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Chemopreventive Properties and Mechanisms of *N*-Acetylcysteine. The Experimental Background

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Abstract The thiol *N*-acetylcysteine (NAC), now under clinical trial for cancer chemoprevention both in Europe (project Euroscan) and in the US (National Cancer Institute), has been shown during the past decade to exert protective effects in a variety of experimental test systems. NAC inhibited spontaneous mutagenicity and that induced by a number of chemical compounds and complex mixtures. Moreover, NAC significantly decreased the incidence of neoplastic and preneoplastic lesions induced by several chemical carcinogens in rodents (mice, rats, hamsters), *e.g.*, in lung, trachea, colon, liver, mammary gland, Zymbal gland, bladder and skin. Our studies provided evidence that multiple mechanisms contribute to NAC antimutagenicity and anticarcinogenicity. They include extracellular mechanisms, such as detoxification of reactive compounds due to the nucleophilic and antioxidant properties of NAC, inhibition of nitrosation products, and enhancement of thiol concentration in intestinal bacteria; trapping and enhanced detoxification of carcinogens in long-lived non-target cells, such as erythrocytes and bronchoalveolar lavage cells; mechanisms working in the cytoplasm of target cells, such as replenishment of GSH stores, modulation of metabolism of mutagens/carcinogens, blocking of electrophiles, and scavenging of reactive oxygen species; and nuclear effects, such as inhibition of DNA adduction by metabolites of carcinogens, inhibition of "spontaneous" mutations, attenuation of carcinogen-induced DNA damage, and protection of nuclear enzymes, such as poly(ADP-ribose) polymerase. In particular, benzo(*a*)pyrene diolepoxide-DNA adducts in rats exposed either to benzo(*a*)pyrene or cigarette smoke were prevented by NAC not only in target organs for carcinogenicity, such as lung and trachea, but also in other organs, such as heart, aorta and testis, where these molecular biomarkers have been tentatively associated with cardiomyopathies, atherosclerosis and hereditary diseases, respectively. The protective mechanisms of NAC are expected to affect not only initiation but also promotion and progression, due to the reiterate involvement of certain key mechanisms in carcinogenesis. Moreover, recent studies demonstrate that NAC can also affect the steps of invasion and metastasis, including the specific inhibition of type IV collagenases degrading basement membranes, inhibition of chemotactic and invasive activities of human and murine malignant cells, delay of primary tumor formation in mice, and inhibition of lung metastases. Evidence was also provided that administration of pharmacological doses of NAC sharply decreases urinary excretion of mutagens in smokers.

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The thiol *N*-acetylcysteine (NAC), an analogue and precursor of reduced glutathione (GSH), has become one of the most promising cancer chemopreventive agents [1], and is currently under clinical trial at both the National Cancer Institute (Chemoprevention Branch) and in Europe (Project Euroscan) [2]. This drug's lack of toxicity is supported by pharmacological experience accumulated for more than 20 years; its use in humans relies on a solid experimental background. In fact, NAC has exerted protective effects against a variety of chemical mutagens [reviewed in 3–5] as well as against induction of preneoplastic and neoplastic lesions in rodents. These include lung adenomas, transitional cell bladder carcinoma and skin papillomas in mice, intestinal tumors, colon aberrant crypt foci, colon carcinomas, Zymbal gland squamocellular carcinomas, liver gamma-glutamyltranspeptidase-positive foci and mammary adenocarcinomas in rats, and tracheal squamocellular carcinomas in hamsters [reviewed in 5].

Chemopreventive agents appear to function through a variety of mechanisms which have been classified in detail [5–8]. We report here a synthetic overview of the effects and mechanisms of NAC investigated in our laboratories, outlined in Figure 1. Moreover, preliminary data concerning protective effects produced by NAC towards intermediate biomarkers in humans are also presented.

EXTRACELLULAR EFFECTS AND GENERAL MECHANISMS

NAC decreases the direct mutagenicity of a variety of chemical compounds in the *Salmonella* reversion test. This effect can be mainly ascribed to the nucleophilic and antioxidant properties of this aminothiol. Test mutagens included captan, folpet, β-propiolactone, hydralazine, quercetin, ICR 191, sodium nitrite, 4-nitroquinoline-1-oxide (4NQO), epichlorohydrin, sodium dichromate, formaldehyde, glutaraldehyde, doxorubycin and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [4,5,9,10]. An experimental database on inhibition of 4NQO mutagenicity was prepared by assaying 90 compounds of various chemical classes. At variance with 17 sulfur compounds lacking a free sulfhydryl group, all thiols and aminothiols, including NAC, inhibited 4NQO mutagenicity [11]. These data were also subjected to structure-activity relationship (SAR) analysis by means of CASE methodology [12]. Activity profiles concerning NAC antimutagenicity have been also prepared [13].

Both NAC and GSH effectively inhibited the mutagenicity of the nitrosation products formed in an acidic environment of the antiulcer drugs ranitidine and famotidine [14]. Moreover, there is indirect evidence that NAC and GSH can enhance thiol concentrations in enterobacteria [10], which may represent an important protective factor in the intestinal lumen.

The oral administration of NAC to rats exposed whole-body to mainstream cigarette smoke prevented massive inflammatory, multiple hyperplastic and metaplastic changes of bronchiolar and bronchial mucosae, and emphysema [15].

TRAPPING AND ENHANCED DETOXIFICATION IN NON-TARGET CELLS

Stimulation of this mechanism is particularly relevant in long-lived cells for transporting or sweeping away carcinogens. Examples are provided by erythrocytes, in which the *in vivo* administration of NAC can enhance GSH stores [16], thereby favoring sequestration of hemoglobin-bound carcinogens and bronchoalveolar lavage cells [17]. Studies in rodents provided evidence that NAC can exert a variety of protective effects in pulmonary alveolar macrophages (PAM, see Fig. 1), which play a crucial role in the defense of terminal airways.

CYTOPLASMIC EFFECTS AND MECHANISMS

We are currently investigating whether NAC may have some influence on the multidrug resistance factor, a cellular mechanism for extruding xenobiotics. Once entered into cells, NAC is deacetylated to yield cysteine, the rate-limiting

I. EXTRACELLULAR EFFECTS AND GENERAL MECHANISMS

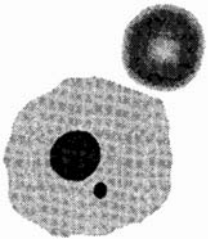
Detoxification of direct-acting mutagens due to the nucleophilic and antioxidant properties of NAC [3-5,7,8]

Inhibition of the mutagenic nitrosation products of aminocompounds (ranitidine, famotidine) formed in acidic environment [14]

Enhancement of thiol concentrations in intestinal bacteria [10]

Inhibition of inflammatory, hyperplastic and metaplastic changes in the respiratory epithelium of smoke-exposed rats [30]

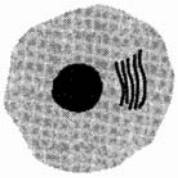
II. TRAPPING AND DETOXIFICATION IN NONTARGET CELLS



Erythrocytes: Enhancement and replenishment of protective GSH stores [15]

Bronchoalveolar lavage (BAL) cells: Normalization of BAL cellularity altered by B(*a*)P [27] and cigarette smoke [30]; Stimulation of detoxifying enzymes (GSH *S*-transferase, diaphorases, etc.) in PAM [17]; enhanced detoxification of direct-acting mutagens in PAM [17]; protection of PAM against cytogenetic damage (micronuclei) induced by B(*a*)P [27] and cigarette smoke [30]

III. CYTOPLASMIC EFFECTS AND MECHANISMS



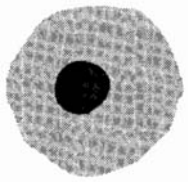
Replenisment of GSH stores [15]

Modulation of procarcinogen metabolism (enhanced formation of reactive metabolites coordinated with their detoxification) [8,15,18,19,25,31]

Blocking of electrophilic compounds [3-5,8,11,12,33]

Scavenging of reactive oxygen species [3,20,21]

IV. NUCLEAR EFFECTS AND MECHANISMS



Inhibition of spontaneous mutations related to DNA repair background [22]

Protection of nuclear enzymes (pADPRP) towards the damage induced by carcinogens [3,23,24]

Enhanced repair of DNA damaged by carcinogens [34]

Inhibition of the formation of DNA adducts by metabolites of 2AAF, BP and cigarette smoke in several organs [3,25-27,32,35]

V. INHIBITION OF MALIGNANT CELL INVASION AND METASTASIS

Inhibition of type IV collagenases (MMP-2 and MMP-9) [38]

In vitro inhibition of chemotaxis and invasion of malignant cells [38]

Inhibition of lung metastases following i.v. injection of NAC-pretreated murine melanoma cells [38]

Delay by oral NAC of primary tumor formation in mice injected with melanoma or lung carcinoma cells, and decrease of lung metastases of melanoma cells [38]

Fig. 1. Outline of the effects and mechanisms investigated in our laboratory, responsible for the prevention of mutation and cancer by *N*-acetylcysteine (NAC) in experimental test systems. Abbreviations: B(*a*)P, benzo(*a*)pyrene; PAM, pulmonary alveolar macrophages.

amino acid in intracellular GSH synthesis. In fact, NAC replenishes GSH stores in cells [16], which is particularly useful when this tripeptide is depleted by toxic agents or cancer-associated viral diseases, such as hepatitis B and AIDS [see 3 and the references therein]. NAC modulated liver-mediated mutagenicity of procarcinogens such as doxorubycin, 2-aminofluorene, 2-aminoanthracene, aflatoxin B_1, cyclophosphamide, the food pyrolysis product Trp-P-2, benzo(*a*)pyrene, cigarette smoke condensate [5,9,16], and mainstream cigarette smoke [11]. Our hypothesis is that thiols may enhance the metabolic activation of procarcinogens, which is, however, coordinated with blocking and detoxification of reactive metabolites, thereby avoiding the accumulation of unmetabolized precursors in the organism.

Rodent studies indicate that NAC can stimulate cytosolic enzyme activities involved in the hexose monophosphate shunt and in the GSH cycle [16,18–20]. Due to its nucleophilicity, NAC and its intracellular derivatives can block electrophilic metabolites. NAC's efficiency at scavenging reactive oxygen species was shown in two different genotoxicity test systems in bacteria. In one, superoxide anion and hydrogen peroxide obtained by the reaction of hypoxanthine with xanthine oxidase and superoxide dismutase were positive in strain TA104 of *S. typhimurium*. Interestingly, NAC, GSH and α-mercaptopropionylglycine also inhibited the spontaneous mutagenicity in TA104 cells [21]. In the second study, NAC inhibited the genotoxicity induced in DNA repair-deficient *E. coli* strains by volatile oxygen species produced by illumination of rose bengal in the presence of molecular oxygen [22]. Moreover, NAC inhibited the mutagenicities of hydrogen peroxide, tested as a laboratory reagent [9], and cumene hydroperoxide [5].

NUCLEAR EFFECTS AND MECHANISMS

Some mechanisms occurring in the cytoplasm are also expected to work in the nucleus. This is particularly true for blocking electrophilic metabolites and scavenging reactive oxygen species.

A study on inhibition of "spontaneous" mutagenicity in *S. typhimurium* strains TA102 and TA104 suggested that the DNA repair background plays a prominent role in determining the genetic instability of these cells. In particular, NAC and other thiols were effective in decreasing the "spontaneous" mutagenicity in strain TA104 [23].

NAC and other thiols, including GSH, α-mercaptopropionylglycine, β-mercaptoethanol and dithiothreitol, protected the DNA of rat hepatocytes *in vitro* from the damaging activity of X-rays and 2-acetylaminofluorene (2AAF) [24]. Moreover, both GSH and NAC exerted protective effects on DNA, as assessed by the alkaline elution technique, in various hepatocarcinogenesis models [24]. NAC also counteracted the depletion produced *in vivo* by 2AAF of the chromatin-bound enzyme poly(ADP-ribose) polymerase (pADPRP), demonstrated by the activity gel technique [25]. Western blot analyses suggested that the loss of enzyme activity was associated with disappearance of the protein band, while Northern blot analyses ruled out any effect on pADPRP mRNA [3,24,25].

Several studies were carried out to evaluate NAC's ability to inhibit the *in vivo* formation of carcinogen-DNA adducts. Using the ^{32}P-postlabeling assay with nuclease P1 enrichment, 3G-N_2-AAF DNA adducts were detected in the liver of Wistar rats treated with 2AAF, supplemented in the diet (0.05%) for 3 weeks [26]. In addition, dG-C_8-AF and other unidentified adducts were more recently detected following butanol enrichment. Co-administration of dietary NAC (0.1%) resulted in a marked decrease of all DNA adducts [27].

Intratracheal instillation of benzo(*a*)pyrene (25 mg/kg body weight) in Sprague-Dawley rats for three consecutive days resulted in a 5-fold increase of micronuclei in PAM and in the appearance of benzo(*a*)pyrene diolepoxide (BPDE)-DNA adducts in both liver and lungs [28]. These adducts were detected by synchronous fluorescence spectrophotometry (SFS), a technique which in humans sharply discriminated the exposure of alveolar macrophages in smokers and nonsmokers [29]. All cytogenetic and molecular alterations induced by benzo(*a*)pyrene were significantly inhibited when rats were pretreated by gavage with NAC 5 h before each intratracheal instillation of the carcinogen [28]. This study was repeated once more, and SFS-positive DNA adducts were detected not only in liver and lung, but also in heart and, in three out of five ani-

mals, in testes. Administration of NAC resulted in a significant inhibition of adduct formation in all organs [27]. It is noteworthy that treating the same rat strain with NAC enhanced GSH levels in testes and epididymis, and inhibited chemically induced dominant lethal mutations [30].

Extensive analyses were carried out in groups of Sprague-Dawley rats exposed whole-body to mainstream cigarette smoke (9–12 cigarettes per day) for up to 40 consecutive days. Such treatment produced severe histopathological damage in the lung as well as cytogenetic alterations in alveolar macrophages, which were prevented by NAC administered by gavage [15]. Changes in pulmonary and hepatic biochemical parameters and in the liver metabolism of several promutagens were also induced by cigarette smoke and modulated by NAC [31]. In addition, SFS-positive DNA adducts detected in heart and lungs of smoke-exposed rats were significantly inhibited by NAC [32]. Further assays demonstrated the formation of these adducts not only in lungs and heart, but also in aorta. No adduct was conversely detected by SFS in liver, brain, or testes. The presence of carcinogen-DNA adducts in the aorta [27] as well as in the trachea [33], nasal mucosa and testis (A. Izzotti, S. De Flora, F. D'Agostini, C.F. Cesarone, in preparation) of rats exposed to cigarette smoke was also shown by ^{32}P-postlabeling. Again, NAC significantly inhibited the formation of adducts in all positive organs.

The selective localization of DNA adducts in different organs depends on several factors, including toxicokinetics, local and distant metabolism, efficiency and fidelity of DNA repair, and cell proliferation rate. For instance, DNA alterations are expected to accumulate in the heart, due to the fact that the poor local metabolism is compensated by "first-pass" effects, low DNA repair, and no cell proliferation [32]. Our working hypothesis is that, while DNA adducts in the lung and trachea may be associated with lung carcinogenesis, those in the heart may be associated with smoke-related cardiomyopathies, and those in aorta with at least a portion of atherosclerotic plaques. An extensive research program on human arteriosclerosis has been implemented in order to explore this hypothesis [34,35]. In any case, it is of particular interest that a single chemopreventive agent, such as NAC, is capable of inhibiting DNA adducts in different organs, where they possibly bear a distinctive pathogenetic meaning.

INHIBITION OF MALIGNANT CELL INVASION AND METASTASIS

The effects and mechanisms so far reported indicate that NAC exerts protective effects towards genotoxic effects and cancer initiation. Due to the reiterated involvement of such key mechanisms as genotoxic effects and oxidative stress throughout the carcinogenesis process, the multiple protective mechanisms of NAC are also expected to encompass the steps of tumor promotion and progression.

Recently, we carried out an extensive study [36] involving a number of experiments in *in vitro* and *in vivo* test systems aimed at assessing whether NAC may also influence invasion and metastasis of malignant cells. Several lines of evidence supported a protective function of NAC in these advanced stages. First of all, NAC inhibited the gelatinolytic activity of K1735-M2 and B16-F10 murine melanoma cells and of C87 Lewis lung carcinoma cells. Both gelatinases A (MMP2, 72 kDa, type IV collagenase) and B (MMP9, 92 kDa, type IV collagenase), known to play a crucial role in degrading the structural collagen of basement membranes (collagen IV), were completely inhibited by NAC, as assessed by zymographic analysis. Moreover, NAC was quite efficient in inhibiting the chemotactic and invasive activities of these cells, as well as of K1735-M4 and human A2058 cells, in Boyden Chamber assays. When B16-F10 cells were pretreated with NAC and injected intravenously in nude mice to bypass formation of the primary tumor, the number of lung metastases decreased to 17.4% of controls. The protective effect was even more dramatic (5.6% of controls) when NAC-pretreated cells were resuspended in medium supplemented with the same thiol. Several experiments were carried out in C57BL/6 mice receiving subcutaneous injections of melanoma cells (B16-F10 or B16-BL6) or intramuscular injections of Lewis lung carcinoma cells. In all cases the oral administration of NAC (0.025-4 g/kg body weight) produced a dose-related delay in the local formation of primary tumors. Moreover, the number of lung metastases significantly decreased in experiments with melanoma cells, but

not in those with Lewis lung carcinoma cells [36].

MODULATION OF INTERMEDIATE BIOMARKERS IN HUMANS BY NAC

As discussed in the previous sections, NAC has been extensively used in studies assessing intermediate biomarkers in animal models. We are now investigating cancer biomarkers in humans. An extremely sensitive biomarker of exposure is evaluating the elimination of mutagens with excreta, such as urine. Following suitable concentration and purification, analysis of several samples showed that the excretion of promutagens in smokers' urine is revealed with outstanding sensitivity by strain YG1024, an *O*-acetyltransferase-overproducing derivative of *S. typhimurium* TA98 [37]. As shown in Figure 2, preliminary analyses provide convincing evidence that urinary mutagenicity is considerably decreased in smokers receiving pharmacological doses of NAC. A similar decrease in urine mutagenicity was observed in two additional smokers. As suggested by other experiments in volunteers, the effect of NAC appears to be rapid and

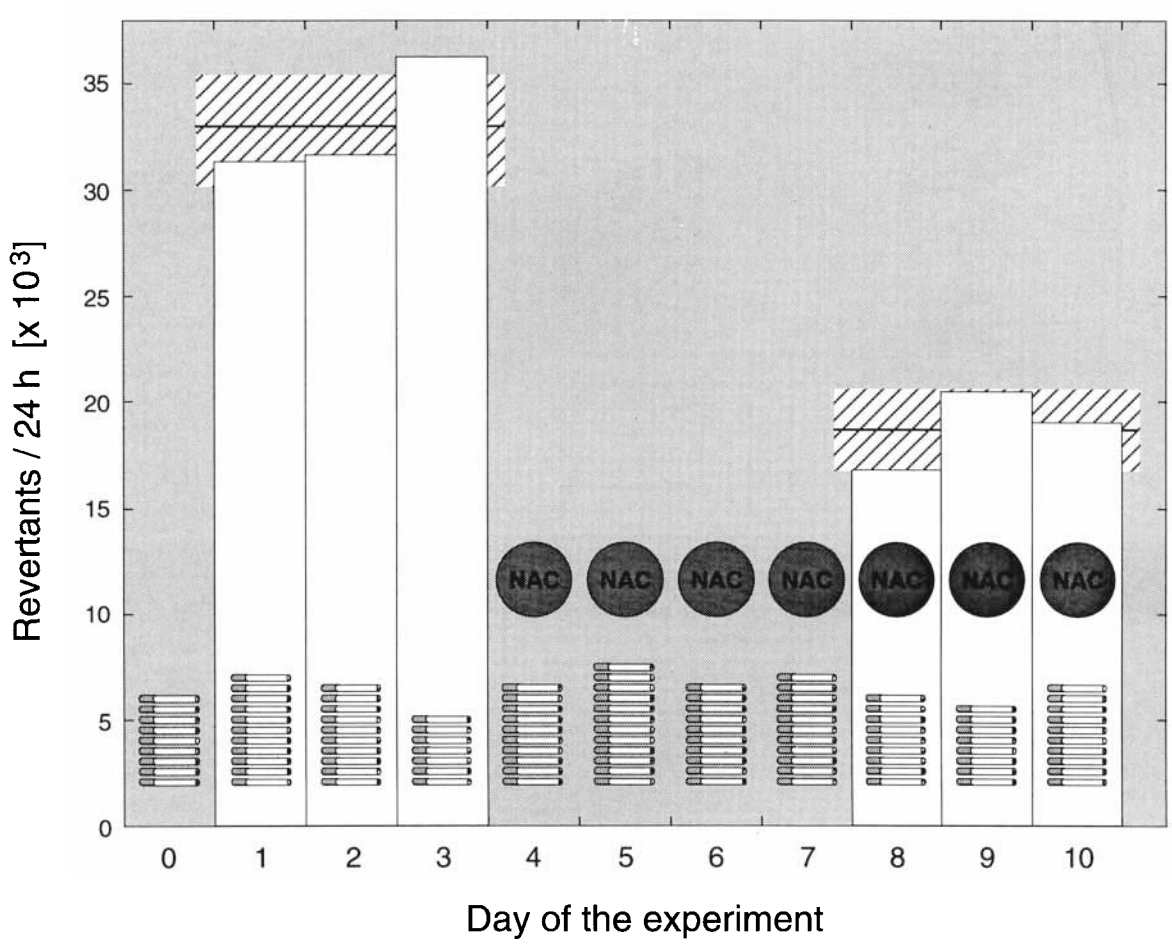


Fig. 2. Decrease of the daily excretion of mutagens with urine in a smoker. The number of cigarettes smoked per day is reported. NAC was given in the form of a water-soluble commercial preparation containing 200 mg of the drug (Fluimicil®, Zambon Group, Verona, Italy). A total of 800 mg was administered per day, 400 mg at wake up time and 400 mg at noon, on the days indicated. Mutagenicity of 24-h urine samples collected on days 1–3 and 8–10 of the experiment was evaluated in strain YG1024 of *S. typhimurium*, in the presence of S9 mix, following concentration of urine by XAD-2 resin columns. The horizontal lines and the slanting dashed areas refer to the means ± SD values calculated within each triplet of specimens (S. De Flora and A. Camoirano, unpublished data).

reversible, being evident from the first day of administration of the drug and disappearing the first day after treatment withdrawal (S. De Flora and A. Camoirano, study in progress).

As previously reported, a variety of biomarkers were affected by the oral administration of NAC in rats exposed to cigarette smoke, including protection of the respiratory epithelium from massive histopathological damage, maintenance of broncheoalveolar lavage (BAL) cellularity, inhibition of clastogenic damage in PAM, modulation of metabolism, and formation of carcinogen-DNA adducts in several organs [15,27,29,31–33]. Therefore, in spite of some uncertainties regarding the interpretation of urinary mutagenicity data, it is most likely that the marked decrease of urine mutagenicity observed in smokers treated with NAC reflects the interception and detoxification by this drug of smoke-associated mutagens in the organism.

CONCLUSIONS

Experimental data summarized in the present review provide sound evidence that the aminothiol NAC exerts a broad array of protective effects and displays a variety of antimutagenesis and anticarcinogenesis mechanisms in experimental test systems. Moreover, preliminary indications in humans indicate that even pharmacological doses of NAC have striking effects on the urinary excretion of mutagens in cigarette smokers.

NAC appears to possess all four requirements [5,7] necessary for a cancer chemopreventive agent to be used in humans: low cost; practicality of use (oral administration); efficacy, as documented by experimental data and by the evaluation of biomarkers in humans; and tolerability and very low toxicity, well established in 30 years of clinical use.

It is premature to suggest the use of any drug for public health intervention addressed towards cancer prevention. However, NAC is already prescribed to patients suffering from respiratory diseases, who for a variety of reasons may be at risk of developing lung cancer. Moreover, administration of a drug like NAC may be particularly useful in cases of GSH depletion which, as indicated by our studies, may also occur in cancer-associated viral infections [reviewed in 3]. Therefore, at present NAC may be tentatively recommended for target chemoprevention in high-risk individuals. The preliminary data obtained in cigarette smokers are quite impressive in this respect.

ACKNOWLEDGEMENTS

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