Form Approved Through 05/2004 OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Application

Do not exceed 56-character length restrictions, including spaces.

LEAVE BLANK—FOR PHS USE ONLY.

Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT
Cyclooxygenase-2 inhibitors & colon cancer prevention

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR — New Investigator ☒ No ☐ Yes

3a. NAME *(Last, first, middle)*
Muldoon, Throckmorton P.

3b. DEGREE(S)
MD PhD

3c. POSITION TITLE
Professor and Director

3d. MAILING ADDRESS *(Street, city, state, zip code)*
Philip G. Hogg Cancer Center
Murray Bldg., Rm 317
1234 Sunnyside Lane
Punxatawny, PA

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Program in Tumor Biology

3f. MAJOR SUBDIVISION
School of Medicine

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: 717-555-1234 FAX: 717-555-5678

E-MAIL ADDRESS: muldoont@som.punx.edu

4. HUMAN SUBJECTS RESEARCH
☐ No
☒ Yes

4a. Research Exempt ☒ No ☐ Yes
If "Yes," Exemption No.

4b. Human Subjects Assurance No.
12345

4c. NIH-defined Phase III Clinical Trial ☐ No ☒ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes

5a. If "Yes," IACUC approval Date

5b. Animal welfare assurance no

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
01/01/2003	12/31/2008	$879,994	$1,279,822	$4,177,259	$6,057,026

9. APPLICANT ORGANIZATION
Name Punxatawney School of Medicine
Address Philip G. Hogg Cancer Center
Punxatawney Health Sciences University
1234 Sunnyside Lane
Punxatawny, PA

Institutional Profile File Number (if known)

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER

DUNS NO. *(if available)*

Congressional District

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name Maynard Krebs
Title Assistant Dean for Grants and Contracts
Address Getty Bldg. Trailer Annex, Suite 1023
Punxatawney Health Sciences University
1000 Sunnyside Lane
Punxatawney, PA
Tel 717-555-4321 FAX 717-555-9999
E-Mail krebsmg@som.punx.edu

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Chatsworth P. Osborne, Jr.
Title Vice President for Finance & Admin.
Address Getty Building, Penthouse 2
Punxatawney Health Sciences University
1000 Sunnyside Lane
Punxatawney, PA
Tel 717-555-8765 FAX 717-555-9999
E-Mail beancounter1@som.punx.edu

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)* — DATE: 06/01/02

15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)* — DATE: 06/01/02

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The proposed multi-center clinical trial seeks to determine whether pharmacological intervention with RK42B, a selective cyclooxygenase-2 (cox-2) inhibitor, can reduce colonic polyp recurrence in human subjects and thereby delay or prevent colonic epithelial cell transformation. The specific aims are to 1) determine whether RK42B reduces the recurrence of adenomatous colonic polyps in humans, 2) investigate whether RK42B reduces prostaglandin E2 (PGE2) content and alters cellular proliferation and apoptosis in morphologically normal colonic mucosa, 3) determine whether RK42B's pharmacokinetics can predict any observed reductions in colonic PGE2 content, and 4) define potential mechanisms of polyp recurrence after prolonged cox-2 inhibition.

Healthy patients who have undergone a colonoscopy to remove all polyps including at least one polyp of greater than or equal to 1 cm in length, or two or more polyps of at least 0.6 cm in length, will be eligible to participate in this trial. Subjects will be randomized to receive placebo or one of two different drug doses administered twice daily over a three-year period. A subset of subjects will undergo flexible sigmoidoscopy with biopsies of morphologically normal colonic mucosa immediately prior to treatment and again after six months of treatment. At the end of the three-year trial, all subjects will undergo diagnostic colonoscopy to detect and remove recurrent polyps and biopsy morphologically normal mucosa. The drug's effect on cyclooxygenases will be assayed using frozen polyps and normal biopsy material. Standard immunohistochemistry assays and molecular assays will be used to monitor proliferative activity, cellular apoptosis, and the presence of mutant forms of p53 and kRas proteins in polyps and normal mucosa. Sample size is based on the assumption that 25% of control subjects will develop an adenomatous polyp by the end of the trial period; 990 subjects will be randomized to enable detection of a 50% reduction in polyp recurrence at a significance level of 0.05 and a power of 0.9.

Because elevated cox-2 activity is commonly associated with clonally expanded transformed cells, positive results could establish RK42B as an effective colorectal cancer chemopreventive agent.

PERFORMANCE SITE(S) *(organization, city, state)*

Punxatawney Health Sciences University, Punxatawney, PA

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Throckmorton P. Muldoon, M.D., Ph.D.	Punx. Hlth. Sci. Univ.	Principal Investigator
Hamilton J. Fonebone, M.D.	PHSU	Co-investigator
Simon Saez, Ph.D.	PHSU	Senior Research Associate
Riley R. Handy, M.S.	PHSU	Sr. Lab. Technician II

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ **Yes** ☐ **No**

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.

RESEARCH GRANT

TABLE OF CONTENTS

Appendix *(Five collated sets. No page numbering necessary for Appendix.)*

Check if Appendix is Included ☐

Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited.

Number of publications and manuscripts accepted for publication *(not to exceed 10)* _____

Other items (list):

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from Form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		244,467	254,246	264,415	274,992	285,992
CONSULTANT COSTS						
EQUIPMENT		12,500				
SUPPLIES		30,000	31,200	32,448	33,746	35,096
TRAVEL		2,000	2,000	2,000	2,000	2,000
PATIENT CARE COSTS	INPATIENT	202,500	225,779	72,600	163,377	127,978
	OUTPATIENT	188,527	196,068	203,911	212,067	220,550
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		200,000	200,000	200,000	200,000	200,000
SUBTOTAL DIRECT COSTS		879,994	909,293	630,174	886,182	871,616
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT					
	F&A					
TOTAL DIRECT COSTS		879,994	909,293	630,174	886,182	871,616

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD *(Item 8a, Face Page)* ——— $ 4,177,259

SBIR/STTR Only Fee Requested					

SBIR/STTR Only: Total Fee Requested for Entire Proposed Project Period
(Add Total Fee amount to "Total direct costs for entire proposed project period" above and Total F&A/indirect costs from Checklist Form Page, and enter these as "Costs Requested for Proposed Period of Support on Face Page, Item 8b.) $

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Personnel: Dr. Muldoon will commit 35% effort to the proposed project, receiving $167,700 per year plus benefits at the rate of 33.8%. Dr. Fonebone will devote 30% of his time to the project, receiving $125,500 per year plus full benefits. Dr. Saez will devote 100% effort to the project, receiving a base salary of $52,750 per year plus full benefits. Mr. Handy, a senior lab technician with a base salary of $42,000 per year plus benefits will devote 100% effort to the project, assisting with sample collection, preparation of solutions, patient record-keeping, and data analysis.

Consultant Costs: None.

Supplies: Costs of surgical supplies, antibodies and ELISA kits represent the bulk of these costs. Routine laboratory supplies (reagents, tubing, syringes, pipettes, etc.) make up the remainder of supply costs.

Travel: Funds are requested for two national meetings to present results to the basic and clinical oncology communities with interests in the areas of colon cancer.

Equipment: Funds are requested for a fluorescence microscope to be used in immunohistochemistry assays.

Other Expenses: Funds to pay human volunteers for participation in the study included in this category.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Muldoon, Throckmorton P.	Professor and Director of Tumor Biology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Duke University, Durham, NC	B.S.	1975	Biochemistry
University of North Carolina, Chapel Hill, NC	Ph.D.	1980	Cell Biology
University of North Carolina, Chapel Hill, NC	M.D.	1982	Medicine
Johns Hopkins University	Residency	1982-85	Oncology
Punxatawney Health Sciences University	Post. Doc	1985-87	Tumor Biology

BOARD CERTIFICATION:

1983 Diplomate of the National Board of Medical Examiners
1985 Diplomate in Internal Medicine, American Board of Internal Medicine
1985 Diplomate in Oncology, American Board of Oncology
2000 Diplomate in Gastroenterological Surgery, American Board of Surgical Medicine

Comments on investigator for review discussion:

Professional Experience: The principal investigator, Dr. Mort Muldoon, is a leading figure in several areas of colon cancer research. He is especially renowned for his seminal contributions to the field of colonic epithelial cell transformation and tumor initiation. Dr. Muldoon is currently a Professor of Medicine and Head of the Tumor Biology Program at PHSU. He rose rather quickly through the ranks at PHSU, from Assistant Professor starting in 1987 to full Professor in 1995. He has also held a visiting professorship at the Sorbonne. He currently heads the tumor biology program at PHSU. He is highly qualified to conduct the proposed research.

Publications: Dr Muldoon has been highly productive as an independent investigator, with over 80 refereed publications since 1987, including 40 publications related to the proposed studies in the last 10 years, mostly in top-rank oncology journals, and a fair number in high-profile general journals such as Science, Nature, and Cell.

Honors , Awards, and Service: Dr. Muldoon has served on numerous editorial boards and scientific meeting planning committees, as well as several grant review panels for the NIH and the American Cancer Society. He also serves on the Scientific Advisory Board for the Howard Hughes Medical Institute, and was very recently elected to the National Academy of Sciences Institute of Medicine. He has been awarded several grants from the NIH over the course of his independent career, including a program project award, now in its 7th year after a recent successful renewal.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Fonebone, Hamilton J., M.D.	Associate Professor of Medicine Associate Professor of Cell Biology & Anatomy

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Oregon State University, Corvallis OR	B.S.	1983	Botany & Plant Pathol.
University of California, San Diego	Ph.D.	1988	Molecular Genetics
University of California, San Diego	M.D.	1989	Medicine
Massachusetts General Hospital, Boston MA	Residency	1989-92	Clinical Oncology
Stanford Univ. School of Medicine, Stanford CA	Post. Doc	1992-94	Developmental Biology

RESEARCH EXPERIENCE AND EMPLOYMENT

1999- Associate Director, Tumor Biology Program, Punxatawney Health Sciences University
1998- Associate Professor (tenured), Department of Medicine, Punxatawney Health Sciences University
1994-98 Assistant Professor, Department of Biochemistry and Biophysics, Northwestern University
1992-94 Postdoctoral Research Fellow, Dept. of Developmental Biology, Stanford University Sch. of Medicine
1983-88 Graduate Research Assistant, Center for Molecular Genetics, Univ. of California, San Diego
1985-88 Graduate Teaching Assistant, Dept. of Biology, Univ. of California, San Diego
1982-83 Research Assistant, Dept. of Botany & Plant Pathology, Oregon State University

HONORS AND AWARDS

1994-97 Junior Faculty Research Award, American Cancer Society
1993-94 Senior postdoctoral research fellowship, American Cancer Society, California Division
1984-87 Predoctoral research fellowship, National Science Foundation

SOCIETY MEMBERSHIP AND PEER REVIEW ACTIVITIES

Member, American Society for Biochemistry & Molecular Biology (since 1995), American Society for Cell Biology (since 1999); Referee, *The Journal of Biological Chemistry*, *Proceedings of the National Academy of Sciences USA* (1994-98); Editorial Board Member, *Molecular and Cellular Biology* (1999 to present), *Cancer Research* (1998-2002); Ad hoc member, NIH PTHB study section (June 1999), U.S. Army Breast Cancer Research Program Molecular Biology-2 (MBY-2) review panel (1995-96); Member, National Cancer Institute Board of Scientific Counselors (2001-2005)

PUBLICATIONS

Over 30 original research papers as an independent investigator since 1994, mostly in front-line journals such as the Journal of Biological Chemistry, Molecular and Cellular Biology, Genes and Development, Cancer Research, Journal of the National Cancer Institute, along with several high-profile papers in Nature and Cell.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Saez, Simon	Senior Research Associate

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Willamette Valley College, Albany, OR	BS	1987	Biology
Univ. of Washington, School of Medicine Seattle, WA	PhD	1993	Genetics
Vanderbilt University School of Medicine Memphis, TN	postdoc	1993-96	Cell Biology

RESEARCH EXPERIENCE:

1988-93 Graduate Research Assistant, Dept. of Genetics, University of Washington SOM
1993-96 Postdoctoral Fellow, Dept. of Cell Biology, Vanderbilt University Medical Center
1997- Senior Postdoctoral Fellow, Tumor Biology Prgm., Punxatawney Hlth. Sci. Univ.

HONORS AND AWARDS:

1987 Biology Senior Research Prize, Willamette Valley College
1993 Alfred E. Neumann Award for Best Doctoral Thesis, Univ. Washington, Sch. Med.
1993-95 Individual NSRA (1 F32 GM10586) yeast cell biology/cell cycle checkpoints

PROFESSIONAL MEMBERSHIPS:

American Society for Cell Biology
American Association for Cancer Research

PUBLICATIONS:

2 first author papers on yeast mating type gene regulation as a graduate student, one in Molecular and Cell Biology and one in Genes and Development

3 first author papers from his postdoctoral work on yeast DNA damage checkpoints at Vanderbilt, one of which appeared in Nature Cell Biology

3 first author papers with Dr. Muldoon since 1998, one on p53 acetylation in the Journal of Biological Chemistry, and two in the Journal of the National Cancer Institute on colon epithelial cell transformation by chemical carcinogens

Dr. Saez has also presented several abstracts at national meetings over the past five years as a research associate with Dr. Muldoon; he appears to be well qualified to contribute to the proposed project

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Dr. Muldoon has 4000 square feet of laboratory space adjacent to his office in the Philip G. Hogg Cancer Center. His lab is continuously staffed by a four full-time research assistants funded from other sources, and by 3-4 graduate students supported by an NCI training grant to the Tumor Biology Program in the Department of Medicine.

Immunohistochemistry and molecular biological assays will take place in Dr. Muldoon's and Dr. Fonebone's laboratories. Dr. Fonebone's laboratory consists of 1,600 sq. ft. in five rooms at the Hogg Cancer Center and is staffed by four full-time research technicians, one Ph.D., and one lab managerr in addition to four post-doctoral MDs.

Embedding and sectioning of fixed tissue for histology will be performed in the histology laboratory funded by Dr. Muldoon's program project grant. Processing of Histologic specimens will be performed by Bert Fogelstein, a research technician employed by the Oncology Division and paid by Core 2 of the Program Project Grant.

Clinical:

Nurse coordinators from oncology clinics at the PHSU Hogg Cancer Center, (Doreen Ellis, R.N.) and Getty Hospital (Maynard Michalis, R.N.) will assist in identifying patients with polyps willing to undergo biopsies and donate blood samples. Complete blood counts and differentials on blood from human volunteers will be performed in the clinical hematology laboratory at the PHSU-affiliated Getty Hospital.

Animal:

Computer:

Office:

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

A. SPECIFIC AIMS

The proposed research seeks to test the central hypothesis that pharmacological inhibition of cycloxygenase-2 (cox-2) activity in human colorectal mucosa can prevent or slow the development of epithelial cell transformation and the onset of invasive colorectal cancer.

The cox-2 enzyme, recently shown to be a key mediator of cell proliferation and apoptosis, is upregulated during times of cellular stress and commonly associated with clonally expanded transformed cells. Because the adenomatous polyp is a useful pathological surrogate for subsequent development of invasive colorectal adenocarcinoma, the project's specific aims are to:

1. test the hypothesis that RK42B, a selective cox-2 inhibitor, can reduce the recurrence of adenomatous colonic polyps in patients and thereby act as an effective colorectal cancer chemopreventive agent;

2. test the hypothesis that RK42B reduces prostagladin E2 (PGE2) content and alters cellular proliferation and apoptosis in morphologically normal colonic mucosa;

3. determine whether RK42B's pharmacokinetics can predict an observed reduction in colonic PGE2; and

4. define potential mechanisms of polyp recurrence in patients after prolonged cox-2 inhibition- such mechanisms may include lack of cox-2 enzyme expression in polyps, lack of drug-induced reduction of PGE2 levels in polyps, failure of the drug to reduce cell proliferation in polyps, failure of the drug to reduce apoptosis in polyps, pretreatment aneuploidy, pretreatment activation of kRas, and pretreatment expression of mutated p53 protein.

B. BACKGROUND AND SIGNIFICANCE

B.1 The adenomatous polyp as a surrogate endpoint in colorectal cancer chemoprevention

As one of the most common forms of cancer and a major cause of cancer deaths in the United States and other developed countries (1-3), colorectal cancer is an important and attractive target for chemoprevention because current data on environmental and dietary risk factors suggest that chemopreventive and/or dietary interventions might reduce or delay the incidence of invasive disease (4-6). A step-wise molecular epidemiologic pathway parallels pathological changes in human patients (reviewed in 7, 8). Such pathological changes might thus be used as pathological surrogates to identify patients at high risk for developing invasive colorectal cancer and test the efficacy of therapeutic interventions.

Adenomatous polyps are widely regarded as precursors of colorectal cancer, reflecting a step-wise model for carcinogenesis that features genetic mutations in specific genes, such as APC, p53, k-Ras, and DNA mismatch repair genes, which parallel morphologic changes as normal epithelium transforms into progressively larger polyps and subsequently into invasive carcinoma (7-14). In rodent models, APC mutations cause intestinal polyposis that progresses to invasive transformed neoplasms (15). Likewise, chemical carcinogens induce adenomas that may progress to invasive colonic neoplasms, a process which can be blocked or delayed by chemopreventive agents (16-21). Extensive clinical data, including data from the National Polyp Study (22), now support the use of adenomatous (neoplastic) polyps as a surrogate endpoint for colorectal cancer development. Indeed, resection of adenomatous polyps can greatly reduce cancer incidence in patients. However, recurrence of adenomatous polyps within three years of surgery occurs in a large fraction of patients (23). A substantial reduction in this recurrence rate by a chemopreventive intervention would therefore represent important surrogate evidence for cancer prevention activity, and could thus save many lives.

B.2 Reduction of human colorectal cancer risk by NSAIDs

In most of the human population-based case-control studies conducted to date, aspirin has repeatedly been found to significantly reduce the risk of colorectal cancer occurrence (24-27). While some such studies have

failed to detect this connection (28), the preponderance of evidence strongly favors an association between aspirin use and risk reduction. Similarly, in rodent models, colonic tumorigenesis by chemical carcinogens can be suppressed by treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, piroxicam, sulindac, ibuprofen and ketoprofen, and in at least some instances suppression works best when the drug is administered prior to or shortly after carcinogen exposure, suggesting that NSAIDs might be most effective in preventing early steps in tumor promotion (16, 17, 29-32. Indeed, suppression of 1,2-dimethylhydrazine (1,2-DMH)-induced carcinogenesis correlated with suppression of cyclooxygenase-mediated metabolic activation of 1,2-DMH (32). A more recent animal study demonstrated that RK42B, a selective cox-2 inhibitor, can suppress aberrant crypts and reduce crypt multiplicity, similar to the effects of sulindac (33). Other studies have shown that aspirin treatment significantly reduced colonic mucosa prostaglandin E2 (PGE2) levels in rats, correlating with reductions in tumor incidence (34). Taken together, the rodent studies provide a strong and consistent body of evidence for NSAIDs' chemopreventive activity against carcinogen-induced colon cancer. Consistent with this, human trials have demonstrated a reduction in colonic polyp formation after NSAID treatment in patients with familial adenomatous polyposis. Unfortunately, polyps recurred after discontinuation of sulindac treatment (35, 36). Effects of NSAIDs on sporadic (non-familial) adenomas are less dramatic but still detectable (37).

B.3 Role of cyclooxygenase (cox) enzyme activity in NSAIDs chemopreventive mechanism

While the mechanism(s) by which NSAIDs prevent colorectal cancer remains unclear, recent data strongly suggest that cox-2 overexpression is a key early step in colorectal carcinogenesis, and additional data indicate that cox-2 inhibition by NSAIDs may be crucial to their chemopreventive action (38). Two cyclooxygenases, cox-1 and cox-2, catalyze both oxidative and reductive reactions in the prostaglandin biosynthesis pathway. The proteins are encoded by different genes but exhibit some structural similarity (39-41). Both enzymes are inhibited to different extents by aspirin, with cox-1 being more sensitive than cox-2, both in terms of the kinetics and extent of inhibition (42, 43). In cells transfected with cox-1, aspirin inhibits both PGE and 15-HETE synthesis, whereas in cells transfected with cox-2, aspirin inhibits PGE synthesis but enhances 15-HETE synthesis. Selective inhibitors for both forms of cyclooxygenase are being developed for clinical use.

While the cox-1 protein is typically detectable by Western blot in normal human colon, cox-2 appears to be variably present and often undetectable (43-45). Unlike cox-1, however, the cox-2 gene is selectively induced by a wide variety of mitogens, tumor promoters, and cytokines (42). Comparing normal human mucosa to colon carcinomas, Smith et al. detected 1.5- to 50-fold induction in Cox-2 gene expression in 12 of 14 carcinomas, whereas no Cox-1 induction was observed in the same samples (46). Moreover, Jones et al. found decreased cox-1 protein levels in 21 of 50 human colon cancer specimens relative to matched normal mucosa, whereas cox-2 protein levels were significantly elevated, relative to normal tissue, in 19 of the same 25 cancer specimens (47). Likewise, invasive colonic adenocarcinomas show enhanced cox-2 protein and mRNA expression relative to adjacent normal colonic epithelium in rodent chemical carcinogenesis models (48). Immunohistochemistry detects weak cox-1 and cox-2 protein expression in cytoplasmic and perinuclear cellular compartments of normal human colonic mucosa epithelial cells, as well as mononuclear, Auerbach's, myentic plexus, vascular endothelial cells and gut smooth muscle cells. By comparison, cox-2 expression is markedly enhanced in cancers, becoming readily detectable in many different types of cells (44).

Genetic evidence also suggests that cox-2 overexpression relates to neoplastic transformation. Crosses between APCd716 and cox-2 knock-out mice have revealed a linear relationship between genetic cox-2 induction and polyp number (49) Moreover, a selective cox-2 inhibitor reduced polyp number in APCd716 mice more effectively than did sulindac, which inhibits both cox-1 and cox-2 equally well (50). In addition, colon cancer cells bearing mismatch repair mutations and transfected with a cox-2::luciferase reporter fusion construct show constitutive cox-2 expression while non-transformed cell do not (51). It is reasonable to postulate that increased cox-2 expression plays a major role in malignant transformation at other anatomic sites as well. Both viral (src) and oncogen (ras) transformed mammary epithelial cells contain increased cox-2 mRNA and protein compared to normal parental cells (52), and increased cox-2 levels have been described in human breast (53) and gastric (54) tumors. Keratinocyte cell lines corresponding to different stages of tumor development constitutively overexpressed cox-2 (52) while normal keratinocyte lines express cox-1 in the

differentiated cells (53). These data suggest that cox-2 expression changes during the carcinogenesis process, with expression jumping to peak levels early on then diminishing during or after transformation.

Current data support the idea that cox-2 is a key player on the pathway of colon carcinogenesis. This might occur through several potential mechanisms:

1) Downstream products such as prostaglandins (PGs) could induce cell proliferation and tumor growth, possibly through paracrine or autocrine signaling via G protein-coupled PG receptors (34).

2) Cox-2 could regulate cellular apoptosis. Indeed, although the mechanism remains unclear, cox-2-associated apoptosis appears to be a crucial and early event in colonic carcinogenesis (55-60).

3) Cox-2 redox activity could create colonic carcinogens, such as superoxide and other reactive moieties formed during the oxidation of arachidonic acid, other fatty acids or bile acids (61-63).

Whatever the molecular and cellular mechanism(s) of cox-2 action in carcinogenesis, data from pre-clinical, clinical, and animal model studies strongly suggest that selective inhibition of cox-2 expression and/or activity could form the basis for an effective colorectal cancer chemopreventive intervention.

C. PRELIMINARY DATA

C.1 RK42B, a selective cox-2 inhibitor for development as a chemopreventive agent

RK42B is a diaryl-substituted pyrazole that acts selectively as a competitive inhibitor of cox-2 enzyme activity (64). In preclinical tests, RK42B's IC50 in SF9 cells expressing human cox-1 and cox-2 was 0.04 micromolar for cox-2 compared to 15 micromolar for cox-1. By contrast, indomethacin's IC50 in this sytem was 0.7 micromolar for cox-1 and 0.2 micromolar for cox-2 (66). In a bacterial lipopolysaccharide human blood activation assay, RK42B's IC50 was 0.16 +/- 0.06 micromolar for PGE2 production (67). RK42B and indomethacin have equivalent acute anti-inflammatory and analgesis activities (67; see also Table 1).

Table 1: RK42B Dose Response Anti-inflammatory Efficacy

In vivo assay	ED50 mg/kg RK42B	ED50 mg/kg indomethacin
Carrageenan paw edema	7.0	3.0
Hargreaves analgesia	35.0	10.0
Adjuvant arthritis	0.3	0.2
Acute ulcerogenicity	>200.0	8.0

Tested as a colonic cancer chemopreventive using aberrant crypt foci in F344 rats as an endpoint, RK42B was active at a diet dose of 1500 ppm, which is nontoxic (67, and Table 2).

Table 2: RK42B Effect on Aberrant Crypt Foci in F344 Rats

Experimental group	Total # ACF/rat	# Foci 1 crypt	# Foci 2 crypts	# Foci 3 crypts	# Foci 4 crypts
azoxymethane treated					
control diet	120+/-15	16+/-6.5	35+/-7.7	34+/-4.6	35+/-7.6
RK42B, 1500 ppm*	71+/-15	10+/-4.5	22+/-6.8	20+/-6.8	18+/-5.8
RK42B, 150 ppm	127+/-13	16+/-4.6	44+/-7.0	35+/-6.8	33+/-6.6
sulindac, 320 ppm*	77+/-14	11+/-6.3	24+/-8.5	21+/-6.6	21+/-5.8
placebo, 1500 ppm	111+/-35	15+/-7.7	34+/-11.8	31+/-10.1	31+/-10.2
saline treated	0	0	0	0	0

* $p<0.05$ from control diet group treated with azoxymethane
data expressed as mean +/-SD number of crypts

Preclinical Efficacy: RK42B's's IC_{50} in SF9 cells expressing human Cox-1 and Cox-2 was 0.04 μM for Cox-2 and 15 μM for Cox-1. Indomethacin's IC_{50} was 0.7 μM and 0.2 μM for Cox-1 and Cox-2 respectively (67). In a bacterial lipopolysaccharide human blood activation assay, RK42B IC_{50} was 0.16±0.06 μM for PGE_2 production (66). RK42B has equivalent acute antiinflammatory and analgesic activities to indomethacin (66) (Figure 1).

As a colonic cancer chemopreventive, using aberrant crypt foci in F344 rats as an endpoint, RK42B was active at a diet dose of 1,500 ppm which was nontoxic (34) (Figure 2):

Table 2: RK42B Effect upon Aberrant crypt Foci in F344 Rats

Experimental Group	Total #ACF/Rat	# Foci 1 crypt	#Foci 2 crypts	# Foci 3 crypts	# Foci 4 crypts
Azoxymethane Treated					
Control Diet	120±15	16±6.5	35±7.7	34±4.6	35±7.6
RK42B , 1,500 ppm	71±15*	10±4.5*	22±6.8*	20±6.8*	18±5.8*
RK42B , 150 ppm	127±13	16±4.6	44±7.0	35±6.8	33±6.6
Sulindac, 320 ppm	77±14*	11±6.3*	24±8.5*	21±6.6*	21±5.8*
Placebo, 1500 ppm	111±35	15±7.7	34±11.8	31±10.1	31±10.2
Saline Treated	0	0	0	0	0

*$p<0.05$ from control diet group treated with azoxymethane
Data are expressed as mean±SD number of crypts

Phase I Clinical Trial: RK42B has been administered to healthy human subjects at single doses ranging from 5 to 1,200 mg in a Phase I trial in a placebo controlled manner (2 placebo/4 active drug/dose level). No toxicity occurred other than mild nausea. Both subjects with mild nausea were in the placebo arm. An MTD of 900 mg was defined due to absorption limitation. No clinical laboratory parameters changed (66).

Multiple Dose Phase I Clinical Trial: RK42B was administered to groups of 9 healthy human subjects at doses ranging from 40 mg, 200 mg, and 400 mg BID for 7 and 14 days with a placebo control group. No serious dose limiting events occurred although elevated hepatic function tests were found in the 40 mg dose group. Upon review, hepatic function abnormalities were attributed to weight gain and not reported. RK42B is safe at a dose level of 400 mg BID. No dose limiting toxicity was identified. PGE_2 ex-vivo concentrations decreased significantly at all doses (66).

Pharmacokinetics: RK42B is rapidly absorbed (Tmax = 2 hr) and is biexponentially eliminated with terminal half life ranging from 8 to 12 hours. AUC is linear to 800 mg. Plasma clearance/F ranges between 500 and 900 ml/min. Plasma protein binding is 97%. Administration with a high fat meal delays absorption but increases bioavailability by 20%-40% (66).

Elimination: RK42B is eliminated by hepatic metabolism (P450 2C9) with less than 5% of the dose excreted unchanged in the urine. It is a medium hepatic extraction drug (66).

Clinical Efficacy: No clinical efficacy data are available as a colon cancer chemopreventive. As an antiinflammatory, RK42B at 200 mg and 400 mg doses was significantly superior to placebo for Global Assessment, Assessment of pain, Joint Tenderness/Pain Score, Duration of Morning Stiffness. Similar data were obtained for treatment of osteoarthritis. RK42B provided significant pain relief in the post-surgical dental pain model (66).

Clinical Toxicity: The safety profile reveals no serious adverse events and is reviewed in the protocol (Appendix 1). Of 169 patients on treatment in a Phase II therapeutic trial, 41 have withdrawn with about equal numbers due to treatment failure or due to adverse event. No serious events and no endoscopic changes were associated with RK42B treatment. Toxicity data are provided in Tables 5 and 6 in the clinical protocol (Appendix 1) (66).

Conclusions: Preclinical and clinical safety data support the development of RK42B as a colorectal cancer chemopreventive. At tolerable doses, RK42B has clinical activity, inhibits PGE_2 and has a promising safety profile.

C. PRELIMINARY DATA

I. Modulation of Colorectal Mucosal Surrogate Intermediate Endpoints from Human Samples

a. Phase I Trial of Aspirin Drug Effect upon Colorectal Mucosal Prostaglandins

We defined the relationship of aspirin dose, pharmacokinetics, and prostaglandin concentration in human colorectal mucosa in order to select a dose that we could document caused drug effect in human colorectal mucosa. Healthy human participants were assigned to a single daily dose of aspirin (placebo, 40.5 mg, 81 mg, 162 mg, 324 mg, or 648 mg) for 14 days. Ten subjects were assigned to each dose level (except 648 mg for which 15 subjects were assigned). Colorectal biopsies via unprepped flexible sigmoidoscopy were performed at baseline, 24 hours after the first aspirin dose and 30 and 78 hours after the last (14th aspirin dose). Biopsies were assayed using a competitive immunoassay for PGE_2 and $PGF_{2\alpha}$ (Cayman Chemical).

The lowest dose to statistically significantly suppress colorectal PGE2 from baseline at 24 hours after the first dose and 24 hours after the last dose was 162 mg or more. At 72 hours after the last dose, there was significant suppression for subjects taking 81 mg or more. The lowest dose to significantly suppress colorectal mucosal $PGF_{2\alpha}$ from baseline at 24 hours after the first dose was 324 mg. After 24 and 72 hours after the last dose, 40.5 mg caused statistically significant suppression (See manuscript, Ruffin et al, Appendix 5).

b. Pharmacokinetics of aspirin dosing

The HPLC procedure of Buskin et al (68) using modifications of Bevitt et al (69) was adapted. Samples were run at GLP standard using known standards in addition to standard curves for aspirin and salicylic acid. The mean half lives of aspirin and salicylic acid for all dose levels were 0.45±0.22 hr and 2.68±1.36 hr. AUCs were linear to dose. No aspirin was detectable in plasma 24 hours after 14 days of aspirin dosing at any doses. Salicylic acid was detected prior to aspirin treatment and occasionally 24 hours after the final aspirin dose. These low concentrations were attributed to dietary intake of salicylic acid in foods. Despite the continued suppression of mucosal prostaglandins, there was no detectable aspirin in any subjects 24 hours after a dose. Detectable concentrations of salicylic acid were rare at 24 hours after a dose and related to pre-aspirin treatment salicylic acid concentrations.

In collaboration with Dr. Jacob Thiessen of the University of Toronto, Faculty of Pharmacy, a unified pharmacokinetic model of absorption and metabolism of aspirin given orally has been developed using time concentration data from 48 subjects for aspirin and salicylic acid obtained in this trial (70).

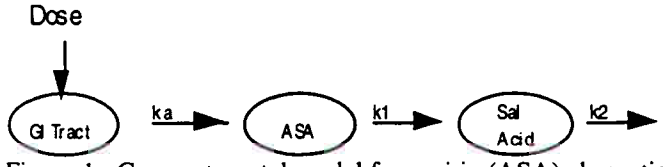


Figure 1: Compartmental model for aspirin (ASA) absorption, metabolic elimination, and salicylic acid (SA) elimination

Equations for this model were simultaneously fit using human plasma aspirin and salicylic data from 48 subjects treated in the Phase I aspirin trial. The model developed employs sampling at 10 minutes, 1.5 hours, and 4 hours after an aspirin dose. The parameters found to predict key parameters with the correlation coefficient (r) or r^2:

Aspirin

$AUC_{(0\text{-inf})} = 0.132 + 0.414(ASA_{0.167}) + 1.878(ASA_{1.5}) + 3.597(ASA_4)$

Prediction: r=0.971; r^2=0.944

$Cmax = -0.062 + 1.193(ASA_{0.167}) + 0.634(ASA_{1.5}) - 1.020(ASA_4)$

Prediction: r=0.974; r^2=0.948

Half life of $k1 = 0.297 - 0.046(ASA_{0.167}) + 0.090(ASA_{1.5}) - 0.138(ASA_4)$

Prediction: r=0.636; r^2=0.404

Salicylic acid

$AUC_{(0\text{-inf})} = 3.528 + 9.317(ASA_{0.167}) - 9.017(ASA_{1.5}) + 9.332(ASA_4)$

Prediction: r=0.987; r^2=0.975

$Cmax = -0.871 + 0.219(ASA_{0.167}) + 0.549(ASA_{1.5}) - 0.408(ASA_4)$

Prediction: r=0.994; r^2=0.987

c. Phase IIa Chemoprevention Trial of Aspirin Drug Effect upon Colorectal Mucosal Prostaglandins and Cyclooxygenases

At the completion of the Phase I trial, we were concerned that the study population was inappropriate (mean age 26 yr.), that all subjects were epidemiologically at normal risk for colorectal cancer, and the cohorts at each dose level were too small. We hypothesized that (a) a once daily aspirin dose given daily for 28 days would suppress colorectal mucosal prostaglandins, (b) Cox-1 protein on Western blot would not be suppressed, (c) that aspirin would reduce crypt proliferation in morphologically normal mucosa, and (d) crypt cellular mucin changes associated with early proliferative change detected by lectin immunohistochemistry would select high risk subjects and would be modulated to that seen in normal human subjects. We studied 84 subjects, age and sex matched for high and normal colorectal cancer risk by obtaining low colonic biopsies via flexible sigmoidoscopy prior to and after 28 days of daily, 80 mg aspirin dosing. High risk was defined as two first degree relatives with colorectal cancer or an adenomatous polyp greater than or equal to 1 cm in size, or a prior history of resected Dukes A or B colorectal cancer.

1. Prostaglandin E_2

Mean PGE_2 for normal risk subjects prior to and after once daily, 80 mg aspirin treatment for 28 days was 11.2 and 5.3 pg/µg protein, respectively ($p<0.0001$). In high risk subjects, mean PGE_2 was 13.1 and 4.7 pg/µg protein, respectively ($p<0.0001$) (Figure 3).

These data confirm that a once daily aspirin dose of 80 mg is sufficient to suppress colorectal mucosal PGE2 in humans (Abstract in Appendix 5).

2. Effect of aspirin upon colorectal mucosal cyclooxygenase protein

Using ovine antibodies to Cox-1 and Cox-2 (provided by SAM JONES Ph.D., Western State University), we have detected and quantified Cox-1 and Cox-2 proteins by Western blot in colorectal mucosa samples from our aspirin treated subjects.

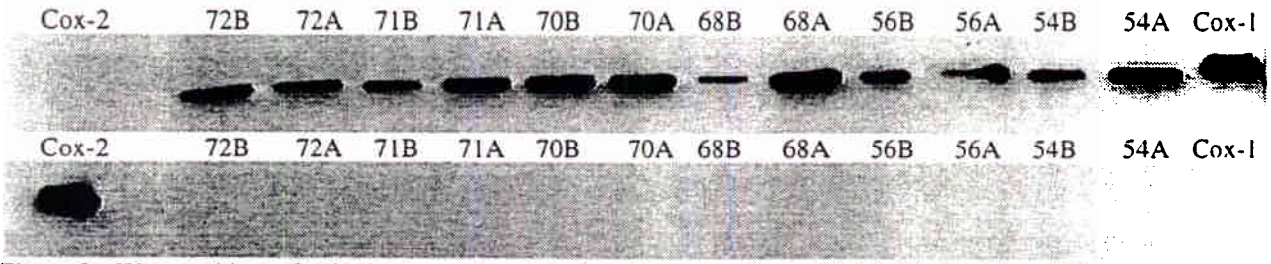


Figure 2: Western blots of colonic mucosal protein from human subjects 54, 56, 68, 70, 71 and 72 prior to (A) and after (B) 14 days of 80 mg once daily aspirin administration for 14 days. The protein from each biopsy was hybridized with Cox-1 antibody, then the same membrane stripped and rehybridized with Cox-2 antibody. Ovine Cox-1 and Cox-2 standards were obtained commercially (Cayman Chemical). The mobility of the human protein is slightly faster than the ovine standards. No Cox-2 protein expression was detected in our subjects.

Integrated densitometry for Cox-1 protein revealed no significant difference prior to or after aspirin treatment.

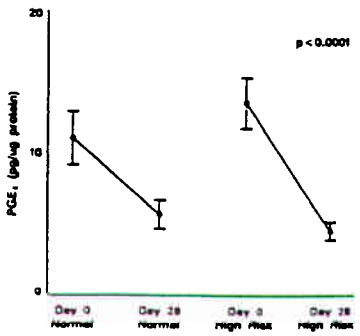


Figure 3: Mucosal PGE2 content prior to and following 28 days of daily, 80 mg aspirin treatment.

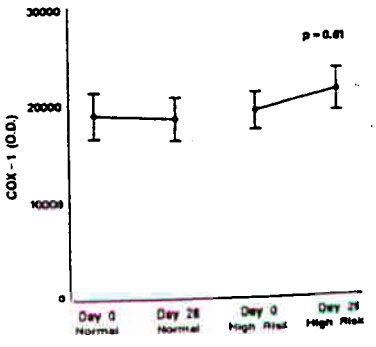


Figure 4: Integrated densitometry for Cox-1 protein from subjects before and after 28 days of daily, 80 mg aspirin treatment.

These data suggest aspirin doses sufficient to suppress PGE_2 in colorectal mucosa does not reduce immunoreactive Cox-1 from the same tissue sample. Immunoreactive Cox-2 protein is not present in morphologically normal colorectal mucosa from normal or high risk human subjects.

3. Colorectal Mucosal Proliferation Indicies in Normal and High Risk Human Subjects: Lack of Effect of Aspirin

We quantified proliferation using PCNA (PC10, Novocastra Antibody) of colorectal epithelial cells in crypts from a subset of 14 normal risk and 14 subjects with adenomatous polyps before and after 28 days of daily, 80 mg aspirin treatment. Samples were fixed in buffered formalin for no more than 24 hours, then fixed in paraffin before immunostaining with PCNA using streptavidin-horseradish peroxidase detection. Proliferation was evaluated by counting the number of PCNA-labeled cells and total number of cells in each of the 5 compartments and total crypt column. A weakly labeled cell was not counted as positive.

Table 3: PCNA Labeling Index of Colorectal Crypts of Aspirin Treated Human Subjects

Crypt Level	*High Risk Pretreatment*	*High Risk Day 28*	*Normal Risk Pretreatment*	*Normal Risk Day 28*
Total Labeling Index	16.6±0.9	16.1±0.9	11.6±0.6	11.2±0.4
Compartment 1 (low)	22.8±2.0	21.6±1.7*	19.5±1.2	18.8±1.3
Compartment 2	17.6±1.7*	18.4±1.2*	12.5±1.4	11.6±1.1
Compartment 3	14.9±1.4*	14.1±1.2*	11.0±1.0	10.2±1.0
Compartment 4+5	13.9±1.2*	13.2±1.0*	7.6±0.8	7.5±0.9

Data are expressed as mean ± SE. *p<0.01 vs normal risk

Proliferation is increased in colonic epithelial crypts in high risk subjects. One month of daily aspirin administration does not reduce proliferative index in the colonic crypts of high risk subjects.(Manuscript Appendix 5)

4. Aspirin Modulation of Colorectal Mucosal Mucins

Lectins are naturally occurring proteins and glycoproteins extracted from plants and other sources that can be used as probes of glycoconjugate structure (71, 72). Alterations in membrane glycoconjugates have long been known to occur with normal cellular differentiation and in cancers. *Amaranthus caudatus* (ACA) binds to the Thompson-Friedenreich antigen (T-antigen) and its sialylated variants which is used as a histochemical probe for proliferating cells in human colonic tissues. *Dolichos biflorus* agglutinin (DBA) recognizes specifically N-acetylgalactosamine and binds to goblet cells of the upper crypt and surface epithelium within the proximal We quantified ACA and DBA binding to colorectal epithelial crypts from a subset of 14 normal risk and 14 subjects with adenomatous polyps before and after 28 days of daily, 80 mg aspirin treatment. Samples were fixed in buffered formalin for no more than 24 hours, then fixed in paraffin before immunostaining with ACA or DBA lectins with fluorescence detection. Weighted scores (0-400 scale validated by Sams et al (73)) for lectin staining were determined in well-oriented crypts.

Table 4: Aspirin Effects upon Lectin Scores and Proliferation in High Risk Human Subjects

	High Risk Pretreatment	*High Risk Day 28*	*Normal Risk Pretreatment*	*Normal Risk Day 28*
ACA Crypts per Subj	17.2±0.5	17.4±0.4	15.8±0.5	16.3±0.5
ACA Weighted Avg	109.1±20.7	63.7±9.9*	101.3±6.7	78.1±10.4*
DBA Crypts per Subj	15.9±1.1	16.1±0.8	16.4±1.1	16.4±0.8
DBA Weighted Avg	330.3±12.4	327±10.6	360±7.5	359±6.1

Data are expressed as mean ± SE. *p<0.01 vs baseline

Aspirin reduces ACA binding to morphologically normal crypt cell mucins in both high and normal risk subjects; however, ACA binding does not help select those subjects without a evidence of a genetic syndrome whose epithelium might be at higher risk than normal (Manuscript, Appendix 5). DBA binding did not differ according to subject risk. We conclude that ACA may help define drug effect in early proliferative cells but does not identify specific high risk subjects when assessed in morphologically normal colonic epithelial crypts.

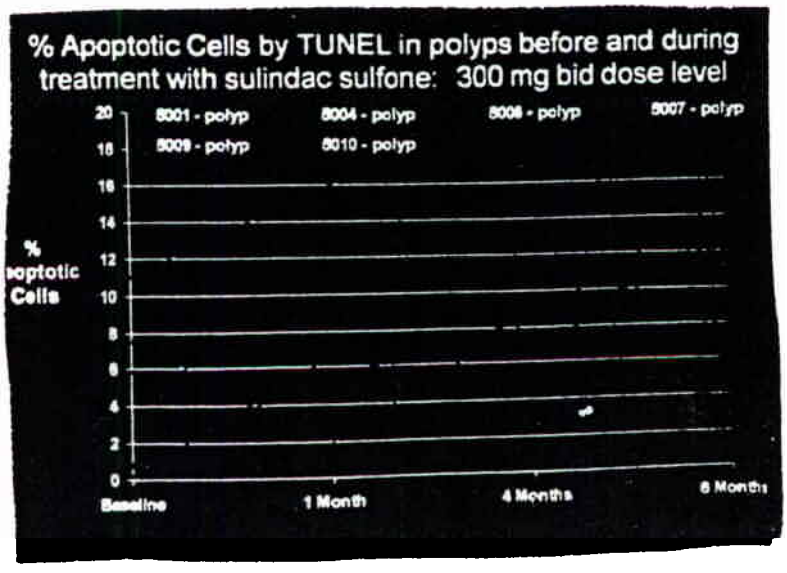
f. Surrogate Biomarkers Human Polyps from a Phase I Clinical Trial of Sulindac Sulfone

During the past two years, Dr. S[illegible] of The Mid State University has been involved with the [illegible] Clinic in a Phase I trial of the toxicity and metabolism of sulindac sulfone in patients with familial adenomateous polyposis. Rectal polyps were collected from treated patients during the trial and analyzed in Dr. S[illegible] laboratory for effects of sulindac sulfone on cell proliferation (Ki67 labeling index), and studies have recently been initiated to examine DNA ploidy in polyps that showed partial regression to determine if the regression is associated with restoration of normal colonic epithelium. In addition, in collaboration with Dr. Gary [illegible] of Cell Pathways, Auro[illegible] we examined the effect of sulindac sulfone on apoptosis in polyps.

Figure 5 and 6 (Color figures 5-6 in Appendix 2) illustrates staining for Ki67 in polyps, respectively. As indicated, the percentage of stained cells in rectal polyps was higher than in normal mucosa. The Ki67 labeling index was higher in polyps taken from patients treated with sulindac sulfone for six months than in zero-time polyps indicating a proliferative stimulus of the drug. Interestingly, this was correlated with the ultimate conversion of dysplastic glands to mucus-producing glands and an increase in the apoptotic index (Figure 6). We conclude that sulindac sulfone stimulates proliferation in cells of dysplastic polyps which then undergo mucus differentiation and apoptosis. The results of these studies were presented at the 1997 Annual Meeting of the American Association for Cancer Research in San Diego and are currently being assembled for publication.

Figure 5:
Ki67 immunostaining of colonic human polyp.

Figure 6:
% Apoptotic cells by TUNEL (Apoptag) before and during treatment with sulindac sulfone (300mg bid)



g. Imaging Algorithm to Quantify Immunohistochemical Markers (See Appendix 3 for Images)

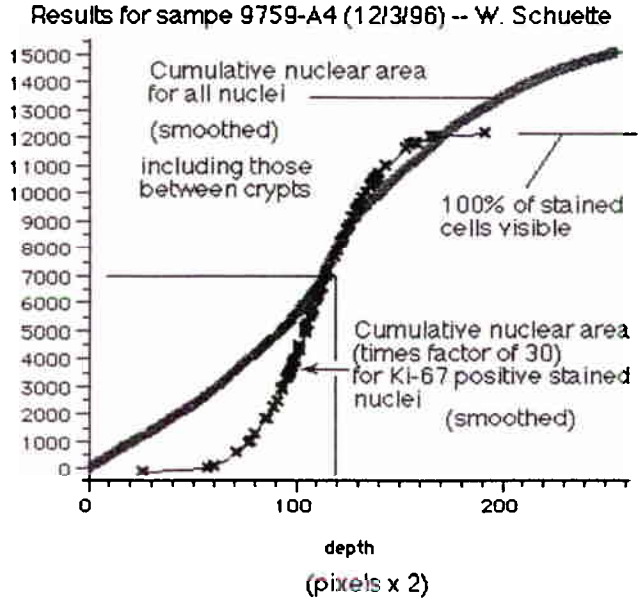
The classical method for determination of cellular indicies presumes locating a "well-oriented crypt", which in turn defines regions in terms of the crypt base(1, 74-76). However, the determination of which crypts are entirely visible and which have been truncated by the 3-D geometry of sectioning is problematic and observer dependent. The resulting selection bias for locating "well-oriented" crypts makes most crypts on most biopsies unusable, especially as the tissue becomes dysplastic, which limits the applicability of the technique, reduces its power to characterize the tissue appropriately, discards most of the visible information on the biopsy, and biases what is measured to the less-dysplastic regions.

We have developed another technique, based on "Euclidean Distance Maps" (EDM's), which is a generalization of the classical method. It effectively reduces to the classical method for non-dysplastic tissue, but it can be applied as well to highly-dysplastic tissue with ill-defined crypts. In addition, all visible nuclei are counted and there is much less room for observer-dependent bias. We believe this will prove a substantially more useful technique and, in addition, it is faster and easier to measure than the classical process.

The proposed technique abandons identifying and selecting individual crypts and the categorizing of them as "well-oriented" and works instead on a tissue-wide basis, counting every nucleus in the image, and identifying the "base of crypt" depth implicitly from distribution curves, as discussed below. This technique is made possible through the use of digital image processing algorithms and standard image-processing software, such as NIH's "Image" program.

For each nucleus in the image categorized as "positive" for a given staining marker the distance from it to the nearest point on the outer surface of the biopsy is determined; a histogram showing number of nuclei vs depth from the surface is generated; the histogram is integrated to show the cumulative number of nuclei as a function of depth, giving a sigmoid curve. The sigmoid curve is fit with a 3-parameter model, characterizing the stained positive nuclei distribution. A similar process characterizes the stain-negative nuclei distribution.

Figure 9: Fitted sigmoid curve for stained and non-stained crypt cells (Ki67)



15,000 square pixels corresponds to about 5000 nuclei.

From the resulting fitted curves, any of the classical measures can be computed, whether it is fraction of the crypts broken into upper and lower halves, or thirds, fourths, fifths. This allows maximum comparison of newly obtained data to the literature, regardless which of several techniques the prior investigators selected.

D. METHODS

I. Overview of Study

We propose a multicenter trial to recruit subjects with at least 1 adenomatous polyp to participate in a randomized, placebo controlled trial of RK42B After successfully completing a one month run-in period, participants will be randomized to one of three arms--placebo, RK42B 200 mg (low dose) and RK42B 400 mg (high dose). The primary endpoint will be polyp recurrence after 3 years of treatment (Aim 1). Pharmacokinetics of RK42B will be evaluated in a subset of studies (Aim 3) and linked to drug effect upon morphologically normal epithelial PGE_2, proliferation and apoptosis (Aim 2). Surrogates of cellular transformation will be studied to define causes for polyp growth in the face of Cox-2 inhibition (Aim 4). Comparison of cellular transformational surrogates in recurrent polyps from placebo treated polyps compared to RK42B treated polyps will provide important information regarding mechanisms of chemopreventive drug action and of cellular transformation in the human gut.

II. Justification of Study Design

a. Justification for Proposing a Pivotal Trial for Colorectal Cancer Prevention with RK42B

Substantial data in animals, humans, and in *in vitro* models cited previously support the fact that many NSAIDs have the potential of preventing colorectal cancer. Such data are only preliminarily available for RK42B . Then why RK42B and not some other NSAID? We believe that RK42B has a very strong potential for being a successful colorectal chemopreventive for the following reasons: First, preclinical data point to the likelihood that Cox-2 and not Cox-1 is the crucial link in NSAID chemopreventive effect. While no single mechanism of chemopreventive activity for any of the NSAIDs has been clearly defined, based upon new data published over the last year and reviewed above (see Significance section), it is reasonable to suggest that selective inhibition of Cox-2 is likely to be a highly effective anticarcinogen and may potentially be a crucial mechanism by which many NSAIDs reduced colorectal cancer incidence in the previously cited human epidemiologic studies. Second, selective Cox-2 inhibition is likely to have reduced toxicity compared to the currently used NSAIDs that are being tested as chemopreventives. In preliminary trials as an antiinflammatory, RK42B toxicity profile has not differed from that of placebo subjects. A moderate sized population trial such as the one proposed here will provide longer term efficacy and toxicity data to determine RK42B usefulness as a chemopreventive for large, high risk populations. Decisions regarding a risk reduction trial may follow. Third, the large numbers of polyps and normal tissue mucosa with an expected polyp recurrence of >200 subjects, two thirds RK42B treated and one third placebo treated, will permit us to probe important chemopreventive mechanism questions in humans. The design of this trial provides us with an opportunity to explore the reasons for anticarcinogenesis treatment failure in humans by studying important surrogates of cellular transformation before and after drug treatment and comparing surrogate markers in placebo controlled subjects to subjects treated with RK42B Finally, this proposal permits us to develop a an expanded safety profile for RK42B in humans focused upon chemopreventive safety using a sophisticated quality of life assessment method as compared to the standard NCI common toxicity scale.

b. Rationale for Molecular and Biochemical Surrogate Endpoints

We are predicting that polyps 1 in 4 study subjects will recur (placebo [estimated recurrence=33%], high [estimated recurrence=21%] and low dose [estimated recurrence=25%] RK42B). The mechanism by which these polyps might recur is unknown. Nevertheless, it is our opinion that certain parameters can be measured to provide clues as to the mechanism of resistance of these polyps to Cox-2 inhibition. There is already evidence that not all early proliferative or preneoplastic lesions must overexpress Cox-2 in order to progress towards transformation. How might clonally expanded or pretransformational cells be resistant to a Cox-2 inhibitor? How would detection and quantitation of molecular and biochemical surrogates provide important inferences into the carcinogenesis process in humans?

1. They can help determine whether cells, while microscopically non-invasive, clonally expanded or normal microscopically have committed to transformation beyond the point that a single, targeted mechanistic chemopreventive intervention is effective. If this is the case, then cells in polyps that recur or have a high likelihood of recurrence are likely to have multiple molecular and

biochemical changes suggestive of LOH and molecular mutation. We propose measurement of cellular ploidy (looking for aneuploidy), kRas, and p53 proteins in polyps prior to and after Cox-2 inhibitor treatment. We expect increased incidence and number of cells in polyps, even those the majority of recurrent polyps will be small, with each or all of these changes which would be indicative of the "late adenoma" stage described by the Vogelstein group (8). Such cells are "committed" to transformation an unlikely to be modified with a chemopreventive agent.

2. They can determine whether sufficient pharmacologic quantities of drug have been delivered to the target and whether the target has been modulated by the drug: This is a pharmacodynamic argument. Measurement of PGE_2 in morphologically normal colonic mucosa pretreatment and after 6 months of treatment will define whether sufficient drug reached the colonic mucosa to cause an effect. Since morphologically normal colorectal mucosa does not express sufficient quantities of Cox-2 protein to detect on Western blot, measurement of prostaglandin product is necessary. As noted in the Significance section, RK42B reduced PGE_2 content in human cells after oral administration. Quantitative PCR will detect expressed Cox-2 mRNA but is expensive for a large number of samples to be generated. We expect to receive sufficient numbers of frozen polyps prerandomization that, with the expected yield of polyps post treatment (257 polyps), we will have sufficient material to match Cox-2 expression by Western blot with polyps from the same subjects pre- and post-treatment. We will also measure PGE_2 in polyps pre- and post-treatment. Drug effect should cause a reduction in PGE_2 in normal mucosa and in the polyp. We expect that recurrent polyps in placebo subjects should express Cox-2 protein and have unchanged PGE_2 content whereas polyps recurrent after RK42B treatment may not express Cox-2 protein prior to or after treatment. If this is indeed the case, we can conclude that Cox-2 expression in the polyp is a useful surrogate biomarker for chemopreventive efficacy and that sufficient RK42B was delivered to the colorectal mucosa to inhibit the target enzyme.

3. They might point to alternative mechanisms of cellular carcinogenesis that, if appropriately targeted, would reduce the likelihood of progression to transformation: Most transformed cells lose proliferative and apoptotic control at some time during the carcinogenesis process. Polyps that recur after celecoxib treatment may have specific defects that are reversible with appropriate chemopreventives. For example, if polyps recurrent after placebo treatment have reduced apoptotic indicies compared to Cox-2 treated polyps, then we can infer that preclinical data regarding Cox-2 apoptotic effects are correct in humans. Combination therapy with antiproliferatives may be warranted. Furthermore, a more detailed exploration of specific biochemical mechanisms of cellular proliferative control (e.g. ornithine decarboxylase enzyme activity, protein kinase C activity, retinoid receptors) or molecular mechanisms (e.g. analysis of DCC, MCC, or oncogenes other than kRas) may be undertaken with frozen polyp samples from this trial in order to develop a better understanding of early mechanisms of transformation in the human gut. Thus, we propose to screen for proliferative and apoptotic activity with Ki67/amaranthin and apotag immunostaining, respectively, in polyps obtained pre and post randomized treatment. These detection methods will provide important insight into reasons for treatment failure with Cox-2 and suggest alternative strategies to reduce or prolong cellular transformation time in the human colon. We have the capability of further exploration of specific proliferative and apoptotic mechanisms with the frozen and fixed tissue available through this trial but have not proposed to do so due to the size of the cohort and the expense of performing these assays. Based upon preliminary data obtained from this cohort, subsets of subject material will be assessed mechanistically in a later proposal.

c. Justification for Large Multicenter Trial

We wish to complete this study in 5 years. A three year treatment duration is necessary to adequately assess polyp recurrence (77). In order to enroll a sufficient number of subjects to adequately address the primary endpoint within the allotted study time, a multiinstitution trial is necessary.

d. Alternative Clinical Trials Strategies

We recognize that other experimental designs might have been chosen. For example, a "two polyp" strategy, (i.e. require subjects have two polyps, remove one polyp prior to study entry, tattoo the second polyp leaving it in place, treat with drug/placebo, then return after 6 months to remove or inspect the location of the remaining polyp) has great appeal as a clinical trials

design. We rejected such a design because the central hypothesis of this project was to determine whether polyp recurrence was prevented by RK42B Requiring a second colonoscopy prior to 3 years complicates the experimental design in a multiinstitution trial and reduces the pool of eligible subjects substantially. Given that polyp prevention remains the primary endpoint, the 2 polyp design will unacceptable prolong enrollment and the project's duration. Some collaborating investigators objected to the 2 polyp design on the basis of ethical concerns of leaving a known adenomatous polyp in the colon for 6 months. They were concerned about the potential of the remaining polyp having invasive cancer. We also considered a study of Familial Adenomatous Polyposis patients rather than subjects with sporadic adenomas. Such a study design also has great appeal because of the ready accessibility of polyps for removal, more frequent surveillance intervals allowing more frequent sampling, and the known association with APC mutations that has been suggested as driving Cox-2 induction. We rejected this design because of accrual concerns--this is a rare lesion with families scattered throughout the USA, most APC subjects are detected as minors and would therefore be difficult to enroll in a trial of this type, and many subjects undergo surgical colectomy as children or teenagers, thus the remaining at risk lower gut consists of a very small rectal shelf used to anastomose the ilium with the anus. We also rejected this design because of our interests in addressing the sporadic polyp, a much more common event in the healthy population, rather than a known inherited but relatively uncommon genetic defect.

II. Description of Clinical Trial: Phase IIb randomized, double blind placebo controlled trial of RK42B as a chemopreventive agent for colorectal carcinoma (Protocol, Appendix 1)

a. Overview

This is a complex, international, multicenter translational trial of 12 clinical institutions, an administrative center, a Statistical and Data Management Office, and a Central Laboratory. Key features of the protocol and clinical methodology are described here. For more detailed descriptions of quality control and data management methods, the reviewers are referred to the appended protocol (Appendix 1).

b. Subject Selection (see Appendix 1 for full protocol)

1. Major eligibility criteria

(a) Males, females (postmenopausal or premenopausal women who agree to use effective birth control)

(b) 18 years of age or older.

(c) Having at least 1 histologically confirmed large bowel adenoma (neoplastic polyp) of ≥1 cm in size removed within 3 months prior to the intake appointment.

(d) Currently disease free with normal bone marrow, hepatic and renal function are eligible for this trial.

2. Major exclusion criteria (See Protocol, Appendix 1 for more details)

(a) Taking medicines that inhibit COX (e.g. steroids, NSAIDs)
(b) Chronic disease with an expected survival of 3 years or less
(c) Malignant diagnosis within 5 years except for basal cell carcinoma
(d) Severe cardiac or pulmonary disease
(e) Hyperplastic polyps in the absence of adenomatous polyps
(f) Prior large bowel resection
(g) Stages A-D colon cancer
(h) Pregnancy
(i) Failure to adhere during the run-in period
(j) Allergy to celecoxib
(k) Known HNPCC and FAP

c. Drug, Dose, Schedule, Duration of treatment

Three armed randomized clinical trial. The arms consist of Placebo, RK42B 200 mg. and RK42B 400 mg. Drugs and placebo will be taken twice daily, in the morning and in the afternoon on an empty stomach.

d. Drug, Dose, Schedule, Duration of treatment, Rationales

Rationale for dose levels: Dose levels were chosen based upon preliminary clinical data for RK42B in rheumatoid subjects. Furthermore, the effective dose used by Reddy et al in the aberrant crypt model translates to a 400 mg, twice daily human dose (34). Data to date suggest that RK42B is an effective antiinflammatory agent at both 200 mg and 400 mg twice daily doses, that

the toxicity profile is acceptable, and that PGE_2 is suppressed (via indirect assay) but thromboxane is not suppressed at these dose levels in humans (66). We propose testing two dose levels to determine whether a lower dose might have equal efficacy to the higher dose, yet be potentially less toxic and, therefore, more efficacious.

Rationale for dose schedule: Although RK42B has long half-life (7-12 hours), preliminary clinical data have used a twice daily dosing schedule. This dosing schedule has resulted in suppression of the prostaglandin drug effect surrogate. Since toxicity at this schedule has been acceptable, we propose the same schedule for use as a chemopreventive.

Rationale for 3 year treatment period: Three years is the recommended surveillance period for colonoscopic evaluation of polyp recurrence. This is a realistically long treatment period that will permit adherence assessment and treatment effects. The colonoscopies performed are considered standard of clinical care.

e. Randomization methodology

After successful completion of the run-in period, subjects are randomized to one of three treatments. Subjects are assigned a subject identification number (SID). The SIDs are sequential within each site. SIDs are assigned to treatments in blocks of six, with each block containing two of each treatment. The Investigational Drug Service at the University of Arizona labels the product with the SIDs before shipping it to the clinical sites. The treatments are assigned in a randomized order to the SIDs by a computer program written by the Statistics and Data Management Office. The assignments are blinded to key personnel within the Office, as well as the clinical and laboratory personnel. Assignments will be kept in the Investigational Drug Service in the University of Arizona Pharmacy which will serve as the 24 hour emergency unblinding site.

f. Placebo and drug preparation procedure

All tablets are distributed by the coordinating center, which will obtain RK42B tablets and placebo from RK42B Co. The tablet calendar packs including the electronic adherence packs will be prepared by RK42B Co. and will each have 31 tablet blisters per pack. A tablet will be used for each day of the month as labeled on the pack. For months less than 31 days, the tablet in day 31(or lower) will not be used. The run-in pack (placebo for all subjects) and one pack every 6 months will be electronically monitored. The filled packs will be shipped to the University of Coordinating Center where they will be labeled with the identification number of the participants and with instructions for administration. They will then be distributed under supervision of the study pharmacist either to the clinical centers on a once yearly basis. Calendar packs are sent to subjects every four months from each clinical site.

g. Endoscopic Procedures

(a) Cleaning colonoscopy at diagnosis: All subjects will have had a complete colonoscopy within 3 months (92 days) of the intake appointment with the entire large bowel visualized (cleaning colonoscopy).

(b) Biopsies for biomarkers pretreatment, 6 months: The subset of subjects agreeing to enter the biopsy protocol will have a flexible sigmoidoscopy without preparation with biopsies prior to the run-in period and 6 months after randomization.

(c) Colonoscopy after 3 years of RK42B: Colonoscopic visualization of the entire large bowel will be performed in each subject 36 months after the cleaning colonoscopy.

h. Justification of procedures and timing of procedures

(a) Colonoscopy: The proposed follow-up schedule is appropriate for this trial for several reasons. First, for subjects with large (≥1 cm) adenomatous polyps, the recommended colonoscopic surveillance interval remains 3 years (77, 78). Second, recent improvements in colonoscopy technique (magnified digital imaging) have probably lessened the problem of missed polyps. Third, because of randomization, polyps overlooked on the baseline exam should occur equally in the RK42B and placebo groups. The impact on analyses using the numbers of polyps seen by year 3 would be a conservative bias in relative effect measures. Finally, RK42B may actually cause adenomas to regress, leaving only truly incident polyps to be found at year 3.

(b) Flexible sigmoidoscopy: Flexible sigmoidoscopy rather than biopsy at colonoscopy to procure morphologically normal colonic mucosa will be performed in a subset of subjects because of concerns that the preparative procedure required prior to colonoscopy (full liquid diet, go litely,

enemas) will disrupt stable colonic epithelial physiology and create artifactual findings. The sigmoidoscopies will be performed without preparation. The biopsies will be taken beyond the endoscope to reduce endoscope induced trauma. Our preliminary data suggest biomarker changes in colonic crypts within 1 month of treatment initiation. We propose a 6 month assessment time in order to test the effects of longer term, steady state treatment.

i. Study Calendar (Table 4)

Month	Pre-Rx	-1[h]	0	4	6	12	16	20	24	28	32	36
History - physical exam[a]	X											X
Eligibility Check List[b]	X											
Demographic Review[c]	X											X
Diet Evaluation[d]	X					X			X			X
Concomitant Medications	X			X	X	X	X	X	X	X	X	X
Toxicity Assessment[e]				X	X	X	X	X	X	X	X	X
Blood Sample[f]	X					X			X			X
Placebo		X										
RK42B or placebo			X	X	X	X	X	X	X	X	X	X
Pharmacokinetics[g]					O[i]							
Colonoscopy	X											X
Flexible Sig with Bx	O[i]				O[i]							
Stool Guiac	X											

X= Required, all subjects; O= Subset special studies at designated Institutions

[a]History and Physical Examination.

[b]Completion of eligibility check list to ensure eligibility.

[c]Demographic review: Demographic questions.

[d]Diet Evaluation: The Fred Hutchinson Cancer Research Center Food Frequency Questionnaire is administered.

[e]Toxicity Assessment: Toxicity assessment form to be filled out.

[f] Blood Sample: 40 ml of blood obtained for the following tests: CBC with differential, platelet count, electrolytes, hepatic function tests, renal function tests, prothrombin and partial thromboplastin times and 5 ml for micronutrient analysis and 5 ml serum storage.

[g]Pharmacokinetics: Study in the Clinical Research Center, blood sampling after AM RK42B dose per Section IIj, applicable only to subjects at University of

[h]Run-In: 1 month run-in phase with placebo with electronic monitor blister pack. Subjects with <80% adherence will not be randomized at the completion of the run-in phase. A two week break between completion of the run-in period and start on trial will be required to obtain the electronic blister pack from the run-in, download and read the data, randomize and ship drug to the clinical center.

[i] To be performed in a subset of subjects--Flexible sigmoidoscopy to be performed on 288 subjects at 4 institutions during run-in period and 6 months after randomization. Pharmacokinetics to be performed at the University of

j. Pharmacokinetic studies

We propose to monitor individual pharmacokinetics of RK42B over long term treatment. Pharmacokinetic sampling coinciding with the 6 month biopsy procedure in 30 subjects from one center (Univ. of Akron) will be performed to address the relationship between RK42B pharmacokinetics and biomarker modulation. Subjects will come to the Clinical Research Center and take their morning dose prior to breakfast. Blood samples will be obtained at the following time points: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, and 12 hours.

k. Tissue and plasma handling procedures

1. Timing of tissue collection procedures: All flexible sigmoidoscopy biopsy specimens will be obtained 8 to 12 hours after the most recent RK42B dose.

2. Size of biopsy samples, assay needs, allocation and prioritization of specimens: Each rectal biopsy obtained during a flexible sigmoidoscopy weighs approximately 5 mg, contains approximately 400 µg protein. When homogenized, this is sufficient for measurement of prostaglandin content and for Western blots. Biopsy samples will be handled in the following order at the sigmoidoscopy and distributed as follows: Bx #1: Prostaglandin assay (frozen), Bx #2: Western blot (frozen); Bx #3-6: Lectins/Ki67/apoptosis/p53 Immunos/H&E (fixed), Bx #7: kRas, Bx #8: future use (frozen).

3. Tissue collection and storage procedures: Frozen = Tissues immediately frozen (within 15 seconds) in liquid nitrogen and stored at -80°C, shipped to Central Laboratory on Dry Ice. Fixed = Tissue placed in 10% buffered formalin immediately after biopsy for 18-24 hours. Tissue samples then removed from buffered formalin and placed in phosphate buffered saline (pH 7.4) (PBS). Shipment to Central Laboratory for paraffinization within 2 weeks of tissue removal.

4. Pharmacokinetic study procedure and specimen handling: Samples obtained in 7 ml heparinized tube, the plasma separated and stored at -20°C until assay.

l. Shipment logistics and verification

1. Tracking and verification: Upon registration, a code will be assigned to the subject which will be used to track samples and data. Following the run-in period a 2 week rest period will be used to download the adherence data, assess adherence, randomize and ship drug supply to the clinical site. Sample data bar code labels will be prepared and shipped to the site with the first, fourth month drug blister pack supply. A sample shipping form containing date, time of biopsy, date, time of shipment to the Central Laboratory, storage time at the shipping site will be accessed at the Web site, completed, printed and bar coded. A copy of this form will accompany samples with shipments to the Central Laboratory. The Central Laboratory will complete a separate form at the Web site and read the bar codes into the computer to match and verify subject codes and dates. All shipment/receipt/assay/completion data will be tracked in the database.

2. Special shipping boxes: Samples will be shipped in a specially provided, double compartment, insulated, reusable box. One compartment will have dry ice and will keep frozen samples frozen for a minimum of 36 hours. The second compartment will be at room temperature for samples in PBS.

3. Box preparation: The Central Laboratory will ship two boxes to each site and have 4 boxes in reserve. Each box when shipped will have a kit containing four tubes with fixatives for the biopsy samples (formalin, PBS), a page of instructions for the order and labeling of biopsy samples, and an overnight express label for easy shipping. The Central Laboratory is at the [redacted] State University.

m. Individual Subject Study Endpoints

1. Completion of the study duration for the assigned time as defined above OR
2. Toxicity considered unacceptable (as defined below, Section "l") OR
3. Subject refusal to continue on with study OR
4. Diagnosis of cancer OR
5. Diagnosis of a polyp OR

n. Toxicity monitoring

Toxicity data are obtained from the 4 month Adverse Reactions form (Appendix 1) or from reports of perceived adverse effects. Complaints are qualitatively and the quantitatively defined using a Quality of Life Instrument developed by the Recruitment and Adherence Core (Appendix 1). A toxicity score is derived from the NCI Common Toxicity Scale.

o. Criteria for dose reduction and toxicity

Subjects with NCI Common Toxicity of ≥3 that are drug related will be removed from study due to drug toxicity. Subjects with NCI Common Toxicity of Grade 2 will remain on study but may require an 8 day drug holiday with restart of the drug. Should a second drug holiday be required, the subject will be removed from study due to toxicity.

p. Justification of Sample Size

1. Total Enrollment: The number of patients recruited for the trial is determined by the primary outcome variable, polyp recurrence. In addition, tissue from all patients at some centers will be harvested for a detailed analysis of intermediate biomarkers.

2. *Test characteristics*: The relationship of the primary outcome variable, recurrence of polyps at three years, will be compared to the three-level treatment variable by means of a Mantel-Haenzel test (which adjusts for between-center effects) at a 5% significance level. The Mantel-Haenzel test is a variant of the standard χ^2 test, and is implicitly-two-sided. The primary outcome variable will be tested based on intention to treat, where subjects who are randomized but do not complete the three-year colonoscopy are considered treatment failures; subjects who complete the initial colonoscopy but are not randomized due to noncompliance during the placebo run-in, or withdraw or drop out for other reasons prior to randomization will not be included in the final analysis. Other than the test for overall treatment effect, between-dose effects will be not be compared as part of the primary analysis, so no adjustment for multiple comparisons will be performed.

3. *Treatment assumptions:* The majority of recurrent polyps will be small (estimated 66% will be ≤0.5 cm (22)) but will be adenomatous. Because of the high likelihood of progression of these recurrent small adenomatous polyps, a 50% reduction in their recurrence in the high dose drug treated group compared to the placebo arm is considered a clinically important outcome. Reported polyp recurrence rates at 1 to 3 years have ranged from 6% to 52% (77, 79-84). The most recent prospective data have reported 32% to 42% adenomatous polyp recurrence 3 years after initial colonoscopy (22). because of expected changes in our experimental subject behaviors (e.g. increased calcium intake, dietary and habit modifications), we assumed a much lower polyp recurrence rate of 25% in the placebo arm. We assumed that 30% of the subjects from each treatment group drop out of the trial between randomization and the three-year colonoscopy, that dropouts are unrelated to treatment success or failure, the recurrence rate is reduced 25% (to 18.75%) in the low-dose RK42B group and 50% (to 12.5%) in the high-dose RK42B group. Finally, we assumed that there is no significant between-center variation in recurrence. By Table 5 below, we have acceptable power for placebo recurrence of 25% or higher.

4. *Calculation:* To calculate the required sample size, the expected proportion of subjects experiencing treatment failure in each dose group under the null and alternative hypotheses is calculated. Under the null hypothesis, the expected proportions of treatment failures in all three dose groups are equal. Under each hypothesis, thirty percent of subjects from each dose group experiencing treatment success and an equal number experiencing treatment failure are assigned to be treatment failures under the intention to treat rule. For a given sample size, the power of a standard χ^2 test is then calculated using the noncentrality parameter determined by the difference in the sample proportions under the null and alternative hypotheses (85). Using this method, it is determined that 990 subjects assigned equally between the three groups will provide power of at least 90% to test the null hypothesis of the primary endpoint. We chose a very conservative assumption of a low baseline recurrence rate, well below that described in the literature. The power of the proposed project will be sufficient to detect smaller differences from a higher baseline recurrence rate. The sensitivity of the assumptions is demonstrated by the following table that displays the power under assumptions adjacent to those listed above:

Table 5: Power Analysis

Baseline Recurrence	% Reduction: 400 mg	% Reduction: 200 mg	Power %
20	50	25	72.9
25	50	25	90.0
30	50	25	97.6
35	50	25	99.6
40	50	25	99.9

5. *Tissue samples for biomarkers:* The number of subjects from whom polyp and normal tissue will be harvested and analyzed for intermediate biomarkers is determined as follows: In order to ensure sufficient recurrent polyp tissue is available for even minimal subgroup analyses, at least forty subjects with recurrent polyps should be harvested. Under the assumptions above, 18.75% of the subjects will complete the second colonoscopy with recurrent polyps and thirty percent of the subjects with baseline biomarker analyses will not complete the trial. Three hundred subjects should therefore have tissue harvested for biomarker endpoints at baseline to ensure that.

out of those subjects, approximately forty will have recurrent polyps that can be harvested for analysis.

q. Data Analysis

1. Addressing Specific Aim #1: To determine whether RK42B reduces the recurrence of adenomatous colonic polyps in humans.

The primary endpoint will be the recurrence of polyps at the end of the study. The hypothesis of no treatment effect will be tested by the Mantel-Haenzel χ^2 test, with all dropouts from the trial assigned to be treatment failures.

In the secondary analysis, parametric (generalized linear) or nonparametric (generalized additive) models will be employed to determine the treatment effect on three-year polyp recurrence. after adjustment for demographic variables (e.g., age and sex), compliance (measured by plasma drug levels, self-report and/or electronic dose monitoring) and baseline biomarkers. If there is a statistically significant treatment effect, an analysis of the effect of individual dose levels will also be performed, with no adjustment for multiple comparisons. The incidence of polyp recurrence will also be estimated.

2. Approach to Specific Aim #3: To correlate RK42B plasma pharmacokinetics with Cox-2 inhibition in polyps, reduction in PGE_2 in polyps and in morphologically normal colonic mucosa.

Calculation of Pharmacokinetic Parameters

Pharmacokinetic parameters for RK42B' will be estimated using standard, biexponential non-compartmental pharmacokinetics (86). A limited sampling model will be developed as described in Preliminary Data)

E_{max} Analysis Method:

While we desire to work with linear or log linear functions, we recognize that most pharmacodynamic correlations to date have been nonlinear. Based upon preclinical data, we expect a nonlinear relationship between our study parameters and biological and biochemical endpoints. The pharmacodynamic model is based upon the sigmoid E_{max} model and fit to the function $E=E_{max} \cdot D^S/(D_{50}{}^S + D^S)$, where E_{max} is the maximum effect attributable to the drug, for example, a lectin, D is a dose of RK42B, D_{50} is the dose that produced 50% of the E_{max} and S is the parameter influencing the slope of the dose effect curve. We will use mixed effects generalized linear models to evaluate the relationships between the treatments and pharmacodynamic parameters.

3. Approach to Specific Aims #2 and #4: Determining effects of RK42B upon biomarkers.

(a) Biomarker variables

The distribution of biomarkers at baseline, relative to demographic and plasma micronutrient variables, will be determined and described by means of linear, and possibly nonlinear or additive regression models.

The change in distribution of biomarkers from baseline as a function of treatment, adjusted for demographics, baseline micronutrients and compliance will be modeled using parametric and/or nonparametric models. These changes will be related to the recurrence of polyps.

(b) Toxicity, Adherence and Dropouts

The adherence of subjects will be analyzed relative to toxicity. Subjects may drop out of the trial due to medication toxicity, an unwillingness to undergo subsequent colonoscopy, loss of interest. Nonadherence of subjects who remain in the trial may be due to medication toxicity. The analysis of adherence in terms of toxicity will include baseline nutritional (food frequency) and micronutrient variables. Subjects who complete the trial will be compared to subjects who drop out in terms of demographic variables as well as the toxicity experienced. The occurrence of adverse events and serious adverse events will be compared to treatment, demographic and baseline micronutrient variables.

IV. Methods of data flow and management, security and confidentiality considerations (Example of Web Based data management appended in Protocol, Appendix 1)

a. Master Data Base

All data will be organized into a relational data base in FoxPro. This data set resides on a Pentium Windows NT server. Standard software for data transfer (e.g., DBMS/Copy) will be used to ensure that all users will be able to obtain current versions of the database as required.

Some of the data sets will be collected at the subject level, some at the visit level and some at the specimen level, so the data manager will be responsible for verifying that appropriate index keys are used in each data set comprising the base to allow for efficient merging of the data. Data will be merged into the master data set as it is entered and cleaned. Treatment identification codes will not be merged into the master data base until the study is unblinded.

b. Derived Data Sets

For each analysis, a rectangular data set will be built by merging appropriate sets from the master base (more than one analysis will use a given set). This rectangular data set will have records at the appropriate level of observation (e.g., subject), and will contain all of the transformations of original variables as specified above and as may be required. Programs will be written to construct these data sets from the master base, so that, if data are added or changed in the master data base, the derived data sets can be reconstructed with minimal effort.

Provisions will be made to allow the production of derived data sets for graphics and reports that can be passed to a variety of programs on any of the major end-user platforms (DOS/Windows, Macintosh or UNIX).

c. Revisions and Corrections

All corrections to primary study documents will be initialed and dated. If computer-readable data is corrected by replacement of a data set, the replaced version of the data set will be retained in an archive. Corrections to electronic data will be stored in a tabular form; that is, for each data set within the master database, an auxiliary data set is retained that records each addition. deletion or correction to records in that data set. The collection of these auxiliary data sets represents an audit trail of corrections to the data base. A single program uses the audit records to update the data base as required.

d. Archives

The file system containing the data base will be backed up onto removable media nightly as part of a standard system backup. In addition, the data base itself will be archived weekly to media which will be kept off-site in a secure place. On-line backups of all data sets will be maintained. In addition, paper reports will be produced of all changes made to the data set. A paper listing of the data set will be produced periodically and kept off-site. Treatment identification codes will be kept on paper and on diskette in a secure place not accessible to blinded personnel (in addition to the sealed envelopes that will be accessible to the appropriate personnel if an individual subject must be unblinded).

e. Security

Subjects will be identified throughout the data base by their unique subject identification numbers (SID). Fields which could identify subjects, such as name, address, or social security number will either not be stored electronically or will be kept on diskettes or tape, but will not be kept on-line, and will be stored under lock and key at all times. All computer systems will be password-protected against intrusion; all network-based intersite communications of confidential information are encrypted. A minimum of paper containing hard-copy of confidential information will be generated.

Sufficient cross-checks will be built in so that a data-corruption or intentional data-alteration problem will be detectable and repairable with a minimum of effort. Computer-virus-protection program will be installed and updated on all study computers. The security of all computers .data files, executable software, diskettes, text macros, and downloads at all laboratory and clinical sites will be checked at the regular visits of the Clinical Research Associates.

Given the constraints required for security, the data will be entered as close in time and place to their generation as can be conveniently arranged, with real-time validation of ID numbers. range-checks and other cross-checks on field values to detect out of range and inconsistent data.

Data collected on remote computer systems will be bundled, encrypted, password-protected and transferred to the University of PUNKATA central site, where it will be securely unencrypted and loaded into the central data repository. Data collected via Netscape forms will be loaded into the central database subject to human review.

V. Experimental Clinical Methodology:

a. Methods to be used to assess and enhance adherence (87, 88)

1. A run-in period: Previous observations have confirmed the value of an initial placebo "run-in period" that precedes randomization. We will use a 1 month run-in. This period would be expected to identify approximately 40% of the subjects most likely not to comply with the intervention. Subjects are identified as nonadherent on the basis of any 1 of the following: (a) subject self-report of less than 80% of the prescribed dose (Initial Interval Questionnaire), (b) pill count based upon blister pack dates/times less than 80% of prescribed dose, and (c) electronic monitoring data from the blister pack (±6 hours of the agreed upon dosing time for each dose). Nonadherent subjects will be excluded from trial.

2. Contact with subjects: The run-in blister pack will be reviewed and electronically read within 1 week of the completion of the run-in period. Subjects will receive their coded, month 1 treatment blister packs for their randomized dose but, if found to be non-adherent electronically or by pill count, the month 1 treatment pack will be withdrawn and subjects removed from study. Coded used blister packs will be returned every 4 months from the subjects. With each group of four-monthly returned blister packs, subjects will return a questionnaire that reviews adherence, toxicity, change in habits, medication intake, and illness. Contact is maintained through telephone contacts on a regular basis and a quarterly newsletter that is sent to all subjects participating in University of PUNKATA chemoprevention projects (Appendix 4).

3. Blister Packs: Drugs are packaged in blister pack cards. Each card consists of 31 doses corresponding to the day of the month. Subjects are encouraged to take the dose on the appropriate day of the month. At adherence evaluations, subjects are requested to report days of drugs that have not been taken. The blister packs will be returned to the RK42B Trial Statistics and Data Management Office for review and comparison to dates drug taken whether tablets are left in the pack.

4. Electronic Monitors (Appendix 6): Electronically monitored blister packs that record the time, date, and dose location each time the blister membrane is broken will be used to monitor subject adherence. Use of the electronic monitor is disclosed to subjects. The monitoring packs will be used for the Run-in 31 day pack and then every 6 months during the 3 year treatment period. A total of 7 electronically monitored months for each subject will be obtained.

5. Confirmation via plasma RK42B concentrations: Once yearly, blood samples will be drawn and assayed for drug concentration. The laboratory and the investigators will be blinded to the results until the completion of the trial. Evidence of drug levels will be used to confirm other adherence detection methods.

Definition of Nonadherence: <80% adherence by any 1 method: self report, pill count, or electronic adherence. Randomized, non-adherence subjects (have successfully completed the run-in period) will be counseled, problems of dosing and toxicity discussed, and alternative drug administration strategies reviewed. A second episode of non-adherence will result in a repeat counseling session. A third episode of non-adherence will result in removal from the trial.

b. Dietary Monitoring

1. Rationale for dietary monitoring: Subjects who are entered on chemoprevention trials may alter their habits (74). Given current information and widespread publicity about fiber, calcium, and other potential chemopreventive micronutrients, it is crucial to monitor food intake and day to day routines of our subjects in order to determine whether they have changed dietary and whether these changes correspond to measured serum micronutrients (total cholesterol, alpha and gamma tocopherol, alpha and beta carotene, lutein, lycopene, betacryptoxanthine). For this purpose, a yearly food frequency evaluation for all subjects. For a subset of 96 subjects in the USA at 2 clinical centers and 96 subjects overseas (Italy and Israel) and micronutrient measurement is proposed. Plasma micronutrient data will be analyzed compared to food frequency data in this cohort.

2. Method of performing diet assessment: The semi-quantitative food frequency questionnaire developed by the Fred Hutchinson Cancer Center (89) is used. The validated instrument includes approximately 100 food items and designated portion sizes. The food frequency data is obtained on a scanning form. Diets are assessed at study entry and completion. Changes in subject oral intake and the micronutrient subset will be documented and analyzed with the primary endpoint of polyp reduction and the secondary endpoints of surrogate biomarker change.

c. Methods Used to Assure Quality Control of Data (See Protocol, Section 13, Appendix 1 for full Q/A-Q/C Methods)

1. Data Entry Quality Control: Data entry will have three types of quality control--*input assistance, global screening and repeated entry*. *Input assistance* will be carried about by ID validation, numerical value checks, cross field validation checks with early capture and instant flagging of deviations. *Global screens* will consist of routine scanning or "grooming" of the entire database with statistics of types of and sources of deviations. *Repeated entry* will be used by at audit visits and reentering the data at the World-Wide Web interface at the individual site.

Data generated from wet laboratories are subjected to the same quality control procedures as well as within laboratory quality control procedures described below (Section VI)

2. Protocol violation procedures: Protocol violations are detected and characterized as:

(a) First Order: Randomization prior to receipt of forms, randomization of an ineligible subject, subject receives wrong calendar packs, drug shipment to subject late, damaged, or lost, subject becomes aware of treatment status, subject misses Year 3 colonoscopy, subject misses 6 month flexible sigmoidoscopy, colonoscopy failure, missing pathology slides, study coordinator or co-investigator unblinded, three episodes of self reported, pill count or electronically monitored non-adherence.

(b) Second Order: Failure to complete Interval Questionnaire within 60 days of due date, baseline blood not drawn, final blood not drawn, missing or unreadable labels on blood tubes, missing clinical center pathology report, drug shipment to clinical center late, damaged or lost, unauthorized signature on a "Follow-up Colonoscopy" form, subject misses Year 3 colonoscopy.

These violations are compiled and reviewed monthly and contact is made with the clinical centers to deal with these problems.

3. Safety and Data Monitoring Committee: A Safety and Data monitoring Committee composed of 3 voting members from outside the study investigative group reviews unblinded results, validates the results and formulates an assessment for the PI and staff. The Committee will consist of the following individuals:

1. John Davis M.D., Towson, Jniversity
2. Scott Lippman M.D., Rochester, Cancer Center
3. Steven Spielberg Ph.D., Tatooine Jniversity

Stop criteria are described in the protocol document, Section 11.

4. Site Audits: On site data audits will be performed by personnel from the University of Chemoprevention Data Center every six months. Audit procedures are described in detail in the Protocol document (Appendix 1). Criteria for dropping a site include three first order or 9 second order violations.

5. Investigator Meetings: Yearly investigator meetings will be held in At this meeting, all investigators and CRAs will review questions and important study issues with the administrative and Statistical and Data Management Office staffs.

VI. Analytical Methodology

a. Proliferation Assays

a1. Amaranthin (Publication in Appendix 5) Sauri Laboratory, transferred from Muldoon, Lab)

Amaranthin immunohistochemistry will be performed as described in the manuscript appended in Appendix 5. Briefly, paraffin embedded tissue sections, 4 µm, are attached to glass slides, deparaffinized and biotinylated lectin is added at a concentration of 0.1 mol/l of amaranthin in a quantity sufficient to completely cover the hydrated tissue (100-200 µl). Amaranthin is rinsed off after a 30 min. incubation, covered with streptavidin-horseradish peroxidase (Zymed

Laboratories) at a concentration of 50 ng/ml for 30 min., rinsed, and the pigmented reaction product developed by the addition of a freshly prepared chromogen reagent solution. The chromogen consists of 3,3'-diaminobenzidine aliquoted at 5 mg/ml in 0.01 M Tris saline, pH 7.5 and frozen at -70°C until use. The aliquot is thawed, diluted 1:10 in 0.01 M Tris saline, pH 7.5 and 3.3 µl 30% peroxide is added to each 5 ml. This solution is allowed to stand for 10 min. and 100-200 µl is applied to the tissue sections that had been incubated earlier with biotinylated lectin and streptavidin-horseradish peroxidase. After 10 min., the slides are rinsed with albumin free PBS. The tissues are briefly counterstained for 50 sec with Gill's 3 hematoxylin, rinsed with water, serially dehydrated using graded alcohols and xylene and embedded in Permount.

3. Reading of slides: Prepared slides are examined under a light microscope (Carl Zeiss) and then imaged using a Roche Imaging Workstation. Each slide is identified with a code number to blind the examiner to the subject.

4. Tissue Scoring: *Quantitation under light microscope*: On each slide, 100-200 crypt cells are independently scored on a scale from 0 to 4 for intensity of color in each crypt and a weighted average determined for each slide as previously described (73). Each crypt is scored based upon the maximal labeling within any cell or cells in the gland, which includes an assessment of goblet, cytoplasmic, and secreted glycoconjugates. The weighted average is calculated by summing the individual scores of all the crypts examined and expressing the sum per 100 crypts, producing a score between zero (all 100 crypts negative) and 400 (all 100 crypts intensely stained).

Quantitation using image analysis and Euclidean Distance mapping algorithm: Images of the stained samples will be digitized on the Roche image analyzer and quantitative data obtained using the algorithm described in "Preliminary Data" and in Appendix 3.

5. Quality Control

By light microscopy using Weighted average scoring: All slides are scored twice on separate days by a single examiner to avoid interexaminer variation. With each group of slides, positive and negative controls are read and scores adjusted based upon the controls. The two scores are entered into the data base and variability of the scores is tracked by the study biostatistician.

By image analysis: The same sample will be quantified three times on three separate days, simple statistics describing variation, precision, kurtosis will be obtained for all samples.

a2. Ki67--Specimen fixation and staining (Swi Laboratory)

1. Specimen preparation: Specimens are fixed overnight in 10% buffered formalin and transferred to PBS between 18 and 24 hours of first biopsy.

2. Staining procedures: Ki67 staining will be performed as in a submitted manuscript (Appendix 5, Bijur et al). Briefly, 4-5 µm tissue sections are deparaffinized and antigen retrieval is obtained by microwaving the slides in 10 mM citrate buffer, pH 6 for 10 minutes. Endogenous peroxidase activity is then quenched with a 1:10 dilution of 30% hydrogen peroxide in PBS for 15 minutes. Nonspecific antibody binding is inhibited by use of 1% non-fat dry milk in PBS for 1 hour. The slides are then incubated with anti-Ki67 antibody (MM1 clone, Novocastra, Burlingame, CA) diluted 1:100 in PBS for one hour in a humidified chamber at room temperature. Secondary, biotinylated, horse anti-mouse antibody will be added at 1:100 dilution for 30 min. after which the sections are incubated with avidin-biotin-peroxidase complexes for 30 min. The sections are then washed, incubated with 3,3-diaminobenzidine for 10 min. and counterstained with Harris hematoxylin for 15 seconds. A negative control is prepared on each slide by omission of the primary antibody.

2. Tissue Scoring:

Quantitation using cell counting: On each slide, 200 crypt cells from at least 20 longitudinally cut crypts are independently scored on a scale from 0 to 4 for intensity of color and number of cells staining positive versus total number of cells in each of 3 crypt compartments (lower, middle, upper). A "positive" is considered a score of 2+ or higher. The index is calculated by dividing the number of cells stained by the total number cells stained for each of five crypt compartments with a sum of the two upper most fifths being identified as ϕ_h.

Quantitation using image analysis and Euclidean Distance mapping algorithm: Images of the stained samples will be digitized on the Roche image analyzer and quantitative data obtained using the algorithm described in "Preliminary Data" and in Appendix 3.

If the computed quantitation method is validated, then all data will be quantified solely using computed imaging quantitation.

5. Quality Control: A random sample of slides are scored twice on separate days by a single examiner to avoid interexaminer variation, however, repeat readings by two histochemical technicians show high intraobserver and intraobserver concurrence. With each group of slides, positive and negative controls are read and scores adjusted based upon the controls. The two scores are entered into the data base and variability of the scores is tracked by the study biostatistician.

b. Assessment of Apoptosis (Sarni Laboratory)

1. Assay Procedure: Colonic biopsies are fixed in formalin for 18-24 hours and oriented prior to being embedded in paraffin. At least five 5 micron sections are cut by the central laboratory from each biopsy to be used for the apoptosis assay. The sections are deparaffinized, the tissue sections are digested in 0.5% (gm/vol) pepsin (Sigma) in 0.1N HCl (30 min., RT), washed in water and quenched in 2% H_2O_2 in PBS (5 min., RT). After washing in PBS, the sections are overlaid with apotag equilibrium buffer and covered with a coverslip (30 Min., RT), then incubated with apotag reaction buffer as described by the manufacturer except that the TdT enzyme is diluted 1:3 in PBS prior to addition to the reaction buffer. After incubation (60 min. 37°C in humidity chamber), the reaction is stopped in apotag stop wash (30 min., 37°C) and the sections are washed in PBS (3x5 min.). The sections are overlaid with Anti-digoxigenin-peroxidase (30 min., RT, humidity chamber), washed, incubated in DAB-H_2O_2 (7 min., RT), and washed in running water. The sections are lightly counterstained with hematoxylin (3-5 sec.), washed in water, dehydrated in ethanol cleared in Hemo De and mounted in Permount. Positive control sections are incubated with 0.1 mg/ml DNAase in DNAase buffer (10 mM Tris-HCl, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 25 mM KCl) for 10 min. RT prior to incubation with the apotag reaction buffer. Negative control sections are incubated with apotag reaction buffer containing no TdT enzyme.

2. Quantitation, quality control of apoptosis assay

Quantitation using cell counting: On each slide, 200 crypt cells from at least 20 longitudinally cut crypts are independently scored on a scale from 0 to 4 for intensity of color and number of cells staining positive versus total number of cells in each of 3 crypt compartments (lower, middle, upper). A "positive" is considered a score of 2+ or higher. The index is calculated by dividing the number of cells stained by the total number cells stained for each of five crypt compartments with a sum of the two upper most fifths being identified as ϕ_h.

Quantitation using image analysis and Euclidean Distance mapping algorithm: Images of the stained samples will be digitized on the Roche image analyzer and quantitative data obtained using the algorithm described in "Preliminary Data" and in Appendix 3.

If the computed quantitation method is validated, then all data will be quantified solely using computed imaging quantitation.

c. Analytical Method for RK42B , Plasma Concentrations (Mega Pharm

This is a proprietary method used by M-P in analysis of Phase I and II samples. The method is a GLP, HPLC method provided by Megapharm (Letter of support from Mega Pharm

d. Prostaglandin E_2 (Muldoon Laboratory)

1. Assay Procedure: A frozen biopsy sample is homogenized in phosphate buffered saline (PBS) at room temperature for 30 sec using a microtube pestle, vortexed for exactly 2 min. and microcentrifuged at 12000 rpm for 30 sec. Only 2 samples at a time are processed to ensure equal thawing and reaction time. The supernatant is transferred to clean marked tubes. 50 µl of the supernatant is applied to the enzyme-linked immunosorbant assay plate in triplicate (Cayman Chemical), incubated 18 hours, color developed and absorbance read on an automated plate reader. Samples are diluted as necessary to fall on the standard curve. Protein content of homogenate is determined by Coomassie Protein Micro Assay (Pierce). The PG concentration is normalized per µg protein.

2. Quality control: Quality control using duplicate standard curves are attached. Correlation coefficient and variances of the standard curves used for assay of rectal epithelium are rejected if the correlation coefficient is <0.95 or the variance is >20%.

Individual subject tissue is assayed in triplicate on the sample plate with the standard curve and 3 sample knowns (in triplicate). The plate reader therefore reads the same color reaction from the standard curve, knowns, and the unknowns. Concentrations are calculated based upon the standard curve. Data obtained between indomethacin-PBS and PBS incubated samples will be compared for baseline and RK42B inhibited PGE_2. If there are large discrepancies, we will continue to use the indomethacin inhibited method.

e. Measurement of Cox-1 and Cox-2 protein (Muldoon Laboratory)

1. Assay procedure: A frozen biopsy sample is placed 0.250 ml of an antiprotease solution, homogenized with microtube pestle (at room temperature), vortexed, sonicated 45 sec and microcentrifuged at 12000 rpm for 30 sec. Total protein content of homogenate is determined by Coomassie Protein Microtiter plate (Pierce). Equal amounts of protein (15 µg) are separated by electrophoresis on 5% stacking and 10% separating SDS polyacrylamide gels. Human COX-1 and COX-2 standard (Cayman Chemical Co.), high molecular weight rainbow markers are also loaded on each gel. After overnight transfer to nitrocellulose membranes, blots are blocked by incubating for 1 hr with 5% non-fat dried milk in Tris buffer saline (TBS) with 0.1% Tween 20, washed 3 x 5 min. in TBS-0.1% Tween 20 then incubated at room temperature for 1 hr with a 1:1500 dilution of human Cox-1(DeWitt's) or Cox-2(DeWitt's) antisera. After washing, blots are incubated for 1 hr with horseradish peroxidase conjugated anti-rabbit IgG. Membranes are washed and developed using ECL chemiluminescent western blotting system (Amersham). The membrane is exposed to ECL-Hyperfilm (Amersham). The resulting bands are quantitated by video densitometry using software from Scion Corp. (Frederick, MD).

2. Quantitation, quality control and standardization of Western blots: A known quantity of a standard COX-1 and 2 and high molecular weight markers is run each time to ensure quality and reproducibility All samples from a subject are run on the same gel for reliable comparisons. Readings are obtained by densitometry as ratios of the sample readings to the subjects pre-treatment control. Blots are digitally imaged with an Javelin Camera, captured to a Macintosh computer, and quantified using NIH Image Freeware.

f. K-Ras and p53 immunohistochemistry in adenomatous polyps

1. Immunohistochemistry. Immunohistochemical analysis will be performed for detection of mutant p53 protein using the standard avidin-biotin peroxidase procedure described above for Ki67. D0-7 and CM-1 antibodies that detect wild type and mutant forms of human p53 protein (Novocastra, Burlingame, CA) will be used at a dilution of 1:100 and 1:1000, respectively after antigen retrieval. We have used this procedure to study oral squamous cell carcinoma and will use the sample procedure recently published (Appendix 5, Gopalakrishnan et al) (90).

f. Mutational analysis of kRas and p53 (Sato Laboratory)

Mutational analysis of K-ras and p53 genes will be conducted in baseline and in recurrent polyps to determine if mutation in these genes is associated with "resistance" to treatment with RK42B The protocol for detection of mutations in these genes in both frozen and paraffin-embedded samples is as follows:

1. Isolation of DNA from frozen samples: High molecular weight DNA will be isolated as previously described by us (Appendix 5, Wang et al)(91).

2. Isolation of DNA from paraffin-embedded samples: To gain access to microscopically abnormal areas in polyps, affected areas will be identified and microdissected from paraffin-embedded tissue sections (Appendix 5, Hongyo et al and Gopalakrishnan et al) (90, 92). Briefly, multiple 10 µm paraffin sections will be cut and applied individually to glass slides. Disposable microtome blades will be utilized and changed following the sectioning of each tissue block to protect against cross-contamination of DNA. Representative sections will be stained by hematoxylin and eosin (H&E). Selected areas of colorectal polyps will be carefully scraped from single unstained sections into a tube with a sterile disposable surgical blade, using the H&E stained section as a visual template. In some cases, immuno-stained (e.g. p53) sections from the same block will serve as visual templates.

3. Polymerase chain reaction (PCR). Partially and entirely intron-based PCR primers will be used to amplify individual exons from the *p53* (exons 5-8) and K *ras* (exons 1 and 2) genes.

Optimized PCR conditions for *p53* and K-*ras* have been previously published by us (Appendix 5, Hongyo et al and Gopalakrishnan et al) (90, 92).

4) "Cold" single-stranded conformation polymorphism (SSCP) analysis: "Cold" SSCP is a novel, rapid, and non-radioactive method which has been shown to be more sensitive in detecting nucleotide changes than traditional SSCP protocols (93). "Cold" SSCP is also more sensitive than direct sequencing in detecting mutations in mixed cell populations, being able to detect <6% mutant alleles compared to ~25% for direct sequencing (93). Another advantage of "Cold" SSCP is the rapid run times. PCR samples can be evaluated in only 2-4 hr. For these reasons, "Cold" SSCP will be used to screen samples for *p53* and K-*ras* mutations, followed by identification of the nucleotide changes by direct sequence analysis. Optimized "Cold" SSCP conditions for *p53* and K-*ras* have been previously published by us (Appendix 5, Hongyo et al and Gopalakrishnan et al) (90, 92).

e) Direct sequence analysis: Purified PCR product will be sequenced on an Applied Biosystem 377 automated DNA sequencer using ABI PRISM™ dye terminator cycle sequencing ready reaction kit. Electrophoresis will be performed at 3000 v for 3.5 hr. and DNA sequences analyzed using ABI sequencing analysis software. All mutations will be confirmed by sequencing both DNA strands. Where "Cold" SSCP analysis indicates the presence of a minor mutant band representing less than 50% of the allelic copies in the original PCR product (detection threshold for direct sequencing), the minor mutant bands will be cut from the SSCP gel, re-amplified by PCR (90, 92) and then sequenced.

VIII. Management of Large, Multisite Consortium

a. Experience in Management of Multisite Consortium Trials

The PI and staff at the University of PUNXATA Cancer Prevention Program are experienced in leading multicenter clinical trials. Dr. Muldoon is PI of a contract funded chemoprevention trial for bladder with fenretinide in the PUNXATA Oncology Group (S9460). The University of PUNXATA Prevention Program serves at the Statistical and Data Management Office for a multicenter chemoprevention trial of DFMO for the prevention of Barrett's esophagus. Dr. Muldoon serves as PI of the DFMO trial. A CCOP Research Base consisting of 5 affiliated midwest CCOPs in Akron and Rye will be funded in June, 1997. This research base is focused solely upon cancer prevention and control. Statistics and Data Management including all aspects of quality control are handled through the Prevention Program Statistics and Data Management Office.

b. Management of the Proposed RK42B Trial

The Prevention Program Statistical and Data Management Office has the initial infrastructure to design and begin management of this large consortium. Additional staff to manage the data and sample flow is requested and justified in the Budget Justification. The justification provides extensive detail regarding staffing requirements and procedures. Additional procedures are found in the Protocol document (Appendix 1, Sections 13, 14, and 15).

VII. Study time plan

Table 6: Subject Enrollment Time Table

	Year 1	Year 1.5	Year 2	Year 2.5	Year 3	Year 3.5	Year 4	Year 4.5	Year 5	Year 5.5
Study Month	0-6	6-12	12-18	18-24	24-30	30-36	36-42	42-48	48-52	52-60
Enrollment	245	245	245	255						
Flex Sigs	192	96								
Pharmacokinetics	48	48								
Complete Study							245	245	245	255

E. Human Subjects (Inclusion of Gender and Minorities In Clinical Trials)

1. Description of proposed involvement of human subjects

a. Characteristics of population: Human subjects who have had an adenomatous polyp resected.

b. Anticipated number: 990 subjects.

c. Age: ≥18 years.

d. Sex: Males and females will be studied. No exclusions on the basis of sex are intended or written in the protocol. We expect a slight predominance of males in this project because of a somewhat higher incidence of colorectal carcinoma in males and because VA Medical Centers will be used to solicit subjects. The sex of subjects entered on study will mirror the populations seen in the Institutional Sites.

e. Ethnic background: Individuals from all ethnic backgrounds will be solicited. No exclusions on the basis of ethnic background are written or intended. The study will not be controlled or stratified on the basis of ethnic background. Race and gender data for the National Polyp Prevention Consortium (another multicenter trial of polyp prevention) and the University of are:

	Male	Female	Caucasian	African American	Hispanic	Native Amer	Asia-Pac
Consortium	64%	36%	83%	6%	7%	1%	3%
Univ	53%	47%	77%	8%	2%	0%	13%

The Consortium has included the Mobile Hospital in Huron in order to enhance minority accrual to trials. Minority accrual is carefully monitored to ensure appropriate representation according to race and gender. Minority and economically disadvantaged populations are solicited for their participation through inner city hospitals (Pueblo and through VA Medical Centers.

f. Health status: Subjects will be healthy.

g. Special classes: No special classes of subjects are included in this project.

2. Sources of research material obtained from human subjects

a. Blood and urine specimens: To be used for drug assay, clinical laboratory tests.

b. Colonic biopsy specimens: To be used for biomarkers.

c. Questionnaires: To be used for demographics, toxicity and dietary monitoring.

3. Plans for recruitment and consent procedures

Subjects will be identified from subjects undergoing endoscopic procedures for adenomatous polyps and who are eligible for entry on to the RK42B Those who have the requisite risk factors or entry criteria (see Protocol or Section "D", Methods) will have a consent obtained. Subjects will not have serious, life threatening illnesses upon protocol entry.

The on site Research Coordinators will solicit subjects, explain the project and obtain informed consent. Complete disclosure of the study procedure, potential risks, potential benefits, and expectations will be provided to human subjects. Consent will be documented by signatures on an IRB reviewed and approved informed consent. The IRB has not waived or authorized any modifications of the consent or documentation of informed consent.

4. Potential Risks

Toxicity from RK42B reported to date include (in at least 5% of subjects treated): headache, diarrhea, rhinitis, nausea, sinusitis, rash, dyspepsia, pharyngitis, abdominal pain, coughing, pruritis, upper respiratory infection, back pain, nervousness, or pain. None of these toxicities reported differed significantly in incidence for subjects taking a placebo medication.

Toxicity from research procedures: Rectal biopsy and colonoscopy-rectal discomfort, bleeding, local infection at biopsy site, perforation, peritonitis. Venapuncture-discomfort at venapuncture site, bleeding, bruising.

5. Procedures for protecting against or minimizing risks

Risks of drug toxicity: Subjects are carefully monitored by the research staff. This includes contacts with research coordinators and the subjects physicians for the duration of study. For any problems that are perceived to represent unacceptable toxicity, immediate physician contact will be obtained and the study will be discontinued.

Risks from research procedures: Subjects will be observed for at least 10 minutes after biopsy procedures. Contact telephone numbers will be provided to ensure that medical staff may be easily reached. If excessive bleeding is evident after the procedure, the lesions will be cauterized. Surgical backup at all consortium sites is available at all times.

Monitoring toxicity: Toxicity assessments are obtained through Interval Questionnaires in interactions with local physicians while subjects are on study. The guidelines for toxicity and unacceptable toxicity are clearly defined and will be adhered to by the research staff. The quality control program ensures that the guidelines are adhered to.

6. Potential benefits versus the risks of study

Potential benefits are the reduction of risk for the development of colorectal cancer in individuals who have a high cancer risk. Such benefits outweigh the risks of taking a daily dose of drugs that are commonly used in the outpatient setting. Since the doses of the proposed drugs are lower than the recommended doses, the likelihood of toxicity is likely to be less than expected. Since subjects are healthy, payment will compensate for their time and discomfort required to undergo the excess procedures.

F. Vertebrate Animals

None

G. Consultants

Three consultants to serve on the Data Monitoring Committee are requested. They are:

S. Joplin M.D., Chemoprevention, Ragtime Cancer Center
J. Davis M.D., Epidemiologist, Towson University
S. Spielberg Ph.D., Biostatistician, Tatoonie University

Data + Safety Monitoring Plan:
The three consultants are to submit the DSMP if the study is funded.

H. Consortium/Contractual Arrangement

This proposal is a 13 Institution, multicenter consortium consisting of 13 clinical sites. 1 central laboratory site and a nutrition science laboratory. The Consortium Institutions at the time of grant submission (it is conceivable that some institutions may withdraw as administrative review is still underway at most consortium institutions) are:

Name of Institution	PI	Location	Function	Estimated Enroll/yr
			Data Ctr, Clin, Lab	48
			Central Lab	0
			Clinical Site	48
			Clinical Site	48
			Clinical Site	48
			Clinical Site	48
			Clinical Site	48
			Clinical Site	30
			Clinical Site	48
			Clinical Site	48
			Clinical Site	48
			Clinical Site	48
			Clinical Site	48
			Clinical Site	48
			Nutrition Sciences	0

The University of Cancer Prevention Program will serve as the research base for this project. The Statistical and Data Management Center, laboratory for cyclooxygenase assays, and a clinical site will be located at the University of

The University will serve as the Central Laboratory under the direction of Gary Ph.D. Dr. ; laboratory will handle all samples generated from this trial. All samples will be shipped to Dr. laboratory. The laboratory will track and distribute all samples for the proposed assays. Dr. laboratory will perform all immunohistochemical and molecular assays on the received tissues.

Clinical sites will recruit, enroll, treat, and follow-up experimental subjects. The subject enrollment expectations are noted above. Budgets requested by each center are determined by the enrollment rates. If a center does not meet its enrollment goals, funding will be reduced and shifted to centers that exceed enrollment goals. Enrollment will close after 990 subjects are randomized.

Four letters of Support follow (NOT Included) from DSMB Committee Members and the Company Supplying RK42B.