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N-Acetyl-*l*-Cysteine

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Abstract The most commonly used chemopreventive agents in the prevention of oral leukoplakia, head and neck cancer, and lung cancer are β-carotene, vitamin A, and other retinoids. One of the few chemopreventive agents not in this group and presently being used in a clinical trial is *N*-acetyl-*l*-cysteine (NAC). NAC, an antioxidant, is used in EUROSCAN, a European Organization of Research and Treatment of Cancer (EORTC) chemoprevention study in curatively treated patients with oral, laryngeal, or lung cancer. The rationale for choosing NAC is based on a variety of experimental data showing its ability to exert protective effects, including extracellular inhibition of mutagenic agents from exogenous and endogenous sources, inhibition of genotoxicity of reactive oxygen species, modulation of metabolism coordinated with blocking of reactive metabolites, protection of DNA and nuclear enzymes, and prevention of the formation of carcinogen–DNA adducts. NAC has also demonstrated an effect on mutagen-induced chromosomal sensitivity assays, and on anticarcinogenicity in experimental animal models. In addition, preliminary data from EUROSCAN show good compliance in treated patients and a low frequency of side effects.

Key words: chemoprevention, head and neck cancer, *N*-acetylcysteine, retinol, second primary tumors, lung cancer, oral cancer, EUROSCAN

The most commonly used chemopreventive agents in the prevention of oral leukoplakia, head and neck cancer, and lung cancer belong to a group of compounds which include β-carotene, vitamin A, and other retinoids. One of the few chemopreventive agents not in this group and which is presently being used in a clinical trial is *N*-acetyl-*l*-cysteine (NAC). NAC, an antioxidant and nucleophile, is being tested in EUROSCAN, a European Organization of Research and Treatment of Cancer (EORTC) chemoprevention study of curatively treated patients with oral, laryngeal, and lung cancer, which started in June, 1988 [1]. In a 2×2 factorial design, retinyl palmitate (300,000 IU) daily for one year and half this dose for the second year, or NAC (600 mg) for 2 years, or both drugs in combination, versus no drugs, are being studied as chemopreventive agents. The rationale for the choice of NAC, based on *in vitro* and animal studies, will be discussed, and preliminary data on EUROSCAN will be presented.

ROLE OF OXIDANTS IN PATHOLOGY

Molecules in biological systems that serve as acceptors of electrons are referred to as "oxidants" or "free radicals." Oxidants play a role in many normal biological functions such as phagocytosis, metabolism, and homeostasis. In recent years the pathogenic role of oxidant damage caused by free radicals has been demon-

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strated with increasing frequency in various experimental models. Oxidants and antioxidants are being extensively studied in respiratory medicine [acute respiratory distress syndrome (ARDS), acute and chronic bronchitis, emphysema, cystic fibrosis], heart disease (reperfusion injury after myocardial ischemia), and acute intoxications (paracetamol poisoning), as well as in oncology (radioprotection and chemoprevention), cancer-associated viral diseases (hepatitis B and C), and AIDS.

Normally, a balance between oxidants and antioxidants exists. In cases of increased oxidative stress and/or decreased antioxidative defense, oxidants can interact with and change normal biological components, causing tissue damage.

Oxidants of importance in humans are derived from three sources: oxidants generated by normal intracellular biological processes, but in excessive amounts, or in a milieu with insufficient defense mechanisms; oxidants released by inflammatory cells into their local environment; and oxidants secondary to foreign bodies, either directly or via an induced production of oxidants in the cell [2].

The mechanisms by which oxidants cause injury are complex. Because of their ability to accept electrons, oxidants can change the structure and/or function of the molecules involved in such reactions. The cell can thus be damaged via an interaction with structural components affecting crucial processes in the cell, such as the genetic system and various enzymatic processes. Oxidants can modify molecules in the extracellular milieu and cause changes in the tissue architecture, molecules important for extracellular defense mechanisms, and mediators involved in cell–cell interactions.

The most efficient antioxidants are the free radical scavenging agents and enzymes. They are present in the intra- and extracellular spaces and function by neutralizing oxidizing agents or by preventing their conversion to more toxic species. These antioxidant systems are represented by (1) enzymes, mainly intracellular, such as the cytoplasmic and mitochondrial forms of superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase; (2) lipid-soluble antioxidant compounds, such as vitamin E; and (3) water-soluble antioxidant compounds, such as vitamin C, macromolecules including ceruloplasmin and transferrin, and small molecules such as methionine, uric acid, and reduced GSH. The latter functions as an antioxidant regardless of its role as an enzymatic co-factor, and plays a fundamental role in protecting the cell against damage caused by hydroperoxides (and other peroxides) by converting from the reduced form (GSH) to the oxidized form (GSSG). This ability leads to the preventive strategy discussed below, *i.e.*, augmentation of the antioxidative protection mechanisms either by antagonizing the formation of free radicals or detoxifying the reactive species that have formed.

ANTIOXIDANTS: MODES OF ACTION

Antioxidant mechanisms are based on blocking processes involving free radicals. In the normal sequence of metabolic events, oxygen is able to accept a total of four electrons, as shown below:

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^- + 2H^+} H_2O_2 \xrightarrow{e^- + H^+} OH^+ \xrightarrow{e^- + H^+} H_2O$$

$$H_2O_2 \longrightarrow H_2O$$

The superoxide anion radical (O_2^-) is produced by the addition of one electron to molecular oxygen. The addition of two electrons leads to the formation of hydrogen peroxide (H_2O_2) which is not, by definition, a free radical, although it is a reactive intermediate. The addition of another electron to hydrogen peroxide forms the highly reactive hydroxyl radical (OH•). By acquiring an additional electron, the hydroxyl radical is converted into water.

Natural defense systems include both enzymatic and non-enzymatic mechanisms. The three most important enzymes involved in free radical scavenging are superoxide dismutase, catalase, and glutathione peroxidase. Superoxide dismutase accelerates the spontaneous dismutation of the superoxide anion to hydrogen peroxide. Catalase, a heme protein located mainly in peroxisomes, catalyzes the degradation of hydrogen peroxide to water and oxygen. Thiol (*i.e.*, SH) groups are essential for defense against reactive oxygen species. GSH, a tripeptide consisting of glutamic acid, cysteine, and glycine, is one of the most important intracellular defense systems. GSH, through the thiol group of cys-

teine, maintains a reducing environment and therefore protects the intracellular constituents from oxidative stress. GSH, as a substrate for the glutathione peroxidase enzyme, removes the oxygen free radicals formed inside the cell. The hydrogen peroxides are reduced to water and the lipid peroxides to their hydroxyl analogues by the transfer of hydrogen atoms which accompanies the formation of GSSG from two GSH molecules. In addition, thiol groups are important for the function of many proteins. To protect thiol groups in proteins, high concentrations of reduced GSH are necessary.

Other natural antioxidants include vitamins C and E, cysteine, and β-carotene. Vitamin C is a hydrophilic vitamin present in the cytosol and extracellular spaces. It is well-known that vitamin C is an antioxidant; less well-known is that vitamin C may act as a pro-oxidant as well. Although it has no oxidative properties itself, vitamin C in combination with Fe^{3+} or Fe^{2+} oxidizes polyunsaturated fatty acids. Vitamin E is lipophilic, located mainly in biological membranes, and is able to interrupt lipid peroxidation chain reactions. Cysteine is involved in various metabolic transformations such as incorporation into GSH, proteins, and Co-enzyme A. β-carotene is important not only as the precursor of vitamin A, but as a scavenger of free radicals as well.

NAC: INDICATIONS, EFFICACY, AND MECHANISM OF ACTION

NAC has been used for many years as a mucolytic drug in patients with acute and chronic bronchitis and as an antidote in paracetamol poisoning. Other pathologic conditions for which NAC is used or is being investigated at present are: acute intoxications (acrylonitrile, paraquat), prevention of hemorrhagic cystitis caused by cyclophosphamide and ifosfamide, lung fibrosis after radiotherapy, ARDS, reperfusion syndrome after myocardial ischemia, rheumatoid arthritis, amyotrophic lateral sclerosis, and AIDS.

The use of NAC as a possible chemopreventive agent is based on its antioxidative/detoxifying properties. In fact, most of the beneficial properties of prophylactically supplied NAC are attributed to its capacity to either reduce extracellular cystine to cysteine, or to be de-acetylated and available extracellularly as either cysteine or cystine. NAC antioxidant actions occur through one of two mechanisms. At intracellular levels, NAC is a precursor of GSH synthesis; NAC easily penetrates the cell where it is deacetylated to form *l*-cysteine, supporting the biosynthesis of GSH. The second mechanism is at the extracellular level where NAC acts directly on oxidant radicals as a nucleophile. NAC also enhances glutathione-*S*-transferase activity [3]. Although the exact mechanism is not understood, this is remarkable in light of the recent discovery of glutathione-*S*-transferase-π as a marker of oral cancer [4].

Evidence for the chemopreventive effect of NAC in animal models came initially from the finding that dietary NAC prevented the formation of urethane-induced lung tumors in mice [5]. Similar results have been found by others (see below).

The efficacy of NAC as a chemopreventive agent was also investigated at the NCI in two *in vitro* studies [6]. Cultured mouse mammary glands were treated with dimethylbenz(*a*)-anthracene (DMBA) and NAC. After 3 weeks the glands were evaluated for presence of hyperplastic alveolar nodules. Hyperplastic nodule formation was inhibited at NAC concentrations between 10^{-9} and 10^{-6} M. Both initiation and promotion stages were inhibited by NAC. In another study, cultured rat tracheal epithelial cells were treated with benzo(*a*)pyrene [B(*a*)P] and NAC. After 30 days the cultures were scored for the presence of transformed colonies. NAC inhibited the appearance of transformed cell colonies by 90–99% at concentrations of 0.3–1 g/ml and was considered to be positive for chemopreventive activity [6].

NAC was also studied in animal systems by NCI. It inhibited *N*-methyl-*N*-nitrosurea (MNU)-induced tracheal squamous cell carcinomas and diethylnitrosamine (DEN)-induced lung adenocarcinomas in the hamster, as well as MNU- and DMBA-induced mammary adenocarcinomas in the rat. NAC had a satisfactory preclinical toxicology profile and is now being tested in a Phase I clinical trial with the aim of establishing the safety and side effects of prolonged administration, studying the pharmacokinetics, and evaluating the biological effects of low dose NAC on plasma intracellular GSH and DNA repair in WBC [6].

Aminothiols, natural (*e.g.*, reduced GSH) and synthetic (NAC), provide typical examples of antimutagenic and anticarcinogenic agents that are known or presumed to work through multiple mechanisms. They are expected to give effective protection against a broad range of mutagenic and carcinogenic agents. In the following sections, the different effects of thiols are summarized.

Extracellular Inhibition of Mutagenic Agents From Exogenous and Endogenous Sources

Carcinogenic agents can be derived from exogenous or endogenous sources. The first barriers against carcinogens are encountered in the extracellular spaces (*e.g.*, gastrointestinal tract, respiratory tract, hematologic system) long before harmful compounds can reach target cells. Cytotoxicity, genotoxicity, and cancer initiation can be prevented either by inhibiting uptake or by facilitating removal of carcinogens, or by inhibiting endogenously formed mutagens.

Ascorbic acid, GSH, and NAC were equally effective in inhibiting the nitrosation products of famotidine and ranitidine, two antiulcer drugs [7]. The nucleophilic and reducing properties of NAC altered the mutagenicity of direct-acting compounds (doxorubicin, epichlorohydrin, sodium dichromate, hydrogen peroxide, 4-nitroquinoline-*N*-oxide, and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine) in the *Salmonella* reversion test without microsomal metabolism [8].

Inhibition of the Genotoxicity of Reactive Oxygen Species

The antioxidant properties of NAC were assessed in bacterial genotoxicity test systems. The interaction between NAC and H_2O_2, which involves the nonenzymatic reduction of the peroxide [8] accompanied by the formation of NAC disulfide, led to the loss of genotoxicity as evaluated by the *Salmonella* reversion test and by a DNA repair test which evaluated the differential lethality in *E. coli* WP2 (wild strain) and CM871 [9]. NAC produced a dose-dependent inhibition of spontaneous mutations in *Salmonella typhimurium* strain TA104. It also inhibited the mutagenicity induced by reactive oxygen species produced by the monoelectronic reductions which follow the oxidation of hypoxanthine in the presence of xanthine oxidase and molecular oxygen [10]. In addition, NAC prevented the genotoxicity of volatile oxygen species generated by the interaction of light with the chromophore rose bengal in repair-deficient strains of *E. coli* [11].

Modulation of Metabolism and Blocking of Reactive Metabolites

Trapping and detoxifying harmful substances in nontarget cells provides an important defense mechanism. These mechanisms prevent the carcinogen from reaching or reacting with the usual target cells [12]. Stimulation of this mechanism is particularly relevant when sequestration occurs in long-living cells (*e.g.*, erythrocytes) or sweeping cells (*e.g.*, alveolar macrophages). An example is the metabolic reduction and loss of mutagenicity of chromium(VI) in these cells. GSH pools are enhanced in rat erythrocytes *in vivo* following NAC administration [13] and provide the main mechanism for chromium(VI) detoxification [14]. NAC treatment of rats enhanced the ability of alveolar macrophages to reduce chromium(VI) *in vivo* [15].

Metabolic activation and detoxification processes are delicately balanced. At intermediate doses, NAC enhances the mutagenicity of promutagens such as B(*a*)P, 2-aminofluorene, cyclophosphamide, aflatoxin B_1, the pyrolysis product Trp-P-2, and cigarette smoke condensate, when assayed in the presence of liver S9 fractions from Aroclor-treated rats. Sublethal doses of NAC eliminated this mutagenicity, demonstrated by adding NAC after the metabolic activation of the promutagens [16]. These assays showed that NAC can not only stimulate metabolic activation of promutagens, but can also block their DNA effects by acting as a nucleophile.

Further evidence that NAC acts as a blocking agent was provided by the enhanced capacity of liver and lung preparations from NAC-treated rats to detoxify direct-acting mutagens [13].

Protection of DNA and Nuclear Enzymes

After DNA damage has occurred, it is still possible to exert protective effects by modulating DNA repair. GSH and NAC protect DNA from the damaging effects of X-rays and

2-acetylaminofluorene (2AAF) *in vitro* [17]. In addition, GSH and NAC exerted protective effects on DNA in various hepatocarcinogenesis models [17].

Inhibition of Carcinogen–DNA Adduct Formation and Clastogenic Effects

Detection of carcinogen–DNA adducts provides a useful method for assessing exposure to carcinogens at the molecular level. Prevention of such events is an important indicator of antigenotoxic activity. The dietary administration of 2AAF produces DNA adducts in the livers of Wistar rats. Co-administration of NAC results in a decrease in the number of DNA adducts formed [18]. Synchronous fluorescence spectrophotometry was used to detect B(*a*)P–DNA adducts in lung and liver. Pretreatment with NAC by gavage decreased adduct formation in the lung; no adducts were detected in the liver [19]. In the same study, NAC completely prevented clastogenic effects (induction of micronuclei) induced by B(*a*)P in pulmonary alveolar macrophages [19]. NAC protected against a variety of whole-body effects due to mainstream cigarette smoke, including histopathological damage in alveolar, bronchial, and bronchiolar mucosae [20]; cytological and cytogenetic changes in bronchoalveolar lavage cells [20]; toxic effects on bone marrow erythrocytes [20]; biochemical changes in lung and liver [21]; and B(*a*)P–DNA adducts in lung and heart [22].

Effects of NAC and Ascorbic Acid on Mutagen-induced Chromosomal Damage in Patients With Head and Neck Cancer

Trizna *et al.* [23] studied the *in vitro* protective effects of NAC and ascorbic acid on mutagen-induced chromosomal breakage using human lymphoblastoid cell lines, as well as freshly cultured lymphocytes from patients with head and neck cancer and healthy controls. Both drugs proved effective in diminishing mutagen-induced chromatid breakage in both established lymphocyte cell lines and freshly cultured lymphocytes. NAC and ascorbic acid decreased the number of breaks per cell up to 73% and 58%, respectively.

Anticarcinogenicity in Experimental Animal Models

The protective effects of NAC have been described in various rodent carcinogenicity models (Table I). NAC decreased the induction of lung tumors in Swiss albino mice when added to the diet from 15 days before to 4 months after an intraperitoneal injection of the carcinogen urethane [5]. NAC also prevented the formation of skin papillomas in mice by DMBA + TPA [24]. In Wistar rats treated with 2AAF according to the Teebor and Becker protocol [25], GSH and NAC added to the diet delayed the development of γ-glutamyl transpeptidase-positive foci in the liver and prevented 2AAF-induced sebaceous

TABLE I. Protective Effects of NAC in Animal Studies

Animal	Tumor	Substance	Ref.
mouse	lung adenoma	urethane	[5]
mouse	skin papilloma	7,12-dimethylbenz(*a*)anthracene + TPA	[19]
rat	intestinal tumors	1,2-dimethylhydrazine	[27]
rat	Zymbal gland carcinomas, liver preneoplastic foci (GGT+)	2-acetylaminofluorene	[26]
rat	mammary adenocarcinomas	N-methyl-*N*-nitrosurea	[28]
hamster	tracheal squamous cell carcinomas	*N*-methyl-*N*-nitrosurea	[28]

squamous cell carcinomas of Zymbal glands [26]. NAC exerted protective effects in a rat colon carcinogenesis model using 1,2-dimethylhydrazine as well [27].

At the NCI, NAC was tested in two animal model systems [28]. MNU was instilled intratracheally and NAC was administered in the diet of male Syrian golden hamsters. Tracheal tumors were assessed after 6 months. In groups receiving 6400 mg/kg NAC from 1 week following carcinogen treatment to the end of the experiment, carcinoma incidence was significantly decreased from 58.6% to 24%. In the second experiment, virgin female Sprague-Dawley rats were treated with MNU (iv) and were fed either 8000 or 4000 mg/kg NAC for 180 days. The tumor multiplicity was reduced from 12.1 tumors/rat in positive controls to 8.6 tumors/rat in animals treated with 8000 mg/kg NAC during MNU treatment, to 9.25 tumors/rat in animals fed 8000 mg/kg after MNU treatment, and to 8.9 tumors/rat in animals fed 8000 mg/kg during the whole period. In animals fed 4000 mg/kg, the tumor multiplicity was 10.7 tumors/rat. The results are awaiting statistical analysis [28].

CHEMOPREVENTION WITH NAC: EUROSCAN

Oral leukoplakia and curatively treated early stage head and neck cancer patients form an ideal population in which to test chemopreventive medication. Fourteen studies using β-carotene, vitamin A, and other retinoids in oral leukoplakia have been reported; however, NAC was not tested. The feasibility of administering NAC as a lozenge for oral leukoplakia is presently being evaluated.

Several clinical chemoprevention trials are presently being carried out with β-carotene, vitamin A, other retinoids, and NAC in patients cured of early stage head and neck and lung cancer. EUROSCAN is by far the largest chemoprevention study for early stage oral, laryngeal, and lung cancer [1]. Retinyl palmitate and NAC are being tested in EUROSCAN. Retinyl palmitate has been used for many years for skin diseases, and the daily dose of 300,000 IU is based on this experience and on the efficacy of this dose in the treatment of oral leukoplakia. This dose yields acceptable side effects, with response rates comparable to those of higher doses.

NAC, as discussed above, is believed to act in early stages of carcinogenesis, preceding and possibly shortly after the occurrence of DNA damage. Vitamin A acts later, in the promotion and progression phases. Thus, the combination theoretically covers nearly the entire carcinogenic process, with no expected interaction with regard to side effects.

The endpoints of EUROSCAN are the number and time of occurrence of second tumors, local/regional recurrences, and distant metastases. In addition, long-term survival rates will be evaluated. The study started on June 1, 1988. The following data were available as of January 1, 1993. Sixty-nine cancer centers from 14 European countries are accruing patients (Netherlands, Italy, Germany, Spain, Croatia, Slovenia, Belgium, Czechoslovakia, Turkey, France, Poland, Portugal, Hungary, Great Britain, and Norway). Of the 2000 patients planned, 1821 had entered the study. On average, 40–50 new patients are entered per month. The accrual of the last patients will end in 1993. Thirty-nine percent of the patients had curatively treated lung cancer; 61%, head and neck cancer; 70%, laryngeal cancer; and 30%, oral cancer.

As of January 1, 1993, there was no evidence of new disease in 1224 out of 1472 patients with sufficient follow-up in the fourth arm (no drug intervention) of the trial. There were local/regional recurrences in 121 patients, distant metastases in 72 patients, local and distant metastases in 12 patients, and second primaries in 43 patients.

Side Effects and Toxicity in EUROSCAN

The following report of side effects is preliminary, since the majority of patients have not reached the end of the intervention period. At each follow-up visit a questionnaire is filled in regarding such side effects as dryness, desquamation, itching, headache, dyspepsia, nosebleeds, hair loss, or others. All side effects, even those mentioned only once, are fed into a computer. Side effects were noted in this manner for 1472 patients with sufficient follow-up as of January 1, 1993, for three out of the four treatment arms [(1) vitamin A and NAC (n = 364); (2) vitamin A (n = 370); (3) NAC (n = 372);

and (4) no drugs (n = 371)] as follows:
No side effects were found in 54, 57, and 79% for treatment arms 1, 2, and 3, respectively. **Detectable yet well-tolerated side effects** were found in 29, 27, and 14%, respectively. **Poorly tolerated side effects** were noted in 7, 6, and 3%, respectively. **Unbearable side effects** were noted in 10, 9, and 3%, respectively. The most common side effects were mucocutaneous complaints, headache, and dyspepsia. It can be concluded from this intermediate analysis of side effects and toxicity that the single *drugs, as well* as the combination treatment, are well-tolerated and that the toxicity is mild as compared with 13-*cis*-retinoic acid (50–100 mg/kg) as reported by Hong *et al.* [29]. NAC is even better tolerated than vitamin A and is devoid of cumulative side effects when taken in combination with vitamin A.

CONCLUSIONS

Based on *in vitro* and animal studies, NAC appears to be an interesting and promising chemopreventive agent. It is one of the few chemopreventive agents not in the group of β-carotene, retinol, or retinoids currently being used in a trial. NAC is being tested in Europe in the successful EUROSCAN study. NAC and retinyl palmitate, alone and in combination, are being tested in a 2×2 factorial design. The accrual of EUROSCAN as of January 1, 1993, was 1821 patients and the total accrual is expected to end this year. NAC is generally regarded as safe and without major side effects. Preliminary analysis in EUROSCAN confirms that NAC is tolerated well in the great majority of patients. If positive results are obtained from EUROSCAN, this chemopreventive agent may be applied on a more routine basis in these extremely high-risk patients, alone or combinations with other agents.

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