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Development of Chemopreventive Agents for Lung and Upper Aerodigestive Tract Cancers

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Abstract The lung and upper aerodigestive tract (oral cavity, larynx, pharynx, upper esophagus) will harbor the greatest proportion ($\approx$20%) of estimated new cancer cases in 1992. The estimated mortality rate is even higher (32%), which is reflected in a 5-year survival rate of only 7% and 12% for esophageal and lung cancer, respectively. Tobacco use appears to remain the major cause of aerodigestive cancers despite efforts at primary prevention—cessation of exposure. Another strategy to decrease this public health problem is secondary prevention or chemoprevention. *Cancer chemoprevention* is defined as intervention with chemical agents before invasion to halt or slow the carcinogenic process; potential agents may include minor dietary constituents and pharmaceuticals.

The main objective of the Division of Cancer Prevention and Control (DCPC), National Cancer Institute, is to develop promising chemopreventive drugs for use in humans. The testing of cancer chemopreventives for efficacy in the clinic differs from that of cancer treatment drugs. Chemopreventive drug trials involve healthy target populations, and the endpoints are reduced cancer incidence or mortality, or increased latency, with no to minimal toxicity. The lung and upper aerodigestive tract represent a unique opportunity for intervention in this setting. Even with cessation of tobacco exposure, the risk of cancer in the entire epithelium remains high for years due to the "field cancerization" effect. Some of the first chemopreventive trials made use of this system due to the availability of a study population with a tissue at demonstrably high risk for malignant progression. Much of the evidence for chemopreventive efficacy is in the oral cavity because of the well-defined epithelial neoplastic progression, the existence of well-established preclinical models, and relative ease of tissue monitoring and sampling. In one of the first randomized trials, Hong and co-workers demonstrated that 13-*cis*-retinoic acid prevents the appearance of second primary tumors in patients previously treated for squamous cell carcinomas of the oral cavity and upper respiratory tract.

Even using a high risk population, chemoprevention trials involve large sample sizes, lengthy duration and follow-up, and high cost. To circumvent these problems, the use of *intermediate biomarkers* as surrogate endpoints is being explored. Intermediate biomarkers are defined as biological alterations in tissue (histological, genetic, biochemical, proliferative, differentiation-related) occurring prior to cancer development. In the oral cavity, studies using modulation of a histological intermediate biomarker, dysplastic leukoplakia, as the endpoint have demonstrated response to a retinoid.

Several problems still need to be addressed in the chemoprevention of aerodigestive cancer, such as the toxicity of some of the agents which have been used (retinoids) and maintenance

of remission after cessation of treatment. In addition, clinical chemoprevention trials in the other sites of the aerodigestive system, such as lung and upper esophagus, are not as well advanced. This is due to sampling and monitoring problems and less well-defined premalignant lesions. The DCPC is developing new strategies for chemopreventive trials in the aerodigestive tract.

Key Words: Chemoprevention, neoplasms, head and neck, lung, esophagus, oral cavity, trachea, intermediate biomarkers, surrogate endpoints, clinical trials, hamster, strain A mice, rat, pharynx, larynx, aerodigestive tract, retinoids

The most common sites of cancer in the total U.S. population are the lung and upper aerodigestive tract (oral cavity, larynx, pharynx, upper esophagus) [1]. As shown in Table I, these tissues will harbor the greatest proportion of new cancer cases (19.6%) estimated for 1992. Mortality from cancers of the lung and upper aerodigestive tract is even higher in all segments of the population, *e.g.*, 39.4% of all cancer deaths in males. This is reflected in the low 5-year survival rates for esophageal (7%) and lung (12%) cancer.

The problems of high incidence and mortality from aerodigestive cancers are compounded by the high rate of local recurrence (30–50%) or second primary tumor (10–40%) following successful treatment of an initial tumor [2]. This phenomenon has been explained by the concept of "field cancerization," which suggests that certain risk factors (*e.g.*, smoking) result in diffuse histological, molecular and biochemical changes in the entire lining of the aerodigestive tract [3]. Thus, the whole tissue is considered to be at high risk for subsequent neoplastic progression.

The factors which confer increased risk can be categorized as lifestyle factors, occupational exposures, disease states, and genetic susceptibility. The major cause of lung cancer in both sexes remains a lifestyle factor—tobacco use. Smoking has been established as a major contributor in more than 100 case-control studies of lung cancer [4]. In men, the relative risk of lung cancer in smokers has been reported to be 12- to 17.4-fold higher as compared with nonsmokers [5,6]. Cancers of the oral cavity have been related to both pipe and cigarette smoking and tobacco chewing, and there is a synergistic effect with alcohol consumption [7].

An additional lifestyle factor which impacts the development of lung cancer is diet. Low dietary or serum vitamin A and/or β-carotene increases the relative risk 2- to 7-fold [8–14]. This factor may also play a role in the etiology of cancers of the oral cavity, larynx and pharynx [15]. Retinoids appear to be necessary for normal growth and differentiation of epithelial cells in the respiratory tract of experimental animals; low dietary vitamin A results in histological changes similar to squamous metaplasia, a putative premalignant lesion [16–18].

Carcinogen exposures related to the workplace are also a major etiological factor in lung and upper aerodigestive cancers. A higher risk of laryngeal cancer has been demonstrated in asbestos-exposed subjects, although this effect may be confined to smokers [20]. It has been suggested that asbestos increases the cellular uptake of carcinogens present in tobacco smoke.

Significant risk for aerodigestive cancers has been related to mining, refining and smelting. Employment in these industries may involve higher exposure to initiating or promoting substances, such as metals, radon, polycyclic aromatic hydrocarbons, coal dust, and irritating gases [19–21]. The interaction between smoking and occupational exposures can further increase cancer risk.

Finally, disease states have been implicated in lung and aerodigestive cancer risk. Viruses such as human papilloma virus (HPV) and Epstein-Barr have been associated primarily with head and neck cancers. Also, inherited predisposition to disease appears to be related to lung cancer

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Table I. Estimated Incidence and Mortality Data for Lung and Aerodigestive Tract Cancers, 1992

	Incidence of New Cancer Cases		
	Total	Male	Female
Lung	14.9%	18.0%	11.7%
Oropharynx	2.7%	3.6%	1.7%
Larynx	1.1%	1.8%	0.4%
Esophagus	1.0%	1.4%	0.6%
Aerodigestive, Total	19.6%	24.9%	14.4%
Prostate	NA	23.4%	NA
Breast	NA	NA	31.9%
	Cancer Mortality Rate		
	Total	Male	Female
Lung	28.1%	33.8%	19.3%
Oropharynx	1.5%	1.8%	1.0%
Larynx	0.7%	1.1%	0.3%
Esophagus	1.9%	2.7%	0.9%
Aerodigestive, Total	32.2%	39.4%	21.5%
Prostate	NA	12.4%	NA
Breast	NA	NA	16.7%

Compiled from [1]

[19]. Genetic susceptibility may be conferred by inherited inactive retinoblastoma gene [22], cytochrome P-450 polymorphisms [23,24] and rare Ha-*ras* alleles [25]. Based on the multistep carcinogenesis model, multiple genetic events are necessary for transformation [26]. Both latent virus infection and inherited genetic predisposition may represent single hits which remain undetected until unmasked by exposure to carcinogens, such as cigarette smoke [27].

From the above discussion, the primary strategy for prevention of aerodigestive cancers would appear to be avoidance of major sources of carcinogen exposure. Public health efforts have decreased smoking prevalence to approximately 30%, based on 1987 statistics [5]. Although mortality due to lung cancer has concomitantly decreased in males below age 45, it is still rising in women and older men in the U.S. [28,29]. Even with cessation of tobacco exposure, however, the risk of cancer in the entire epithelium remains high for years due to the "field cancerization" effect. After 10 years, former smokers still have a risk of death from lung cancer 4.7-fold higher than nonsmokers [30]. A secondary prevention strategy—chemoprevention—is designed to interfere with the biological response to carcinogen exposure in populations at high risk for neoplastic progression. *Cancer chemoprevention* is defined as in-

tervention with chemical agents before invasion to halt or slow the carcinogenic process.

CHEMOPREVENTIVE DRUG DEVELOPMENT AT THE NCI

The main objective of the Division of Cancer Prevention and Control (DCPC), National Cancer Institute (NCI), is to develop promising chemopreventive chemicals as drugs for human use. The strategy for this effort has been described in detail previously [31]. Briefly, the process begins with the identification of potential chemopreventive drugs (pharmaceuticals, natural products or minor dietary constituents) from surveillance and analysis of the literature [32] and from the DCPC Testing Program. Data on both efficacy (*i.e.*, biological activities that either directly or indirectly indicate inhibition of carcinogenesis) and toxicity are gathered from both sources.

In the Preclinical Testing Program, a battery of four *in vitro* efficacy assays using human and animal cells is used to select promising agents for further testing. Two of the *in vitro* assays which will be discussed below are relevant for testing agents for efficacy against aerodigestive tract cancers: the rat tracheal epithelial cell (RTE) assay and the human lung tumor A427 cell line. A panel of animal screening assays which are target organ-specific are then used to assess efficacy *in vivo*. Relevant assays for this discussion include inhibition of *N*-methyl-*N*-nitrosourea (MNU)-induced tracheal squamous cell carcinomas and *N,N*-diethylnitrosamine (DEN)-induced lung adenocarcinomas in the hamster [33]. Traditional preclinical toxicity tests are also performed in two species, especially if the agent is not a pharmaceutical. The scientific rationale for all of the systems used in the DCPC have been described previously [34, 35].

The most promising and least toxic potential drugs enter the clinical phase of testing. Phase I clinical trials are designed to investigate human dose-related safety, pharmacokinetics, and metabolism of the drug. Both Phase II and III clinical trials are designed for the determination of cancer chemopreventive efficacy. Phase II trials are small scale, placebo-controlled studies which may include modulation of intermediate biomarkers as study endpoints, as discussed in the section, **Importance of Intermediate Biomarkers in Chemopreventive Drug Development**. Phase III trials involve a large target population, with cancer incidence reduction as the endpoint.

It should be emphasized that conceptual differences exist between clinical testing of cancer chemopreventive drugs and cancer chemotherapeutic drugs. Chemopreventive drug trials involve healthy target populations, although these may be populations at increased risk, and the endpoints are reduced cancer incidence or mortality, or increased latency [36]. In contrast, cancer chemotherapeutics are tested in cancer patients, with decreased tumor size and increased disease-free survival as the endpoints. However, the real difference is in the level of toxicity which is acceptable. In the treatment of advanced cancer, severe toxicity is permissible; in chemoprevention, only no to minimal acute and chronic toxicity are admissible.

The drug development effort at the DCPC has been in progress for about 6 years. Approximately 200 agents are on test in *in vitro* screens; more than 100 agents are on test in animal efficacy screens. There are approximately 20 agents for which reasonable toxicity data are already available or for which DCPC is evaluating toxicity. The best of these agents are coming into Phase I and Phase II clinical trials [37].

PRECLINICAL TESTING BY DCPC

As described previously, the Preclinical Testing Program at DCPC includes both *in vitro* and *in vivo* assays for chemopreventive efficacy. *In vitro* efficacy assays are based on inhibition of morphological transformation or anchorage-independent growth, and are short-term and relatively inexpensive. The RTE assay measures potential chemopreventive efficacy as inhibition of benzo(*a*)pyrene [B(*a*)P]-induced formation of colonies of morphologically altered cells in primary cultures of isolated rat tracheal epithelial cells [38,39]. Effective agent activities are those that increase glutathione levels or enhance conjugation (bismuthiol I, *N*-acetyl-*l*-cysteine, oltipraz), alter cytochrome P-450 activity, display nucleophilic activity, or inhibit oxidation [all-*trans*-retinoic acid, *N*-(4-hydroxyphenyl)-retinamide (4-HPR)] [39,40].

Table II. Rat Tracheal Epithelial (RTE) Cell Assay Results versus Hamster Lung and Trachea Assay Results

RTE ASSAY RESULT	HAMSTER LUNG/TRACHEA ASSAY RESULT +	HAMSTER LUNG/TRACHEA ASSAY RESULT −
+	*N*-Acetyl-*l*-cysteine Bismuthiol I 4-HPR Oltipraz 13-*cis*-Retinoic Acid Selenite, Sodium	Anethole Trithione Ascorbyl Palmitate Vitamin E Succinate PEG
−	Diallyl Disulfide	DFMO Ellagic Acid Ibuprofen Molybdate, Na 2-Oxothiazolidine 4-Carboxylate Vitamin E Acetate

Positive Predictive Value of RTE: 6/9 = 67%
Negative Predictive Value of RTE: 6/7 = 86%
Accuracy of RTE Result: 12/16 = 75%

The human lung tumor A427 cell line assay evaluates chemopreventive activity as inhibition of anchorage-independent growth (cancer cell phenotype) by an agent added at the time cells are seeded in a semisolid agarose medium. A partial list of agents effective in this assay includes retinoids, β-carotene, diallyl disulfide, phenethyl isothiocyanate and some of the nonsteroidal antiinflammatory drugs [41].

The short-term *in vitro* assays are used to select and prioritize agents for *in vivo* testing. The value of the RTE assay in predicting inhibition of both MNU-induced tracheal and DEN-induced lung carcinogenesis in the hamster appears to be high. As shown in Table II, efficacy both *in vitro* and *in vivo* was obtained with 6/9 agents tested—a positive predictive value of 67%. The RTE assay also predicted the *in vivo* results for 6/7 agents (predictive value, 86%) shown to be negative in the animal screens (data not shown). The non-concordant results appear to result from differences in metabolism or bioavailability of the agents *in vivo*. The high predictability of the RTE assay facilitates selection of agents for animal efficacy testing. The latter is more costly, but establishes efficacy of an agent in preventing experimental cancer, provides information on target organ specificity and toxicity, and satisfies the FDA requirement for agents to progress to clinical trials. The results from the Preclinical Testing Program at DCPC for the most promising agents for chemoprevention of lung and upper aerodigestive tract carcinogenesis are outlined in Tables III and IV.

Combinations of potential chemopreventive drugs are also being assessed in preclinical test-

Table III. Promising Agents for Inhibition of Lung and Upper Aerodigestive Tract Carcinogenesis in the Preclinical Testing Program at DCPC: *In Vitro* Assays

Agents	A427	JB6	RTE	MMOC	HFE	HFF	Gap
N-Acetyl-*l*-cysteine	NE	NE	+	+P	+		OT
Bismuthiol I	NE	NE	+	NE	+		(NE)
4-HPR	+	(NE)	+	+	+	+	OT
Oltipraz	+	NE	+	+	+	NE	(NE)
13-*cis*-Retinoic Acid	+		+				
Selenite, Sodium	NE		+	+	+	+	(NE)

Assay Class: A427, Human lung tumor A427 cells; **JB6**, Mouse JB6 epidermal cells; **RTE**, Rat tracheal epithelial cell assay; **MMOC**, Mouse mammary gland organ culture assay; **HFE**, Human foreskin epithelial cell assay; **HFF**, Human foreskin fibroblast assay; **Gap**, Gap junction assay.
Test Results: +, Chemopreventive activity observed; **+P**, Chemopreventive activity observed during promotion phase; **NE**, No chemopreventive effect observed; **(NE)**, No chemopreventive effect observed, preliminary result; **OT**, Agent is on test or is scheduled for testing.

ing. Since little or no toxicity is acceptable in clinical chemoprevention trials, one approach for decreasing the toxicity from an individual agent is the administration of combinations of agents. Treatment with two or more efficacious agents would require lower doses of each, and thus decrease the potential for toxicity. Also, efficacy may be enhanced by combining agents which are active by different mechanisms or at different stages of carcinogenesis. In some cases the combined effects are synergistic or additive, producing an even greater reduction in cancer incidence or increase in latency. Combinations which have synergistically inhibited carcinogenesis in either hamster model include oltipraz plus β-carotene and/or 4-HPR; β-carotene plus vitamin A; and 4-HPR plus difluoromethylornithine (DFMO). The β-carotene and vitamin A combination is now in clinical trials.

IMPORTANCE OF INTERMEDIATE BIOMARKERS IN CHEMOPREVENTIVE DRUG DEVELOPMENT

For chemopreventive drug development, one of the most difficult aspects is the long period required for many cancers to develop, and, consequently, the apparent requirement for long clinical trials to test the efficacy of chemopreventives. One approach to this problem is the identification of intermediate biomarkers for evaluating clinical efficacy. *Intermediate biomarkers* are biological alterations in tissue between initiation and tumor invasion. It is hypothesized that modulation of one or more intermediate biomarkers by a chemopreventive agent(s) would interrupt carcinogenesis. Validation of a biomarker as a surrogate endpoint for clinical trials would be obtained when the final endpoint, cancer incidence, is also decreased as a result of this modulation.

Evaluation of intermediate biomarkers as trial endpoints instead of cancer incidence allows chemoprevention trials to be of shorter duration, use fewer subjects, and be lower in cost. They may also allow use of serum or a small tissue sample to monitor response. In addition, they allow determination of effective doses for Phase II trials and rationale for Phase III trials, and may provide basic scientific contributions to understanding the mechanisms of carcinogenesis. Clearly, much work remains to be done in identifying and validating appropriate intermediate biomarkers. One of the

Table IV. Promising Agents for Inhibition of Lung and Upper Aerodigestive Tract Carcinogenesis in the Preclinical Testing Program at DCPC: *In Vivo* Assays

Agents	Lung			Colon			Mammary			Skin		Blad	Pros	Panc	Lymph	Esoph
	DEN	MNU	NNK	Crypts	Mouse	Rat	DMBA	MNU	Trans	DMBA	B(*a*)P					
N-Acetyl-*l*-cysteine		+	OT	(NE)		+		+			OT	+				
Bismuthiol I	+			(NE)				NE				+				
4-HPR	+	NE	OT	OT	NE		+	+	OT	+		NE	OT	OT	OT	NE
Oltipraz	+	+		(+)	+	+	+	+	OT	+		+	OT	OT		
13-*cis*-Retinoic Acid		+ B(*a*)P*		OT												
Selenite, Sodium	+	NE		(+)	NE		+			NE		NE				

Assay Class: Lung/DEN, Hamster lung (DEN-induced); **Lung/MNU**, Hamster lung/trachea (MNU); **Lung/NNK**, Strain A/J mouse (NNK); **Colon/Crypts**, Foci of aberrant crypts in rat colon; **Colon/Mouse**, Mouse colon (MAM); **Colon/Rat**, Rat colon (AOM); **Mammary/DMBA**, Rat mammary glands (DMBA); **Mammary/MNU**, Rat mammary glands (MNU); **Mammary/Trans**, Transgenic mouse (c-*neu*/c-*myc*) ; **Blad**, Mouse bladder (OH-BBN); **Pros**, Rat prostate (MNU/hormone); **Panc**, Hamster pancreas (BOP); **Skin**, Mouse skin (DMBA); **Lymph**, TG.EB Oncomouse (IgH enhancer/c-*myc*); **Esoph**, Rat esophagus (NMBA).
ChemicalAgents:AOM, azoxymethane; B(*a*)P, benzo(*a*)pyrene; BOP, *N*-nitrosobis(2-oxopropyl)amine; DEN, diethylnitrosamine; DMBA, 7,12-dimethylbenz(*a*)anthracene; MAM, methylazoxymethanol; MNU, *N*-methyl-*N*-nitrosourea; NMBA, nitrosomethylbenzylamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; OH-BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine.
Test Results: +, Chemopreventive activity observed; **(+)**, Chemopreventive activity observed, preliminary result; **NE**, No chemopreventive effect observed; **(NE)**, No chemopreventive effect observed, preliminary result; **OT**, Agent is on test or is scheduled for testing.
* Induced by B(*a*)P/ferric oxide particles [64].

main thrusts of the DCPC drug development activity is to review the current status of early markers and to develop research strategies for identifying and validating intermediate biomarkers for lung and upper aerodigestive tract carcinogenesis.

To model the role of intermediate biomarkers in cancer, it is useful to classify them into the following groups: premalignant lesions/histologic changes, proliferation-related, differentiation-related, genetic, and biochemical. This classification scheme has been applied to biomarkers in various tissues such as colon [31], prostate [42] and bladder [43]. Table V is a representative listing of potential intermediate biomarkers in the lung and aerodigestive tract classified in this manner. However, it should be noted that the terms "biomarker" and "marker" in the cancer literature can refer to several concepts which should be distinguished from intermediate biomarkers. This issue has been discussed previously [31].

In chemopreventive drug development strategy, histological premalignant lesions are an important starting point. As described recently [44], they may provide a measurable endpoint for clinical trials, as well as define a high risk tissue in which other intermediate biomarkers can be developed and validated. In this volume, Drs. Koss and Kaugars discuss the significance of dysplasia to the malignant potential of the clinical oral cavity lesion, leukoplakia. Similar lesions are obtained in the hamster cheek pouch model described by Dr. Gimenez-Conti. Finally, Dr. Crissman recommends use of the term squamous intraepithelial neoplasia (SIN), grade III, as a replacement for both severe dysplasia and carcinoma *in situ* (CIS) in the upper aerodigestive tract.

Loss of control of cellular proliferation is a basic component of carcinogenesis. In most experimental models of carcinogenesis, decreasing the proliferation rate results in decreased cancer incidence or multiplicity, or lengthened latency period. For example, proliferation-related markers appear to be very important in the colon [45]. Epidermal growth factor receptor (EGF-R) levels and ornithine decarboxylase (ODC) activity have been investigated in various stages in the histological development of hamster buccal pouch carcinoma (Gimenez-Conti, this volume). Elevated TGF-α and EGF-R expression appear to occur in both squamous cell carcinoma of the head and neck and in normal-appearing adjacent mucosa, as outlined by Dr. Grandis in these proceedings.

As cells differentiate, a specific pattern of expression of cellular components such as proteins and carbohydrates occurs. Since cancer cells undergo aberrant patterns of differentiation, it is likely that cellular components characteristic of differentiation will be modified in premalignant states. For example, during abnormal development of colonic epithelial cells, the expression of certain cell surface or secreted carbohydrate conjugates may be altered [46,47]. In the aerodigestive tract, altered expression of integrins and blood group antigens on cell surfaces has been reported by Dr. Carey (this volume).

The accumulation of genetic changes within a single cell has been proposed to be responsible, at least in part, for the development of cancer [26]. The importance of genetic instability is illustrated by the induction of mutations and chromosomal aberrations by most carcinogens [48], the detection of karyotypic variation in many solid tumors [49], and the higher incidence of cancer in individuals with compromised DNA repair [50]. Gross genetic changes which may be useful intermediate biomarkers include alterations in cellular DNA content (aneuploidy, DNA index), nuclear aberrations, and altered patterns of gene expression. In these proceedings, Dr. Hittelman describes chromosomal alterations detected by premature chromosome condensation, and Drs. Garewal and Benner analyze the utility of micronucleus measurements as biomarkers. Other changes, such as mutations, may take place at the gene level. Dr. Sidransky discusses the possible appearance of p53 mutations and/or overexpression in premalignant lesions of the esophagus and lung. Finally, detection of DNA adducts is described in three articles by Drs. Stern, Turteltaub, and Perera, included later in this volume.

Biochemical markers such as increased levels of enzymes and other proteins have also been associated with early stages of carcinogenesis. An obvious example is the increase in serum levels of prostate specific antigen (PSA) in the presence of prostatic intraepithelial neoplasia [51]. Dr. Schantz has investigated alterations in the emission spectra of intrinsic tissue fluores-

Table V. Examples of Intermediate Biomarkers in the Lung and Upper Aerodigestive Tract by Class

Histological and Premalignant Lesions
Dysplasia in Oral Leukoplakia/Erythroplakia
Bronchial Atypical Metaplasia/Dysplasia
Esophageal Dysplasia, Carcinoma *in situ* (CIS)
Esophageal Squamous Papilloma
Squamous Intraepithelial Neoplasia (SIN)
Genetic
Micronuclei: DNA Content
Activated Oncogenes (*e.g.*, H-*ras*)
Inactivated Tumor Suppressors (*e.g.*, p53)
Macromolecular DNA Adduct Formation
Nuclear Alterations (*e.g.*, Hyperchromasia)
Proliferation-Related
Growth Factor Overexpression (*e.g.*, TGFα, EGF-R)
Ornithine Decarboxylase Activity Enhancement
Ki67 Nuclear Antigen
PCNA
Differentiation-Related
Altered Keratin Expression (*e.g.*, Involucrin)
Transglutaminase I
Altered Cell Surface Protein Expression (*e.g.*, Integrins)
Altered Blood Group Antigens
Biochemical
Intrinsic Tissue Fluorescence
Peptidyl Glycine α-Amidating Monoxygenase Activity

cence as a method to identify biochemical changes in the nitrosomethylbenzylamine (NMBA)-exposed rat esophagus. Also in this volume, Dr. Mulshine describes a prospective lung cancer trial using peptidyl glycine α-amidating monooxygenase activity as a marker of the capacity to produce growth factors.

Once potential intermediate biomarkers are identified, it is important to establish criteria for selecting those to be used in clinical trials. Some of the major considerations are [31]: Is the marker differentially expressed in normal and high-risk tissue? Can the marker be modulated by chemopreventive agents? At what stage of carcinogenesis does it appear? Does the assay for the marker provide acceptable sensitivity, specificity, and accuracy? How easily can the marker be measured? Can it be obtained by non-invasive techniques? Is it technically difficult to measure? For most organs, it is hard to

find many markers that fill some or all of these criteria. This lack of validated markers obviously means that more development is needed. It also suggests that batteries of markers will probably be used until more are validated. Ideally, modulatable biomarkers for chemoprevention should occur as early in carcinogenesis as possible. Paradoxically, the earlier in carcinogenesis that the marker is measured, the less predictive value the marker is likely to have. This suggests that histologic lesions must serve, at least initially, as the gold standard for validation of other markers.

EVALUATION OF INTERMEDIATE BIOMARKERS AS SURROGATE ENDPOINTS IN THE PRECLINICAL TESTING PROGRAM

To further develop intermediate biomarkers, the DCPC is evaluating some as potential surrogate endpoints in both preclinical models and Phase II trials, and anticipating many more such trials in the next few years. Three preclinical models of aerodigestive carcinogenesis are in use this year which allow the investigation of intermediate biomarkers. First, the 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced hamster buccal pouch assay is a model of human oral carcinogenesis. Multiple topical applications of the carcinogen produce squamous cell carcinomas preceded by hyperkeratotic and dysplastic lesions similar to oral leukoplakia. The lesions develop in a specific sequence over a short time period (12 weeks) and can serve as histological biomarkers. In addition, the appearance of γ-glutamyltranspeptidase (GGT)-positive foci has been investigated as an intermediate biomarker; these foci are detected as early as three days after the first DMBA dose [52]. In the Preclinical Testing Program, the incidence and grade of dysplasia and the incidence and size of GGT-positive foci will be evaluated as potential surrogate endpoints in this model using the agents, aspirin, carbenoxolone, β-carotene, DFMO, 4-HPR, piroxicam and 13-*cis*-retinoic acid.

Two lung models will also be initiated this year as part of the intermediate biomarker effort: the hamster carcinogen pellet implant model and the strain A/J mouse model. The hamster lung implant model involving sustained release of B(*a*)P (10% w/w) from *in situ* silicone pellets is a model of chronic human exposure [53; Hammond and Benfield, this volume]. At 150 days following implantation of the pellet in the right bronchus, >90% incidence of epidermoid carcinomas similar to human non-small cell lung cancer is obtained at the implant site. Histological progression is also observed at the site: hyperplasia, squamous metaplasia, squamous metaplasia with increasing atypia, carcinoma *in situ*, microscopic carcinoma (primarily squamous cell), and palpable carcinoma. Histopathological analysis will provide morphological markers to be compared with final cancer incidence and multiplicity. The DCPC will be testing modulation of these lesions by 4-HPR and oltipraz.

The A/J mouse strain is extremely sensitive to both spontaneous and carcinogen-induced formation of lung tumors [Stoner, this volume]. A single dose of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), produces a progression characterized by alveolar hyperplasia, solid and papillary adenomas, and carcinomas. Maximal adenoma yield occurs at only 16 weeks. Activated K-*ras* has been demonstrated in hyperplastic lesions and adenomas [54]. This year, the DCPC will be starting preliminary studies to determine the presence of potential intermediate biomarkers (p53, *myc*, proliferating cell nuclear antigen, retinoblastoma gene expression, and K-*ras* mutations) in normal-appearing tissue as well as premalignant lesions (adenomas) in exposed mice. Following these studies, modulation of relevant biomarkers will be investigated using the agent phenethyl isothiocyanate.

In all of these studies, parallel agent treatment groups will be included to analyze the effect on cancer multiplicity and incidence. It is hoped that modulation of intermediate biomarkers will correlate with decreased cancer incidence and, thus, validate their use as surrogate endpoints. The goal is to utilize these biomarkers as surrogate endpoints in future clinical chemoprevention trials.

CLINICAL TESTING BY THE DCPC

The carcinogen-exposed epithelium of the lung and upper aerodigestive tract is at demonstrably high risk for neoplastic progression due to the "field cancerization" effect, and this risk

Table VI. DCPC-Sponsored Phase II and Phase III Clinical Chemoprevention Trials

Site	Chemopreventive Agent	Population
Lung	Vitamin A β-Carotene	CARET Study Heavy Smokers (2) Cigarette Smokers Men with Asbestosis Men exposed to Asbestos
	13-*cis*-Retinoic Acid β-Carotene	Chronic Smokers Women Smokers
Oral/Head & Neck	Vitamin A	Oral Precancer
	13-*cis*-Retinoic Acid	Oral Leukoplakia Previous Head & Neck Cancer
	β-Carotene	Oral Precancer Oral Leukoplakia
All Sites	β-Carotene	U.S. Physicians

remains high for years even without continued exposure. Thus, three types of study populations are available: (1) individuals at high risk for aerodigestive cancer due to exposure to carcinogens such as tobacco smoke or asbestos; (2) patients with a successfully treated primary cancer of the lung or upper aerodigestive tract; and (3) patients with premalignant lesions. As shown in Table VI, the DCPC is utilizing all of these population categories in ongoing Phase II and Phase III clinical trials.

In order to avoid delaying the clinical portion of the Testing Program until preclinical results were available, the first generation of agents was selected on the basis of published data from epidemiology studies and early human trials [cited in 55], as well as animal efficacy experiments. As shown in Table VI, the agents presently on test in the clinic are retinoids or precursors: vitamin A (retinol), 13-*cis*-retinoic acid, and β-carotene. As mentioned previously, retinoids are required for normal differentiation and growth of the aerodigestive tract epithelium, and epidemiological data shows an inverse relationship between retinoids and aerodigestive cancer risk. In addition, Hong *et al.* [56], in one of the first randomized chemoprevention trials, demonstrated that 13-*cis*-retinoic acid prevents the appearance of second primary tumors in patients previously treated for squamous cell carcinomas of the head and neck.

Much of the human evidence for chemopreventive efficacy is in the oral cavity and pharynx because of the existence of well-established preclinical models (*e.g.*, DMBA-induced hamster buccal pouch carcinogenesis), the obvious ease of tissue monitoring and sampling, and a well-defined histological progression. For the same reasons, some intermediate biomarkers (*e.g.*, premalignant lesions/histological changes) have begun to be evaluated in human trials. In the oral cavity, the histological biomarker is dysplastic leukoplakic lesions. Published data have demonstrated that this biomarker can be modulated in clinical trials of retinoids and β-carotene [57,58]. Problems identified in these studies include a high rate of recurrence after the end of the chemopreventive protocol and the toxicity of certain agents (*e.g.*, 13-*cis*-retinoic acid). Thus, the DCPC-sponsored clinical trials are evaluating the response of dysplasia in oral leukoplakia to lower doses of 13-*cis*-retinoic acid and β-carotene.

Another widely studied biomarker in both human and hamster oral cavity has been the frequency of micronuclei. Micronuclei are DNA fragments unconnected to the nucleus which are a measure of clastogenesis. Although the background frequency appears to vary greatly between populations, higher counts do occur in high risk populations, *e.g.*, betel quid or tobacco chewers. Agents such as β-carotene and/or vita-

min A appear to modulate the frequency of micronucleated cells in exfoliated human oral cavity cells; however, suppression of this marker has not correlated with clinical remission of existing leukoplakia or inhibition of new lesions. Thus, the DCPC is evaluating the correlation between altered micronucleated cell frequency and modulation of dysplasia within oral leukoplakia in a clinical trial of β-carotene.

Initial studies of additional biomarkers have been done by Dr. Hong and co-workers; however, it is unclear at this time which may be useful. For example, the proposed proliferation biomarker, transglutaminase I, synthesizes cross-linking of proteins (involucrin) in the final stages of squamous differentiation. Staining for transglutaminase I in tissue sections appears to decrease from normal tissue to oral lesions with mild dysplasia; it disappears in severe dysplasia, CIS and poorly differentiated carcinoma. Further, transglutaminase I expression is modulated by the agent, 13-*cis*-retinoic acid. More work needs to be done on the correlation between this response and a decrease in cancer rate, however, since it is possible that transglutaminase I is merely a marker of epithelial maturation and differentiation, rather than malignant potential.

In contrast, less progress has been made in clinical trials for chemoprevention of lung cancer. Lung epithelium is not as accessible as oral cavity epithelium, and sampling and detection of premalignant and malignant lesions is a serious problem. Bronchial squamous metaplasia and/or dysplasia and sputum atypia have been suggested as premalignant conditions, and thus as histological intermediate biomarkers. Squamous metaplasia refers to replacement of the ciliated columnar bronchial epithelium with squamous epithelium. The metaplasia index (MI) is used as a measure of the extent of this condition [59]; it is calculated as the percentage of histological sections with metaplasia in biopsy specimens. Metaplasia is widespread in smokers, and may have low specificity as a histological intermediate biomarker. In the DCPC study of 13-*cis*-retinoic acid in smokers listed in Table VI, patients with an MI >15 or dysplasia are randomized to placebo or agent. Modulation of these endpoints will be correlated with other genetic, proliferation and differentiation markers.

In clinical trials, compliance with invasive procedures (bronchoscopy) can be low, so sputum cytology has been suggested as an alternative. Atypical sputum cells are classified by cell size and nuclear abnormalities. Although atypia (dysplasia) is a specific indicator of premalignancy, certain problems are inherent in evaluating cells shed into sputum. For example, the site from which the cells were shed is unknown, all lesions may not be represented, and cells of the peripheral lung may be unrepresented. In addition, variability in classification of cells can be high between observers. The incidence, prevalence and modification of sputum atypia are being assessed in a population of men exposed to asbestos in the workplace in a DCPC study on retinol and β-carotene; this data will also be related to the final endpoint, lung cancer (Table VI).

PROGRESS AND APPROACHES IN CHEMOPREVENTION OF LUNG AND UPPER AERODIGESTIVE TRACT CANCER

Progress continues to be made in identifying potential drugs for the chemoprevention of lung and aerodigestive cancer. The DCPC has tested a number of agents in the MNU-induced trachea and DEN-induced lung carcinogenesis models in the hamster. Of these, six promising agents (Table II) have been identified from the *in vitro* screens and both of the hamster efficacy models. These represent the second generation of agents to enter the clinical phase of the Testing Program. A new Phase II trial of 4-HPR, a retinoid with low human toxicity, is already in the planning stages. Oral leukoplakic lesions will be biopsied at the beginning and the end of the trial to determine the agent's effect on the grade of dysplasia.

Two major strategies are contemplated for future chemoprevention trials. The first strategy involves short-term (6 month) Phase II trials with reversal of premalignant lesions and/or other intermediate biomarkers as the endpoints. In the oral cavity, the short-term Phase II trial will involve oral leukoplakia patients whose lesions have been characterized by biopsy and histopathology. Excision of dysplastic lesions will be postponed for chemopreventive agent treatment; at the end of the study, the leukoplakia will be excised to evaluate any effect on the

extent of dysplasia. As many intermediate biomarkers as possible will be followed with standardized sampling and assessment. Suggestions from the hamster buccal pouch model include abnormal expression of GGT, ODC activity, transglutaminase I, EGF-R and cytokeratins K1 and K14. Modulation of such markers will be correlated with clinical and histological response of the lesion to a chemopreventive agent. Possible agents include *N*-acetyl-*l*-cysteine (preclinical testing and toxicology completed), 4-HPR, and *oltipraz*. 4-HPR, in particular, has demonstrated clinical effects in preliminary results from a randomized trial in Milan, Italy, of patients with a previously excised oral leukoplakia [60; Chiesa *et al.*, this volume]. After one year, more relapses or new lesions occurred in the control group (12/41) than in the 4-HPR group (3/39). This agent appears to be better tolerated than other retinoids, such as 13-*cis*-retinoic acid.

Future Phase III trials will test the agents in a large high risk group such as patients with a previously excised dysplastic leukoplakia. In this case, the endpoint would be squamous cell carcinoma of the oral cavity. Another Phase III trial would be a large study of patients successfully treated for a previous early stage oral cancer. In 1988, a trial with a similar design, EUROSCAN, began accrual of 2,000 patients "cured" of early stage oral, laryngeal or lung cancer in 14 European countries [61]. The patients are being followed for second tumors, recurrences and long-term survival. *N*-acetyl-*l*-cysteine and/or vitamin A treatment is being used to inhibit both the initiation and promotion phases of carcinogenesis, and to provide a non-toxic regimen. A panel of markers (unidentified) will be evaluated in a subset of patients to determine their feasibility as surrogate endpoints [62]. Interim results are discussed by Dr. De Vries in this volume.

In the lung, future short-term Phase II trials will have a different purpose. Since the premalignant lesions have not been well-defined, the trials will involve a high risk population (*e.g.*, prior resected laryngeal or stage I lung cancer) who will undergo invasive sampling procedures such as bronchoscopy or endoscopy, with sputum collection by lavage. Those patients with documented metaplasia/dysplasia will be accrued. The study population will be followed to correlate the premalignant lesion with the development of a second primary cancer. As many molecular biomarkers as possible will be concomitantly evaluated. The Phase III trial to follow will utilize a similar cohort, modulating the identified premalignant lesion (detected non-invasively if possible) with a promising agent. Problems with this approach still exist however. The rate of lung cancers even in this high risk group may be only 10–15% over 5 years. The number of patients needed will be high, and the effectiveness of lung cancer detection even by bronchoscopy is low [62]. At this time, the DCPC-sponsored Carotene and Retinol Efficacy Trial (CARET) is accruing such a study population at very high risk for lung cancer [63]. The goal is recruitment of >4200 asbestos workers who are current/former smokers and >13,500 smokers. These populations are randomized to a placebo or β-carotene and vitamin A (retinyl palmitate) arm, and are monitored for development of lung cancer. A Phase II pilot study demonstrated that these essentially asymptomatic populations will adhere to a regimen of β-carotene and vitamin A.

In conclusion, significant progress has been made in identifying potential drugs for chemoprevention of lung and upper aerodigestive tract cancers. Five clinical trials are ongoing in the oral cavity and seven have been initiated in the lung. Since the oral cavity has a well-defined premalignant lesion, more progress has been made in evaluating intermediate biomarkers as potential surrogate endpoints. This progress may be more rapid in the future due to the development of molecular probes for certain intermediate biomarkers and by computer-assisted means of analyzing cytomorphology objectively. In the lung, progress will be assisted by identification of premalignant lesion(s) for each type of cancer, as well as by dependable means of detection.

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