylation of target proteins. Lovastatin induced an accumulation of unmodified Ras and caused an up-regulation of both RhoB and Rap1A (Holstein *et al.*, 2001). HMG-CoA reductase inhibitors, including Lovastatin, decrease the overall synthesis of final products derived from the isoprenoid biosynthetic pathway, including cholesterol, dolichol and ubiquinone (Corsini *et al.*, 1995). In one study, researchers examining the effects of Manumycin A, an FPP analogue, found that the mechanism of action of Manumycin A did not involve the biosynthesis of intermediates of the cholesterol biosynthetic pathway and did not affect the synthesis of cholesterol

(Nagase *et al.*, 1996). Furthermore, Manumycin A is cytotoxic and induces apoptosis in a human cell line derived from a colorectal cancer which expresses a wild-type *K-ras* gene (Di Paolo *et al.*, 2000; Yeung *et al.*, 2000). This effect is obtained by inhibition of ras farnesylation that reduces signal transduction through the MAPK (the mitogen-activated protein kinase)/ERK (Signal-related kinase) pathway, without affecting protein geranylgeranylation or the synthesis of final products of the cholesterol synthesis (Di Paolo *et al.*, 2000). The analysis of add-back experiments in HCT 116 or HT-29 cells showed that Manumycin A, as a farnesylation inhibitor, had no effect, similar to the results of Arglabin-DMA on protein farnesylation and geranylgeranylation. It was shown that Arglabin-DMA did not affect the biosynthesis of end products of isoprenoid metabolism, and the effect of Arglabin-DMA on cell growth does not require the presence of a mutated *ras* gene. Although results with two FTIs (Manumycin A and Arglabin-DMA) in add-back experiments were not modified specifically by either FPP or GGPP, it may also be possible that Arglabin-DMA, as a non-FTI, may have anti-tumor effects. However, we can not conclude that Arglabin-DMA had no effect on farnesylation.

One would predict that Arglabin-DMA may inhibit the growth of tumor cells by blocking signaling pathways emanating from Ras. One of the best characterized of these pathways is the MAPK cascade, which is activated following Ras/Raf interaction, or via the phosphatidylinositol 3-kinase (PI 3-kinase; PI(3)K) pathway. Small G proteins such as Ras, Rho, and Rac have been shown to regulate a wide spectrum of cellular functions, including cytoskeletal organization, membrane trafficking, transcriptional activation, and cellular transformation (Gibbs and Oliff, 1997; Porter *et al.*, 1998). Many types of

extracellular signals, especially those involving activation of receptor tyrosine kinase and integrin receptors, trigger activation of small G proteins, which in turn activate a variety of signalings. Ras, which is frequently mutated in human tumors, activates several signaling pathways, including the Raf/mitogen-activated protein kinase cascade and the PI 3-kinase/Akt pathway, resulting in malignant transformation in rodent fibroblasts (van Weering *et al.*, 1998; Jiang *et al.*, 2001).

Protein farnesylation, catalyzed by a FTase, plays important roles in the membrane association and protein-protein interaction of a number of eukaryotic proteins. Recent development of FTase inhibitors (FTIs) has led to further insight into the biological significance of farnesylation in cancer cells. A number of reports point to the dramatic effects FTIs exert on cancer cells. These can be classified into four distinct categories. First, FTIs inhibit anchorage-independent growth of a wide variety of cancer cells (James *et al.*, 1993; Kohl *et al.*, 1993). Second, FTIs may cause G₀/G₁ cell cycle arrest, G₂/M cell cycle arrest, or have no effect on cell cycle progression changes, depending on the cell line examined (Vogt *et al.*, 1997). Third, FTIs induce apoptosis of cancer cells when the cells are exposed to low serum conditions or are inhibited in their attachment to the extracellular substratum (Tamanoi *et al.*, 2001). Four, FTIs are reported to affect the actin cytoskeleton and cell morphology (Suzuki *et al.*, 1998; Tamanoi *et al.*, 2001). Clinical trials of FTIs are not complete; thus, how they selectively inhibit malignant cell growth remains uncertain. Our studies showed that in many respects, Arglabin-DMA looks like other chemical FTIs, but not as a FTI inhibiting farnesylation.

Given all these considerations, the potential role of Arglabin-DMA in the inhibition of tumor cell growth certainly deserves further investigation.

A striking morphological effect of Arglabin-DMA is its ability to cause cells to round up and detach in both colon tumor cell lines (HCT 116 and HT-29). Annexin V and Apoptosis dye-uptake assays were applied to detect early apoptotic cells with altered membranes to cause translocation of phosphatidylserine molecules to the exterior surface of the cell membrane. These assays detected a significant increase in apoptotic cells in the floating group when compared to other groups (untreated group or attached group exposed with Arglabin-DMA). Although early phase apoptotic cells were detected in the attached groups in the Annexin V assay, these were not documented through photography due to the fact that the cells were dimly stained. However, the Apopercantage assay was used to show more effectively that the membrane had been altered and that early phase apoptosis occurred in attached cells.

During the late phase of apoptosis, specific endonucleases create DNA fragments with double-stranded breaks, cleaving them into so-called domain-sized fragments (50-300 bp). The TUNEL assay allowed for identification of fragmented DNA. These assays showed that untreated cells or adherent cells treated with Arglabin-DMA did not exhibit this late apoptosis phenotype. As seen in the DNA fragmentation assay, Arglabin-DMA induces DNA fragmentation in the late phase of apoptosis in floating but not attached HCT 116 and HT 29 cells. After exposure to Arglabin-DMA in one of apoptosis-

detection assays, identification of dying apoptotic cells, early and late phase of apoptotic cells increased greatly in the floating group, and nucleic in the early phase of apoptosis were seen in the attached group. This demonstrated the ability of Arglablin-DMA to induce the early and late apoptosis in floating cells and the early phase of apoptosis in attached cells. We did not conclusively determine whether or not detection of apoptosis in floating cells is due to cells detaching, or if the detaching cells were the result of apoptosis. However, measurement of cell viability after Arglablin-DMA challenge shows that the early-detached cells can reattach upon replating in the absence of Arglablin-DMA and grow identically to untreated control cells (data not shown). This suggests that apoptosis precedes detachment. The results indicate that the early phase apoptotic cells affected by Arglablin-DMA in the two colon tumor cell lines probably were detached and then preceded to the late phase of apoptosis. Therefore, Arglablin-DMA may lead to apoptosis, then to changes in the adherence properties of cells, causing them to be shed from the substrate prior to identifiable apoptosis. This apoptosis may contribute to the antitumor effects of Arglablin-DMA *in vivo*.

We propose that the growth matrix-detached fraction of HCT 116 or HT-29 cells treated *in vitro* with Arglablin-DMA were apoptotic. The results suggest that this apoptosis, in response to the loss of cell-to-cell and/or cell-to-matrix communication, may be analogous to "anoikis," (Frisch and Francis, 1994). Anoikis is the term for detachment-induced cell death: cells undergo apoptosis upon disruption of the interactions between integrins and proteins of the extracellular matrix. Integrins are receptors that bind extracellular matrix proteins to the outside of the cell. The

extracellular matrix is responsible for tissue organization throughout the body. It is also becoming clear that matrix proteins regulate certain aspects of gene expression and the cell cycle. They also regulate apoptosis in epithelial cells (Frisch and Francis, 1994). Anoikis also occurs when epithelial cells are either not bound to the matrix via integrins, or bind to the wrong integrins (Boudreau *et al.*, 1995).

The tumor suppressor gene, p53, regulates apoptosis following a variety of stress stimuli (Bennett, 1999). P53-dependent apoptosis activates the apoptosome, a multimeric complex consisting of Apaf-1, cytochrome *c*, dATP/ATP and procaspase-9, via the so-called intrinsic pathway (Soengas *et al.*, 1999). Our data demonstrated that floating cells exposed *in vitro* to Arglabin-DMA enter apoptosis. To examine the relationship between p53 and apoptosis, we proposed that Arglabin-DMA may induce p53-independent apoptosis. The drug may be able to induce an extrinsic pathway of apoptosis or may mediate a cross-talk mechanism between the apoptosis pathways, through Bid, one of the Bcl-2 family. It was reported that epithelial cells undergo death receptor-dependent apoptosis when detached from the matrix, a process termed anoikis. Also, the cleavage of Akt/protein kinase B (PKB) by death receptor signaling contributes to anoikis (Bachelder *et al.*, 2001). Anoikis was found to be activated through the Fas pathway by FasL, the so-called extrinsic pathway. FasL/Fas interaction, Fas-FADD complex formation, and caspase-8 activation precede the bulk of anoikis in endothelial cells, and inhibition of any of these events blocks anoikis (Aoudjit and Vuori, 2001). Other results have indicated that the death receptor adaptor molecule, FADD, may be involved in anoikis, as a dominant-negative truncated FADD, containing only the death domain, inhibits anoikis.

Anoikis was also shown to be accompanied by an early activation of caspase-8, as would be expected for death receptor activation (Frisch and Screaton, 2001).

In epithelial cells of the intestine and other organs, activated Ras induces resistance to anoikis (Shao *et al.*, 2000). Resistance to anoikis may facilitate the survival of cancer cells during systemic circulation, aiding the formation of metastases (Douma *et al.*, 2004). The nature of the survival signals activated by integrin engagement and usurped by oncogenic Ras are still unclear. Recent studies have implicated that adhesion or transformation by ras oncogenes have been shown to protect epithelial cells from apoptosis through activation of PI 3-kinase and Akt/PKB (Khwaja et al, 1997). A Rho family member, Rac 1, inhibits anoikis in epithelial cells. Rac 1-mediated cell survival strongly depends on PI 3-kinase activity. PI 3-kinase, acting through Akt/PKB, a subfamily of serine/threonine protein kinases, is implicated as a key mediator of the aberrant survival of Ras-transformed epithelial cells in the absence of attachment, and mediates matrix-induced survival of normal epithelial cells (Coniglio *et al.*, 2001; Frisch and Francis, 1994). Recently, the neurotrophic receptor tyrosine kinase (RTK), TrkB, was identified as a potent and specific suppressor of caspase-associated anoikis and linked it to the promotion of metastases. TrkB triggered the activation of PI 3-kinases which stimulate Akt/PKB, and this too was active in the TrkB-producing cells. The result was a blockade of caspase-associated anoikis and related forms of cell death. PI 3-kinases also drive numerous other cellular functions associated with metastasis (Liotta and Kohn, 2004; Douma *et al.*, 2004). It would be of interest to characterize Trk B effects in the Arglabin-DM-treated cells.

In general, inhibition of cell growth by FTI in human tumor cells results in G₀/G₁ cell cycle block, G₂/M cell cycle arrest, or has no effect on cell cycle progression (Vogt *et al.*, 1997). One aspect of FTI biology that is poorly understood is the ability of these drugs to induce cancer cell growth arrest at the G₂/M phase of cell cycle (Mazzocca *et al.*, 2003). In the present study, both human colon tumor cell lines showed cell accumulation at the G₂/M phase of the cell cycle following Arglabin-DMA treatment. Flow cytometry results indicated that these cells were tetraploid. Effective growth inhibition by FTI L-744,832 correlated with the accumulation of cells with a tetraploid (4n) DNA content and high levels of cyclin B1/cdc2 kinase activity, implying cell cycle arrest downstream from the DNA damage-inducible G₂/M cell cycle checkpoint (Song *et al.*, 2000). Treatment of a lung cancer cell line (A-549) with the protein farnesyl transferase inhibitor Lonafarnib (SCH66336) resulted in an increase of G₂/M phase cells. It was shown that prophase and metaphase cells accumulated (Jiang *et al.*, 2000). The inhibition of CENP-E farnesylation by FTI results in the alteration of the microtubule-centromere interaction during mitosis and results in the accumulation of cells prior to metaphase (Ashar *et al.*, 2000). It would be that Arglabin-DMA may associate with proteins involved to cell cycle or other farnesylation proteins to induce human colon cancer cell growth arrest at the G₂/M phase of cell cycle.

As discussed previously, Arglabin-DMA appears to induce apoptosis in a p53-independent manner; p53, a tumor suppressor protein, acts also in late G₁ phase. If DNA damage has occurred, p53 accumulates in the cell nucleus and induces the p21-mediated inhibition of cyclin D/cdk. Mdm2, by facilitating the nuclear export/inactivation of p53,

becomes part of an inhibitory feedback loop that inactivates p21-mediated G₁ arrest. p53 prevents the cell progressing to the S phase (Momand *et al.*, 2000). Also, p53 participates in the regulation of G₂/M. The progression into M phase requires Cdc2 which can be inhibited by p21 (Cyclin dependent kinase inhibitor), GADD45 (a p53-regulated stress protein), or 14-3-3 σ (adapter protein) . p53 regulates the expression of these inhibitory proteins to induce growth arrest (Hermeking *et al.*, 1997; Liu and Kulesz-Martin, 2001). Both cell lines, having different Ras and p53 status, similarly induce cell cycle arrest at the G₂/M phase. It is therefore assumed that Arglabin-DMA may affect the arrest of G₂/M phase in cell cycle without involvement of the p53 protein.

In flow cytometry analysis after the exposure of Arglabin-DMA for one day, our results did not show DNA content characteristic of apoptotic cells (e.g., position of the sub-G₁ peak on the DNA content frequency histograms). In analysis of DNA fragmentation, the floating groups, after treatment with Taxol in HCT 116 or HT 29 cells, showed ladder bands of DNA after one day incubation. However, DNA ladder bands were not found in the floating group with Arglabin-DMA. It may be reasoned that flow cytometry analysis does not show the sub-G₁ peak on DNA content frequency histograms.

CONCLUSION

We conclude that Arglabin-DMA has specific cytotoxic effects in the two human colon tumor cell lines and in comparison to other antitumor drugs, less cytotoxicity to normal cells *in vitro*. Also, the drug has a marked effect on the morphology and the growth of both mutant *K-ras* and wild-type *ras* human colon tumor cell lines. Arglabin-DMA does not affect the biosynthesis of the cholesterol pathway. Our studies do not support that Arglabin-DMA acts to inhibit tumor cell growth as a FTase inhibitor. One possible explanation is that Arglabin-DMA may inhibit the processing of other farnesylated proteins or may block signaling pathways emanating from Ras. However, it certainly deserves further attention. In our studies, Arglabin-DMA induces apoptosis after cellular rounding and detachment as a striking morphological effect in human colon tumor cell lines. The mechanism involved may be anoikis as cells undergo apoptosis upon disruption of the cell-matrix interaction. This apoptosis is induced in a p53-independent manner. It may also be induced via an extrinsic (death receptor) pathway. Finally, Arglabin-DMA induces G₂/M cell growth arrest in human colon tumor cell lines (HCT 116 and HT-29). The utility of this drug in combating cancer remains an attractive, possibility, although more work will be required to elucidate its mechanism of action.

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