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Intraepithelial and Postinvasive Neoplasia as a Stochastic Continuum of Clonal Evolution, and Its Relationship to Mechanisms of Chemopreventive Drug Action

Charles W. Boone, MD, PhD[1], Gary J. Kelloff, MD[1], and Lawrence S. Freedman, PhD[2]

[1] Division of Cancer Prevention and Control, Chemoprevention Investigational Studies Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892
[2] Division of Cancer Prevention and Control, Biometry Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Abstract The progression of intraepithelial and postinvasive neoplasia depends on the occurrence of clonal evolution, defined as the continuous development of mutations and selective clonal expansions in the neoplastic cell population. The two continuously repeating events of clonal evolution, mutation and clonal expansion, occur at unpredictable times and locations. Therefore the neoplastic process is best characterized as a stochastic, *i.e.*, probabilistic, continuum. The *rate* of intraepithelial neoplastic progression is continuously driven by the *dosage level* of exposure to mutagens and mitogens. For example, in chronic smokers the length of time before development of lung cancer depends on the number of cigarettes smoked per day.

A commonly held misconception is that human carcinogenesis develops after an initial short period of mutation followed by a long period of stimulated proliferation (the multistage model). This incorrect idea derives from the sequential nature of the consecutive two- or three-step operational protocols imposed on experimental animal models by the experimenter. In reality, human carcinogenesis develops as the result of simultaneous and continuous exposure to mutagens and mitogens over the entire period of tumor development. A recent example is the finding that the intraepithelial neoplasia of colorectal adenomas continuously progresses through serial waves of mutation and clonal expansion.

The rational design of chemopreventive agents should be based on blocking the two parameters which continuously drive neoplasia: mutagenesis and mitogenesis. In addition to blocking exposure, chemopreventive agents may act at many points during activation and DNA adduction of mutagens, or during stimulation of the proliferation signal pathway by mitogens. Based on the chemopreventive strategy of blocking mutagenesis and mitogenesis, chemopreventive agents are classed as either antimutagenic or antimitogenic. A third class, the antioxidants, are both antimutagenic and antimitogenic, and operate by the common mechanism of breaking free radical chain reactions initiated by reactive oxygen species. In the program of the Chemoprevention Investigational Studies Branch, Division of Cancer Prevention and Control, National Cancer Institute, preclinical development of antimutagens, antimitogens, and antioxidants is well under way, and some of these agents are highlighted here.

Key words: Clonal evolution, intraepithelial neoplasia, mitogenesis, mutagenesis, neoplastic progression

Address correspondence to Charles W. Boone, MD, PhD, Program Director, DCPC, National Cancer Institute/NIH, Chemoprevention Investigational Drug Unit, CPRP, 9000 Rockville Pike, EPN Suite 201, Bethesda, MD 20892.

INTRAEPITHELIAL NEOPLASIA, THE PRECANCEROUS (PREINVASIVE) PHASE OF NEOPLASIA

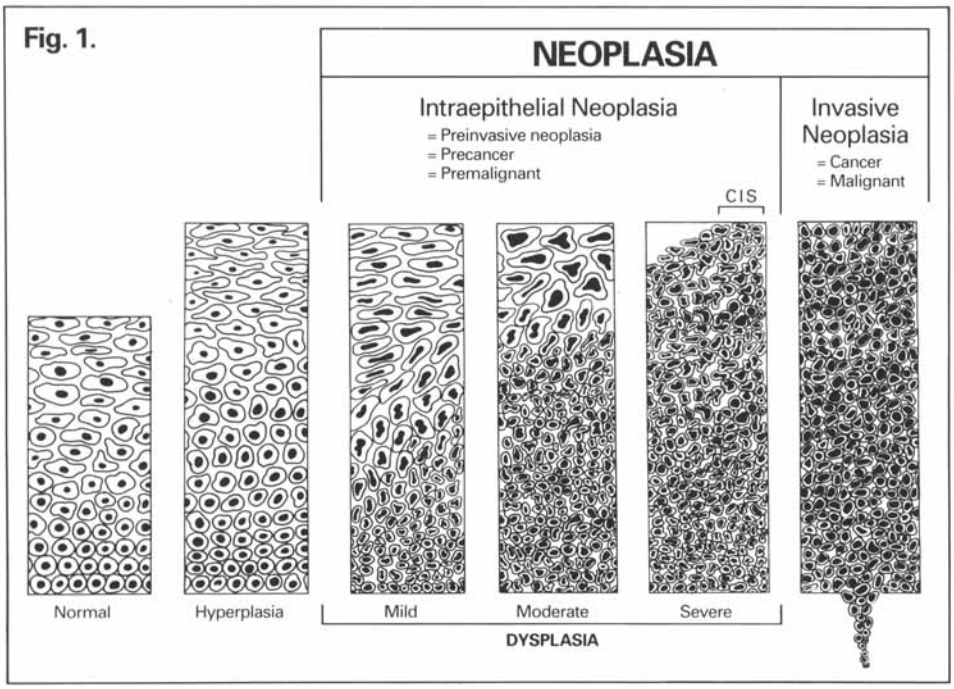
Pathology Terminology

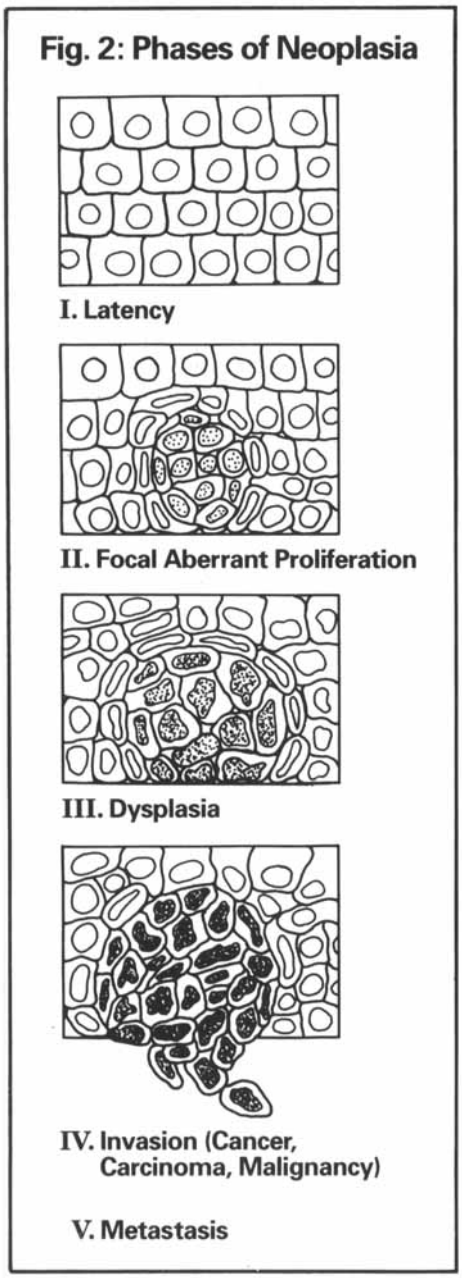
The term intraepithelial neoplasia applies to all phases of focal abnormal new growth within the epithelial compartment before ultimate invasion and metastasis. Following Foulds [1, vol 1, p. 69], initial genetic changes are included in the term. The diagram in Figure 1 illustrates the terminology associated with this definition. It shows that the diagnosis of carcinoma, synonymous with malignant neoplasm or cancer, depends on finding neoplastic epithelial cells invading across the basement membrane more than it does on specifying a qualitative change in the cells themselves.

Uncertainty exists regarding the mechanistic meaning of the terms "malignant" and "cancer," because some cancer researchers use them to denote an unspecified qualitative change in a grossly observed neoplasm, as opposed to a specific change in the microscopic topography of intraepithelial neoplastic cells relative to the basement membrane, *i.e.*, invasion. Common examples of the former usage are the terms "malignant conversion" applied to a hypothetical qualitative change of a papilloma in the mouse skin-paint model of carcinogenesis, and "malignant transformation" applied to a focus of piled-up cells in tissue culture. When the cells of a tissue culture focus are implanted subcutaneously *in vivo*, they often form a tightly cohesive cellular mass with sharp, smooth, non-infiltrating borders that never exhibit local invasiveness or metastasis. Animals bearing these tumors are permanently "cured" by simple excision, no matter how large the tumors become. This kind of *in vivo* behavior defines cells as neoplastic, but not malignant, a distinction that is infrequently specified, if considered at all.

Phases of Intraepithelial Neoplasia

Figure 2 illustrates the evolution of intraepithelial neoplasia through three phases, followed by two more phases of extraepithelial neoplasia, *i.e.*, invasion and metastasis. The phases of neoplasia are defined not as discrete steps, but rather as milestones in an evolutionary continuum. The first, or *latent phase*, follows the mutation of an epithelial cell (mutation in the broad sense of a heritable genetic alteration, including gene amplifications and karyotypic abnormalities) in





which the cell appears microscopically normal, but is committed to expand clonally at a later time. The second phase starts with *focal aberrant proliferation*, consisting of normal-appearing cells that exhibit disorganized architecture, frequently including compression of surrounding normal tissue. This phase may also be called predysplasia. Aberrant crypts of the colon, which in some cases are associated with the appearance of dysplasia and adenocarcinoma [2], are examples of this phase. *Dysplasia*, the third phase of intraepithelial neoplasia, starts with the appearance of dysplastic changes (described below) in the focally proliferating, disorganized cells of the second phase. Dysplastic growth is graded into three categories depending on its extent above the basement membrane, as shown in Figure 1, or simply into two categories of low grade and high grade. In some cases, when the dysplastic cell population extends to the full thickness of the epithelium, it is called carcinoma *in situ*, in recognition of the higher cancer risk associated with this pattern. It has been well established that severe dysplasia and carcinoma *in situ* cannot be reliably distinguished [3]. In spite of this fact, and in spite of the unnecessary alarm premature use of the word "carcinoma" may cause in the patient, the term "carcinoma *in situ*" appears irrevocably entrenched in the practice of medicine, particularly in the case of ductal and lobular carcinoma *in situ* (DCIS, LCIS) of the breast. The two final phases of neoplasia are *invasion*, which starts when dysplastic cells migrate across the basement membrane, and *metastasis*, characterized by individual cells and cell groups separating from the main tumor mass and disseminating via vascular and lymphatic channels to form secondary growths at distant sites.

FOUR IMPORTANT BIOLOGICAL PROPERTIES OF INTRAEPITHELIAL NEOPLASIA

Progression

Foulds originated the expression "tumor progression" to describe the propensity of a neoplastic cell population to develop "by way of permanent, irreversible qualitative changes in one or more of the characters of its cells" [1, vol 1, p. 69] that are "determined at an early stage" and as "comprehensively applicable to the development of tumors from beginning to end" [4, pp. 335–336]. He describes progression (in his terminology, "from one B lesion to another") [1, vol 2, p. 80] as occurring during what is now called intraepithelial neoplasia. Slaga and colleagues [5–8] have recently shown, in the two stage initiation-promotion model using SENCAR mice, that neoplastic progression begins early during the promotion step and is characterized by increasingly severe aneuploidy and dysplasia, and that

progression is probably occurring in the majority of lesions at different rates. The presence of severe aneuploidy during the promotion step of the two-stage model is clearly at variance with the conventional multistage model of carcinogenesis, which portrays the stage of promotion as being reversible [9], whereas aneuploidy is irreversible. Also at variance with the multistage model is the finding that progression begins early during the promotion step, rather than after it [10].

Following Foulds, we will apply the term "progression" to denote the propensity of neoplastic cells at all stages of neoplasia, from the first genetic alterations to late metastasis, to evolve irreversibly through states of increasing deviation from normal structure and function, which are associated with increasing size, growth rate, and invasiveness.

Clonal Evolution

The phenomenon of clonal evolution in neoplasia, first described by Nowell [11], has been reviewed in relation to intraepithelial neoplasia [12]. Briefly, it is the continuous development within a proliferating neoplastic cell population of mutated variants able to escape ambient growth control mechanisms and form clonal expansions which compete with each other on the basis of growth rate. An outstanding recent example of clonal evolution is the demonstration by Vogelstein, *et al.* [13,14] that, during the progression of intraepithelial neoplasia on the surface of adenomatous polyps of the colon, there occurs an accumulation of lesions in growth-associated genes which appear to be "a continuum resulting from successive waves of clonal expansion."

Critical growth-related point mutations, amplifications, and chromosomal aberrations may arise in any cell of the neoplastic population, and within each cell these mutations may arise in any of a number of locations. Thus, the number of alternative paths to a unique set of accumulated critical gene mutations is likely to be very large. Clearly, clonal evolution is a stochastic, *i.e.*, probabilistic, multipath progression. This could explain why Vogelstein's group found the sequence and frequency of each mutation in colonic polyps to be so variable, and why they found that accumulation of all four of the common genetic lesions they describe (*i.e.*, *ras* gene mutations and allelic deletions of chromosomes 5q, 17p, and 18q) occurred in less than 10% of colorectal carcinomas [14]. It follows that the frequency of any given gene mutation in the tumor cell population is likely to vary from tumor to tumor and even in different parts of the same tumor—a point which becomes important when considering the sensitivity of surrogate endpoint biomarker (SEB) assays based on genetic alterations.

It is clear that clonal evolution is the underlying mechanism of neoplastic progression [15]. Expanding mutated clones can frequently be directly visualized within a carcinomatous tissue as sharply defined foci with altered architectural pattern, the so-called "focus in focus" phenomenon [16].

Acceleration of Progression

If selection of mutated neoplastic clones occurs on the basis of growth rate, it follows that during intraepithelial neoplasia the mean growth rate of the neoplastic cell population should increase as progression proceeds. Richart [17] confirmed this for cervical intraepithelial neoplasia by showing that the tritiated thymidine labelling index increases with the grade of dysplasia, from 4% in mild dysplasia to a remarkable 45% in severe dysplasia. (The labelling index of invasive tumors can be expected to be less than this because of poorer nutrient supply and gas exchange). In the case of DCIS, the labelling index of low grade DCIS is 1.5% and 5% in high grade DCIS [18], compared to 0.8% in normal ductular epithelium during the late secretory phase of the menstrual cycle, and 0.2% at other times [19]. Thus, an abnormally high proliferation rate tends to correlate with the extent of neoplastic progression and may serve as a useful SEB (see below).

The Druckrey Phenomenon

Druckrey [20] first quantitatively demonstrated an inverse relationship between the dose level of continuously administered carcinogen and the length of the tumor latent period. As illustrated in Figure 3, an increasing daily dose of carcinogen causes a decrease in the duration of the latent period. Since the tumor latent period is a reciprocal expression of the rate of neoplastic

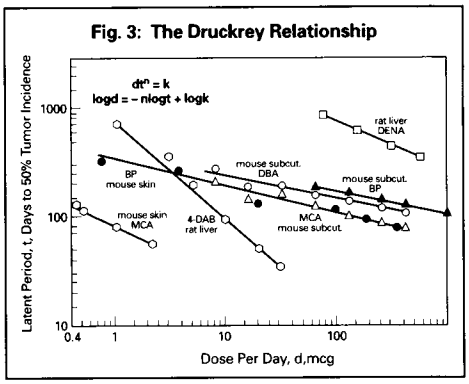
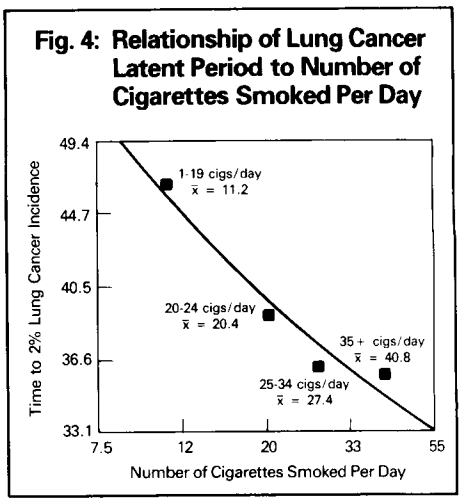


Fig. 3. BP: benzo(*a*)pyrene; 4-DAB: *N,N*-Dimethyl-4-aminobenzene; MCA: methyl cholanthracene; DBA: 7,14-dimethyldibenzo(*a,h*)-anthracene; DENA: diethylnitrosamine.



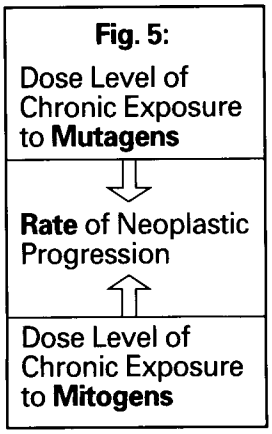
progression, an equivalent statement of the Druckrey phenomenon is that the dose level of continuously administered carcinogen drives the rate of neoplastic progression. The relationship is a remarkably precise analytical one described by the formula: $dt^n = k$, where d is the daily dose, t the 50% tumor latent period, and n and k are constants characteristic of the system.

The mechanism of the Druckrey phenomenon may be given as follows. Most carcinogen molecules are simultaneously mutagenic and mitogenic. Mutagenic activity increases the rate of appearance of expanding mutated clones. Mitogenic activity produces two effects: a larger target population with increased chance of mutation, and a larger fraction of cells in the more mutation-prone S phase.

In animal models of carcinogenesis, administration of either an antimutagen, such as phenethylisothiocyanate [21], or an antimitogen, such as difluoromethylornithine (DFMO) [22], results in prolongation of the tumor latent period. This is additional experimental evidence that the parameters of mutagenesis and mitogenesis both drive the rate of neoplastic progression.

THE RATE OF CLONAL EVOLUTION IN HUMAN CARCINOGENESIS IS DRIVEN BY THE LEVEL OF CONTINUOUS AND SIMULTANEOUS EXPOSURE TO MUTAGENS AND MITOGENS

Humans are commonly exposed continuously and simultaneously to mutagens and mitogens.



Examples are chronic exposure to combustion products of fossil fuels and cigarettes, both of which contain mutagenic and mitogenic molecules, plus weak irritants which induce epithelial proliferation associated with reactive inflammation. The Druckrey relationship, developed in animal models of carcinogenesis, also applies to humans. Figure 4 demonstrates the case of chronic smokers, where the estimated latent period before 2% of smokers develop lung cancer is seen to be inversely related to the number of cigarettes smoked per day (derived from [23] using the calculation methods of Druckrey [20]). Thus, in humans, the rate of intraepithelial neoplastic progression is continuously driven by simultaneous chronic exposure to mutagens and mitogens (Fig. 5).

THE BERENBLUM TWO-STAGE MODEL OF CARCINOGENESIS IS PART OF THE PHENOMENON OF CLONAL EVOLUTION

Berenblum [24] postulated in 1947 that carcinogenesis develops through two consecutive stages of initiation and promotion, based on experiments of skin-painting mice with dimethylbenz(*a*)anthracene (DMBA) and croton oil (later TPA, see below). Carcinogenesis is now depicted as proceeding through as many as three distinct consecutive stages [9,10]: initiation (irreversible genetic alteration), promotion (reversible induction of clonal expansion), and progression, which is associated with conversion (change from benign to malignant). Promotion is now characterized as having an initial stage of conversion to promotability by a Stage I promoter (not to be confused with later occurring conversion to malignancy) and a subsequent stage of continuing promotion by a weak Stage II promoter [25]. Stage I promoters produce conversion to promotability even when applied 1–2 weeks prior to initiation. Progressor agents cause acceleration of cancer development when applied after promotion [26]. Both initiators and progressor agents have been characterized as mutagenic, whereas promoters are mitogenic. There now appears to be widespread advocacy of the consecutive three-stage mechanism (see, for instance, the diagram of carcinogenesis in [27]).

DMBA, like the majority of carcinogens, is intensely mitogenic as well as mutagenic [28]. Berenblum could develop the initiation-promotion model of carcinogenesis because he was able to lower the dose of DMBA to the point where it was still mutagenic but no longer mitogenic. At this point carcinomas no longer appeared unless the DMBA was followed by twice- or thrice-weekly applications of croton oil, an intense irritant stimulant of hyperplasia. Croton oil was later replaced by a pure derivative, the well-known 12-*O*-tetradecanoylphorbol-13-acetate (TPA). As expected, if the dose of DMBA in the two-stage model is increased, either as a single dose [29] or in continuous multiple doses, carcinomas are again produced without the need for promotion. The use of higher doses of carcinogen converts the two-stage, or incomplete model, into the so-called complete model of carcinogenesis in which carcinogen alone is sufficient.

It is clear that the Berenblum two-step model of carcinogenesis is a specialized experimental protocol of operational steps which separate the usually concurrent events of clonal evolution, mutation and clonal expansion, into a consecutive sequence now called initiation and promotion. Although the Berenblum protocol continues to be enormously heuristic and valuable as an experimental method for dissecting molecular mechanisms, it does not accurately fit general human experience as a model of carcinogenesis. It is rare for humans to undergo both short-term and low-dose exposure to a pure mutagen followed by long-term exposure to a pure mitogen.

Thus, the common perception of human carcinogenesis as a sequence of discrete consecutive steps or stages, in fact, derives from the series of consecutive operational steps imposed on animal models by the experimenter. Carcinogenesis in humans is rarely seen to pass serially through the consecutive sequences of initiation, promotion, and progression with malignant conversion. Instead, it appears to progress from the outset as a stochastic continuum of multiple concurrent mutations and clonal expansions driven by continuous and simultaneous exposure to mutagens and mitogens from the environment and from endogenous sources. As described above, the rate of progression through this continuum is driven by the dose level of exposure to mutagens and mitogens. In the usual three-stage mouse skin-painting experiment, the investigator first applies an initiator, then a promoter, then a progressor agent. If both the initiator and the progressor are simultaneously mutagenic and mitogenic, inducing concurrent mutations and clonal expansions of mutated cells, and the promoter is mitogenic, one can appreciate the conceptual merging of the three-stage model with the more broadly applicable paradigm of a stochastic continuum of clonal evolution driven by mutagens and mitogens.

SEBs BASED ON THE MICROSCOPIC CRITERIA OF DYSPLASIA

SEBs are urgently needed to replace the endpoint of cancer incidence reduction in clinical trials of chemopreventive agents, which cost millions of dollars and require large cohort size (thousands of subjects) and long study duration (5–10 years). SEBs based on the microscopic criteria of dysplasia have been recently devel-

oped [30]. The advantage of dysplasia-based SEBs is that they are not markers **of** neoplasia, they **are** neoplasia; and by definition, they are seen with the highest frequencies compared to other markers which depend on genetic changes, altered states of differentiation, or altered regulatory pathways.

MICROSCOPIC CRITERIA OF DYSPLASIA

The criteria of dysplasia, based on a consensus derived from the literature, have been reviewed previously [12]. Briefly, they are increased nuclear size, abnormal nuclear shape, increased nuclear stain uptake, pleomorphism (increased variability of nuclear size, shape, and stain uptake), increased mitoses, abnormal mitoses, and abnormal or absent maturation. Abnormal mitoses are the hallmark of aneuploidy, because they are associated with unequal allocation of chromosomes to daughter cells during cell division. In glandular epithelia such as breast and prostate, an additional criterion is the presence of an increased number of nucleoli showing enlargement, abnormal shape, and increased stain uptake. The first four criteria above taken together make up the nuclear grade, which is the main component of a commonly used grading system for breast carcinoma [31].

GRADING SYSTEM FOR DYSPLASIA OF DCIS OF THE BREAST

Figure 6 depicts the histomorphologic grading system for DCIS as devised by Lagios [32]. It consists of three components: nuclear grade, presence or absence of necrosis, and histologic pattern. The closely related grading system of mild, moderate, and severe dysplasia is shown for comparison.

VALIDATION OF DYSPLASIA AS AN SEB

The certain diagnosis of dysplasia attested to by a panel of expert pathologists does not need further validation as an SEB. Appreciation of this important point, which requires a knowledge of the role of dysplasia in the biology of neoplasia, can save the time and expense of clinical trials to "validate" dysplasia. The dysplastic phase of intraepithelial neoplasia is not a marker **of** neoplasia, it **is** neoplasia, and is an integral part of

Fig. 6: Criteria for Grading Ductal Carcinoma of the Breast

Type	Dysplasia	Nuclear Grade	Differentiation Pattern	Necrosis	Name
I	severe	high	none	3+	Comedo
II	severe	high	glands, papillary	3+	Cribriform, papillary
III	moderate	inter-mediate	glands	+/–	Cribriform, intermediate
IV	mild	low	micro-papillary	0	Micropapillary

Fig. 7: Subjective Criteria of Dysplasia Transformed to Continuous Variables by Image Analysis

Subjective	Image Analysis
Nuclear Grade	**Nuclar Parameters**
• nuclear size	nuclear area ± σ^2
• nuclear shape	nuclear shape factor ± σ^2
• nuclear stain uptake	DNA density (Feulgen) ± σ^2
• variation in above (pleomorphism)	mean σ^2
Mitotic Rate (no. of mitoses)	**S-Phase Fraction**
Abnormal Mitoses	**DNA Aneuploidy**
Nucleoli	**Nucleoli**
• size, shape, number	— area, shape factor, number
• pleomorphism	— mean σ^2

the causal pathway to clinically manifest cancer in the same sense as the fetus is an integral part of the causal pathway to the adult. A valid causal relationship does **not** require that every focus of dysplasia progress to clinical cancer. Some foci of dysplasia may regress, just as, in the analogy to fetal development, some fetuses may regress. But viewed retrospectively, virtually every epithelial cancer will have progressed through a dysplastic stage, and eliminating dysplastic foci in an epithelium can be expected to decrease the incidence of carcinoma. For example, elimination of adenomatous colorectal polyps, which are essentially dysplasia on a fibrovascular stalk, has been shown to be associated with a reduced incidence of adenocarcinoma of the colon [33].

Validation of an SEB has been quantitatively characterized using the concept of attributable proportion [34], which may be expressed as $AP = S[(I_1 - I_0)/I_1]$ [35], where AP is the attributable proportion, which varies from zero and one, S is the proportion of cancer cases with the biomarker, I_1 is the incidence of cancer cases with the biomarker, and I_0 is the incidence of cancer cases without the biomarker. The factor $[(I_1 - I_0)/I_1]$ may also be calculated from the identity $(1 - 1/R)$, where R is the relative risk defined as I_1/I_0. Practically all epithelia exhibit dysplasia at some point during cancer development. Therefore, the proportion of cancer cases associated with dysplasia at some point, S, approaches 1.0. For the

same reason, the incidence of cancer cases not associated with dysplasia, I_0, approaches zero, causing the factor $[(I_1 - I_0)/I_1]$ to approach 1.0. It is clear that, by the criterion of attributable proportion, dysplasia is completely validated as an SEB because both of the factors contributing to the calculation of AP are equal to 1.0.

USE OF COMPUTER-ASSISTED IMAGE ANALYSIS (CAIA) TO INCREASE THE QUANTIFIABILITY AND PRECISION OF SEBs BASED ON THE MICROSCOPIC CRITERIA OF DYSPLASIA

Figure 7 shows how CAIA can be used to precisely quantify the microscopic criteria of dysplasia [see also 30]. Each subjectively estimated criterion is converted by CAIA into a continuous, precisely measured variable with a mean and a variance. There appear to be three parameters derived from the microscopic criteria of dysplasia and measured by CAIA that offer exceptional potential as SEBs. They are as follows:

(1) The nuclear pleomorphism index. By CAIA, the variables of nuclear size, nuclear shape, and nuclear stain uptake each have a mean and variance. The pleomorphism index is calculated as the mean of the three variances. Extensive pleomorphism is a fundamental hallmark of neoplasia.

(2) Abnormally high proliferation index. CAIA can be used to determine the frequency distribution and intensity of chromogenic antibody probes associated with PCNA, Ki-76, BrdU uptake, or S-phase fraction determined by DNA densitometry of Feulgen-stained tissue. Ultrafast static image analysis approaches the speed of flow cytometry for this purpose, without the loss of information produced by the tissue homogenization required for flow cytometry.

(3) Aneuploidy. In addition to depicting the frequency and type of aneuploidy in the conventional DNA histogram, CAIA can also display an image of a histologic section in which all the aneuploid cells are marked (James and Sarah Bacus, CAS, Inc., personal communication). From this image, the pathologist may determine at a glance the presence of nests of aneuploid cells in an epithelium. Such nests are an extremely strong indication that a neoplastic process is present.

MECHANISMS OF ACTION OF CHEMOPREVENTIVE COMPOUNDS

The graph in Figure 4 may be used to illustrate the primary basic strategy of chemoprevention. Giving someone who smokes 20 cigarettes per day a chemopreventive agent which blocks the chemical effects of cigarette smoke would decrease the effective dose of cigarettes and therefore increase the latent period before appearance of lung cancer. This effect of giving chemopreventive agents to extend cancer-free lifespan is the basic strategy of chemoprevention. A comprehensive review of chemopreventive agents under development by the Chemoprevention Investigational Studies Branch has been published [37].

As discussed above and illustrated in Figure 4, the rate of intraepithelial neoplastic progression in humans is driven by the level of exposure to mutagens and mitogens. Chemopreventive agents may therefore be classed as antimutagenic or antimitogenic, depending on whether they block exposure to or the effects of, mutagens or mitogens. Pro-oxidants, or compounds which cause increased production in the cell of superoxide, singlet oxygen, hydrogen peroxide, and hydroxyl free radicals [together called reactive oxygen species (ROS)], are both mutagenic and mitogenic [38]. The antioxidant agents which block them are conveniently handled as a third class of chemopreventive agents, because they act through a similar mechanism, even though strictly speaking the antioxidants are combined antimutagens and antimitogens. The classification of chemopreventive agents as antimutagenic or antimitogenic corresponds to their classification by Wattenberg as blocking and suppressing agents [39].

SOURCES OF MUTAGENS, MITOGENS, AND PRO-OXIDANTS

The major sources of mutagens and mitogens are outlined in Figures 8 and 9. Important intracellular sources of ROS are leaky membrane flavoproteins present in various electron trans-

Fig. 8: Sources of Mutagens

ATMOSPHERIC — Combustion products of:
- fossil fuels → PAH, e.g., benzo(a)pyrene
- tobacco → PAH, nitrosamines, e.g., NNK
- refuse → PAH, heterocyclic amines, e.g., IQ, PhIP

DIETARY
- fried, broiled meats → heterocyclic amines, e.g., IQ
- nitrates in water, vegetables → nitrite by oral bacteria, plus dietary amines → nitrosamines

ENDOGENOUS
- infidelity of DNA replication
- reactive oxygen species (ROS)

Fig. 8. PAH: polycyclie aromatic hydrocarbons; IQ: 1,2-amino-3-methylimidazo(4,5-*f*)-quinoline; PhiP: 2-amino-1-methl-6-phenylimidazo(4,5-*b*)pyridine; NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

Fig. 9: Sources of Mitogens

EXOGENOUS
- many "complete" carcinogens
- "promoters" (mitogens), e.g., arsenic, human papilloma virus
- chronic irritation, e.g., abrasions, asbestos, silica
- chronic inflammation due to infection e.g., chronic viral hepatitis → liver cancer, schistosomiasis → bladder cancer

ENDOGENOUS
- hormones, e.g., estrogen, testosterone
- growth factors, e.g., EGF, TGFβ, HER-2/neu
- lipid peroxidation due to ROS

Fig. 9. EGF: epidermal growth factor; TGFβ: transforming growth factor-β.

port chains in the cell, as illustrated in Figure 10. The function of flavoprotein molecules in electron transport chains is to separate pairs of electrons into a stream of single electrons, effected by a riboflavin molecule attached to the flavoprotein. Single electrons tend to leak from the riboflavin to nearby oxygen molecules in the membrane or cytosol to form superoxide. The leak reaction is catalyzed by ionic iron [38] or by heterocyclic amines derived from pyrolyzed amino acids in the diet [40]. Examples of leaky membrane flavoproteins are cytochrome P-450 reductase, cytochrome b_5 reductase, and coenzyme Q reductase. A major extracellular source of ROS is the "respiratory burst" reaction of macrophages and neutrophils during the inflammatory reaction (Fig. 11). Nitric oxide is also produced, and can react with superoxide to produce peroxynitrite, a stable molecule that may diffuse long distances before it splits to form hydroxyl free radicals [41,42].

Fig. 10: Cellular Sources of Pro-oxidants

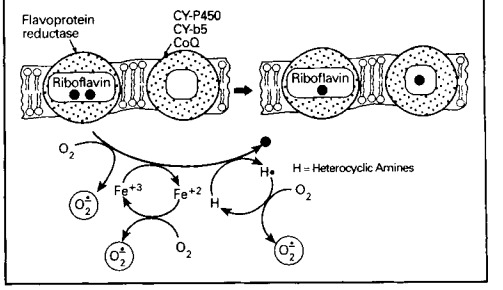


Fig. 11: Respiratory Burst Reaction

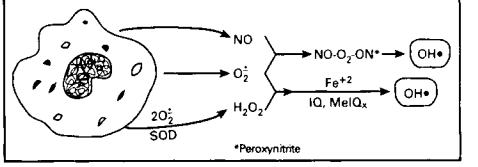


Fig. 11. SOD: superoxide dismutase; IQ: 1,2-amino-3-methylimidazo(4,5-*f*)quinoline; MeIQx: 2-amino-3,8-dimethylimidazo(4,5-*f*)quinoxaline.

MECHANISMS OF CHEMOPREVENTION WITH ANTIMUTAGENIC AGENTS

The induction of glutathione-*S*-transferase is a prominent mechanism of action for a major group of antimutagenic chemopreventive agents (Fig. 12). Other Phase II xenobiotic detoxifying enzymes are also induced by these agents. Isothiocyanate and diallyl sulfide also act by inhibiting the cytochrome P-450 isozyme, which catalyzes the formation of carcinogenic epoxides from polycyclic aromatic hydrocarbons. Prostaglandin-H synthetase, present in colon and bladder epithelium, contains a hydroperoxide site which also activates procarcinogens in the so-called co-oxidation reaction [42].

MECHANISMS OF CHEMOPREVENTION WITH ANTIMITOGENIC AGENTS

Figure 13 lists some important examples of antimitogenic chemopreventive agents. Antimitogenic agents may be classified on the basis of where they block the signal pathway for mitogenesis. Tamoxifen blocks at the initial receptor

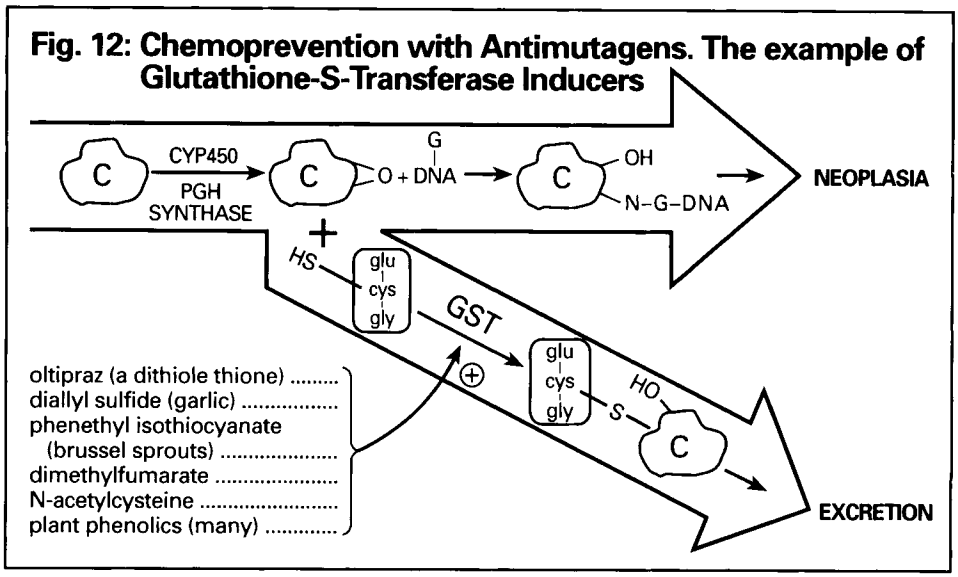


Fig. 13: Chemoprevention with Antimitogens

- ornithine decarboxylase inhibitor: difluoromethylornithine (DFMO)
- anti-estrogen: tamoxifen
- anti-testosterone: proscar
- pro-differentiation: retinoids
- anti-inflammatory: aspirin, ibuprofen, piroxicam
- anti-proliferation signal pathway
  - — limonene: • inhibits isoprenylation of G proteins • inhibits CoQ synthesis
  - — CAI (GTP analogue): inhibits G protein function
  - — genistein (isoflavone ATP analogue): tyrosine kinase inhibitor
  - — flavonoids, e.g., quercetin (ATP analogue): PKC inhibitors

level, carboxyamide-amino-imidazole (CAI) [43] and quercetin at the protein kinase level, retinoids at the transcription level, and DFMO and finasteride (Proscar®) at the effector level (ornithine decarboxylase and testosterone-5α-reductase activity, respectively). The mitogenesis associated with inflammation appears to be due to many factors, among which is the production of prostaglandins. Among their many activities, prostaglandins are autocrine signal molecules, so non-steroidal anti-inflammatory drugs may be tentatively placed in the category of blockers of signal molecule production.

MECHANISMS OF CHEMOPREVENTION WITH ANTIOXIDANTS

Figure 14 diagrams the major mechanism of action of antioxidant chemopreventive agents, which is to stop free radical chain reactions through the ability to become a stable, unreactive free radical molecule. After the antioxidant molecule surrenders one electron of an orbital pair to a nearby free radical molecule participating in a chain reaction, the remaining electron in the orbital delocalizes to form a large number of resonance hybrids with concomitant increase in stability and decrease in free radical reactivity of the antioxidant molecule. Lipophilic antioxidants stop lipid peroxidation of cell membranes, a commonly occurring free radical chain reaction known to produce mutagenic and mitogenic molecules [38]. Hydrophilic antioxidants scavenge ROS in the cytosol.

SUMMARY

The progression of intraepithelial and post-invasive neoplasia depends on the occurrence of clonal evolution, defined as the continuous development of mutations and selective clonal expansions in the neoplastic cell population. The two continuously repeating events of clonal evolution, mutation and clonal expansion, occur at unpredictable times and locations. Thus, the neoplastic process is best characterized as a stochastic, *i.e.*, probabilistic, continuum.

The rational design of chemopreventive agents should be based on blocking the two parameters which continuously drive neoplasia: mutagenesis and mitogenesis. In addition to blocking exposure, chemopreventive agents may act at many

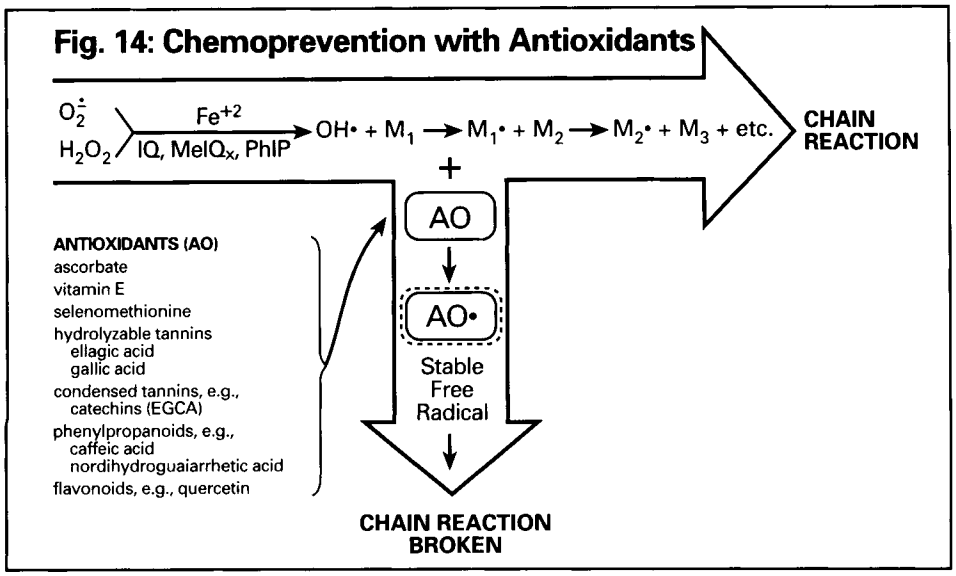


Fig. 14. IQ: 1,2-amino-3-methylimidazo(4,5-*f*)quinoline; MeIQx: 2-amino-3,8-dimethylimidazo(4,5-*f*)quinoxaline; PhiP: 2-amino-1-methl-6-phenylimidazo(4,5-*b*)pyridine; M: molecule.

points during activation and DNA adduction of mutagens, or during stimulation of the proliferation signal pathway by mitogens.

SEBs are urgently needed for use in clinical trials of chemopreventive agents because the currently used endpoint of cancer incidence reduction is forbiddingly expensive, labor intensive, and time consuming. The use of CAIA promises to improve the sensitivity and precision of SEB assays and assist in the development of SEBs based on the microscopic criteria of dysplasia, *e.g.*, the nuclear pleomorphism index, aneuploidy, and the proliferation index.

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REFERENCES

1. Foulds L. (1975) "Neoplastic Development." Vols 1 & 2, New York: Academic Press.
2. Wargovich MJ, Harris C, Chen C, Palmer C, Steele V, Kelloff G. (1992) Growth kinetics and chemoprevention of aberrant crypts in the rat colon. J Cell Biochem 16G(Suppl):51-54.
3. Anderson MC. (1987) Premalignant and malignant diseases of the cervix. In Fox H (ed): "Haines and Taylor Obstetric and Gynecological Pathology." New York: Churchill Livingston, pp 225–277.
4. Foulds L. (1954) The experimental study of tumor progression, a review. Cancer Res 14:327–339.
5. Aldaz CM, Conti CJ, Klein-Szanto AJP, Slaga TJ. (1987) Progressive dysplasia and aneuploidy are hallmarks of mouse skin papillomas: Relevance to malignancy. Proc Natl Acad Sci (USA) 84:2029–2032.
6. Aldaz CM, Conti CJ, Larcher F, Trono D, Chesner T, Whitehead T, Slaga TJ. (1988) Sequential development of aneuploidy, keratin modifications, and gamma-glutamyltransferase expression in mouse skin papillomas. Cancer Res 48:3253–3257.
7. Conti CJ, Aldaz CM, O'Connell JO, Klein-Szanto AJP, Slaga T. (1986) Aneuploidy, an early event in mouse skin tumor development. Carcinogenesis 7:1845–1848.
8. Gimenez-Conti IB, Bianchi AB, Fischer SM, Reiners JJ Jr, Conti CJ, Slaga TJ. (1992) Dissociation of sensitivities to tumor promotion and progression in outbred and inbred SENCAR mice. Cancer Res 52:3432–3435.
9. Pitot HC. (1993) The molecular biology of carcinogenesis. Cancer 72:962–970.
10. Hennings H, Glick AB, Greenhalgh DA, Morgan DL, Strickland JE, Tannenbaum T, Yuspa SH. (1993) Critical aspects of initiation, promotion, and progression in multistage epidermal carcinogenesis. Proc Soc Exptl Biol Med 202:1–8.
11. Nowell PC. (1976) The clonal evolution of tumor cell populations. Science 194:23–28.
12. Boone CW, Kelloff GJ, Steele VE. (1992) Natural history of intraepithelial neoplasia in humans with implications for cancer chemoprevention strategy.

Cancer Res 52:1651–1659.
13. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. (1988) Genetic alterations during colorectal tumor development. N Engl J Med, 319: 525–532.
14. Fearon ER, Vogelstein B. (1990) A genetic model for colorectal tumorigenesis. Cell 62:759–767.
15. Nowell PC. (1986) Mechanisms of tumor progression. Cancer Res 46:2203–2207.
16. Scherer E, Feringa AW, Emmelot P. (1984) Initiation-promotion-initiation. Induction of neoplastic foci within islands of precancerous liver cells in the rat. In Borzsonyi M, Lapis K, Day NE, Yamasaki H (eds): "Models, Mechanisms, and Etiology of Tumor Promotion." Lyon, France: IARC Scientific Publication No. 56, pp 57–66.
17. Richart RM. (1963) A radioautographic analysis of cellular proliferation in dysplasia and carcinoma *in situ* of the uterine cervix. Am J Obstet Gynecol 86: 925–930.
18. Meyer JS. (1986) Cell kinetics of histologic variants of *in situ* breast carcinoma. Breast Cancer Res Treat 7:171–180.
19. Meyer JS. (1977) Cell proliferation in normal human breast duct, fibroadenoma, and other ductal hyperplasias measured by nuclear labelling with tritiated thymidine. Human Pathol 8:67–81.
20. Druckrey H. (1967) Quantitative aspects in chemical carcinogenesis. In R Truhaut (ed): "Potential Carcinogenic Hazards from Drugs." UICC Monograph Series, Vol. 7, New York: Springer-Verlag.
21. Doerr-O'Rourke K, Trushin N, Hecht SS, Stoner GD. (1991) Effect of phenethyl isothiocyanate on the metabolism of the tobacco-specific nitrosamine-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by cultured rat lung tissue. Carcinogenesis 12:1029–1034.
22. Thompson HJ, Ronan AM, Ritaco KA, Meeker LD. (1986) Effect of tamoxifen and *d,l*-2-difluoromethylornithine on the growth, ornithine decarboxylase activity and polyamine content of mammary carcinomas induced by 1-methyl-1-nitrosourea. Carcinogenesis 7:837–840.
23. Doll R, Peto R. (1978) Cigarette smoking and bronchial carcinoma among regular smokers and lifelong non-smokers. J Epidemiol Commun Health 32:303–313.
24. Berenblum I, Shubik P. (1947) A new, quantitative approach to the study of the stages of chemical carcinogenesis in the mouse's skin. Brit J Cancer 1:383–391.
25. Furstenberger G, Kinzel V, Schwarz M, Marks F. (1985) Partial inversion of the initiation-promotion sequence of multistage tumorigenesis in the skin of NMR1 mice. Science 230:76–78.
26. Hennings H. (1991) Malignant conversion, the first stage in progression, is distinct from phorbol ester promotion in mouse skin. Basic Life Sci 57:31–39.
27. The Surgeon General's Report on Nutrition and Health. (1988) U.S. Dept. of Health and Human Services, DHHS (PHS) Publication No. 88–50210, p 189.
28. Iverson S, Edelstein JM. (1952) On the mechanism of experimental carcinogenesis. 4. The early mitotic effect of 9,10-dimethyl-1,2-benzanthracene and of 1,2,5,6-dibenzanthracene on epidermal cells of the mouse ear. Acta Path Microbiol Scand 30:213–222.
29. Terracini B, Shubik P, Della Porta G. (1960) A study of skin carcinogenesis in the mouse with single applications of 9,10-dimethyl-1,2-benzanthracene at different dosages. Cancer Res 20:1538–1542.
30. Boone CW, Kelloff GJ. (1950) Intraepithelial neoplasia, surrogate endpoint biomarkers, and cancer chemoprevention. J Cell Biochem 17F(Suppl):37–48.
31. Bloom HJG. (1950) Prognosis in carcinoma of the breast. Br J Cancer 4:259–288.
32. Lagios MD. (1990) Duct carcinoma *in situ*, pathology and treatment. Surg Clin North Amer 70:853–871.
33. Selby JV, Friedman GD, Quesenberry CPJ, Weiss NS. (1992) A case-control study of screening sigmoidoscopy and mortality from colorectal cancer. N Engl J Med 326:653–657.
34. Schatzkin A, Freedman LS, Schiffman HH, Dawsey SM. (1990) Validation of intermediate endpoints in cancer research. J Natl Cancer Inst 82:1746–1752.
35. Rothman KJ. (1986) "Modern Epidemiology." Boston: Little, Brown, and Company, p 38.
36. Kelloff GJ, Boone CW, Steele VE, Fay JR, Sigman CC. (1993, in press) Inhibition of chemical carcinogenesis. In Arcos J, Argus M, and Woo Y (eds): "Chemical Induction of Cancer: Modulation and Combination Effects." Boca Raton, FL: CRC.
37. Halliwell B, Gutteridge JMC. (1989) "Free Radicals in Biology and Medicine." Second Edition, Oxford: Clarendon Press.
38. Wattenberg LW. (1985) Chemoprevention of cancer. Cancer Res 45:1–8.
39. Sato K, Akaike T, Kojima Y, Ando M, Nagao M, Maeda H. (1992) Evidence of direct generation of oxygen free radicals from heterocyclic amines by NADPH/cytochrome P-450 reductase *in vitro*. Jpn J Cancer Res 83:1204–1209.
40. Hogg N, Darley-Usmar VM, Wilson MT, Moncada S. (1992) Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. Biochem J 281(Pt 2):419–24.
41. Radi R, Beckman JS, Bush KM, Freeman BA. (1991) Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys 288(2):481–487.
42. Marnett, LJ. (1992) Aspirin and the potential role of prostaglandins in colon cancer. Cancer Res 52:5575–5589.
43. Felder CC, Ma AL, Liotta LA, Kohn EC. (1991) The antiproliferative and antimetastatic compound L651582 inhibits muscarinic acetylcholine receptor-stimulated calcium influx and arachidonic acid release. J Pharmacol Exp Ther 257:967–971.