Mechanisms of N-acetylcysteine in the prevention of DNA damage and cancer, with special reference to smoking-related end-points

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Although smoking cessation is the primary goal for the control of cancer and other smoking-related diseases, chemoprevention provides a complementary approach applicable to high risk individuals such as current smokers and ex-smokers. The thiol N-acetylcysteine (NAC) works per se in the extracellular environment, and is a precursor of intracellular cysteine and glutathione (GSH). Almost 40 years of experience in the prophylaxis and therapy of a variety of clinical conditions, mostly involving GSH depletion and alterations of the redox status, have established the safety of this drug, even at very high doses and for long-term treatments. A number of studies performed since 1984 have indicated that NAC has the potential to prevent cancer and other mutation-related diseases. N-Acetylcysteine has an impressive array of mechanisms and protective effects towards DNA damage and carcinogenesis, which are related to its nucleophilicity, antioxidant activity, modulation of metabolism, effects in mitochondria, decrease of the biologically effective dose of carcinogens, modulation of DNA repair, inhibition of genotoxicity and cell transformation, modulation of gene expression and signal transduction pathways, regulation of cell survival and apoptosis, anti-inflammatory activity, anti-angiogenic activity, immunological effects, inhibition of progression to malignancy, influence on cell cycle progression, inhibition of pre-neoplastic and neoplastic lesions, inhibition of invasion and metastasis, and protection towards adverse effects of other chemopreventive agents or chemotherapeutical agents. These mechanisms are herein reviewed and commented on with special reference to smoking-related end-points, as evaluated in in vitro test systems, experimental animals and clinical trials. It is important that all protective effects of NAC were observed under a range of conditions produced by a variety of treatments or imbalances of homeostasis. However, our recent data show that, at least in mouse lung, under physiological conditions NAC does not alter per se the expression of multiple genes detected by cDNA array technology. On the whole, there is overwhelming evidence that NAC has the ability to modulate a variety of DNA damage- and cancer-related end-points.

Introduction

The primary goal in the prevention of cancer and other mutation-related diseases is the avoidance of exposure to recognized risk factors. Strengthening of the host defence mechanisms provides a complementary preventive approach, which is particularly important when targeted to high risk individuals. This strategy, referred to as chemoprevention, has found broad applications for the control of risk factors in cardiovascular diseases, and deserves greater emphasis in the prevention of cancer (1,2). The intake of protective factors can be achieved by means of both dietary measures and pharmacological agents.

Reduced glutathione (GSH) plays a central physiological role in maintaining the body homeostasis and in protecting cells against oxidants, toxicants, DNA-damaging agents and carcinogens of either exogenous or endogenous source. Unfortunately, the large GSH molecule is not transported efficiently into cells. Furthermore, L-cysteine, which is the rate-limiting amino acid in the intracellular synthesis of this tripeptide (γ-glutamyl-L-cysteinyl glycine) is toxic to humans (3). The thiol N-acetyloy-L-cysteine (NAC) is readily deacetylated in cells to yield L-cysteine thereby promoting intracellular GSH synthesis (4). Besides this activity as a GSH precursor, NAC is, per se, responsible for protective effects in the extracellular environment, mainly due to its nucleophilic and antioxidant properties, which influence the toxicokinetics of xenobiotics (4). N-Acetylcysteine, introduced as a mucolytic agent in the 1960s, has found very extensive clinical applications in the therapy and prophylaxis of respiratory diseases including, for example, acute bronchitis, chronic bronchitis and its acute exacerbations, acute respiratory distress syndrome and influenza-like syndromes (5,6). Moreover, NAC use has been proposed for the treatment of a variety of diseases sharing alterations of the redox status and GSH depletion as common pathogenetic determinants (7). These applications also include use of NAC as an antidote towards acute intoxications caused by paracetamol overdosage and by a variety of poisons as well as towards the toxicity of anticancer drugs such as doxorubicin, cyclophosphamide and ifosfamide (4).

Since 1984 (8), a number of studies provided evidence that...
Effects on cell survival, apoptosis and tissue inflammation

Protection towards cytotoxic effects (see references in the relevant text section)
Modulation of apoptosis (see references in the relevant text sections)
Prevention of apoptosis-dependent alopecia induced by cigarette smoke (68)
Anti-inflammatory activity (69) due to inhibition of COX-2 (28,29) and of cytokine production and secretion (70,71)
Normalization of smoke-altered bronchoalveolar lavage cellularity (72)

Inhibition of tumour promotion and progression

Inhibition of H$_2$O$_2$-induced phosphorylation of connexin 43 and disruption of gap junctional intercellular communications (73)
Protection towards smoke-induced histopathological and functional alterations in animal models (74–78)
Inhibition of vascular endothelial growth factor (VEGF) production (79,80)
Anti-angiogenic activity (81)
Enhancement of the immune response of peripheral blood T cells (82)
Improvement of immune functions in asymptomatic HIV-infected subjects (83)
Enhancement of the immunogenic potential of tumour cells (84) and of natural killer (NK) activity of mononuclear cells (85)
Inhibition of progression to malignancy of lung tumours in mice (86)
NAC has antigenotoxic and anticarcinogenic properties in a variety of experimental models (for examples, see reviews in refs 4 and 9–15). More recent studies have shown a broad involvement of NAC in modulating different stages of the carcinogenesis process. The number of citations available from MEDLINE under the query term ‘acetylcysteine’ has grown from 162 (quinquennium 1966–70) to 188 (1971–75), 238 (1976–80), 437 (1981–85), 686 (1986–90), 970 (1991–95), 1904 (1996–2000), to a total of 4600 by the end of 2000. Clearly, just a part of these studies refer to the antigenotoxic and anticarcinogenic properties of NAC, but it is impressive to see how this molecule is being increasingly used as a ‘diagnostic’ reagent for evaluating DNA damage- and cancer-related mechanisms, for instance as a prototype antioxidant, modulator of apoptosis, regulator of signal transduction, etc.

Due to the vastness of this subject, it has become very difficult to generate a comprehensive overview of the relevant literature. In the present article we will first propose a general outline of the mechanisms of NAC in modulating DNA damage and carcinogenesis, and then we will focus on specific mechanisms and effects related to cigarette smoke (CS), either mainstream, sidestream or environmental, CS condensates (CSC) and CS constituents, as evaluated in in vitro test systems, experimental animals and humans. In particular, NAC was challenged with a variety of chemical families of DNA damaging and/or carcinogenic compounds which have been detected in CS (16). These included tobacco-specific nitrosamines and other N-nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic amines, aldehydes, metals and agricultural chemicals. Moreover, emphasis is given on the effects of NAC towards reactive oxygen species (ROS) and modifiers of the redox status, which are known to play a crucial role in carcinogenesis and other CS-related pathological conditions (7,17), as well as in the biology of ageing (18).

**Outline of NAC mechanisms and effects in DNA damage and carcinogenesis**

For the rational application of chemopreventive agents, it is essential not only to establish their safety and efficacy, first in pre-clinical models and then in clinical trials, but also to elucidate their mechanism of action. We previously discussed the rationale for cancer chemoprevention and proposed detailed classifications covering a broad array of mechanisms of inhibitors of mutagenesis and carcinogenesis (2,4,19–25). Several chemopreventive agents, also including NAC, are known or suspected to work via multiple mechanisms, which is expected to render them more efficient towards a wider range of carcinogens and situations at risk. It is also possible, by taking into account mechanistic considerations, to combine different agents working with complementary mechanisms. Examples of combined chemoprevention with NAC and other agents will be reported in this article.

Table I summarizes the mechanisms and protective effects of NAC towards DNA damage and carcinogenesis, many of which will be commented on in more detail in the following sections focusing on CS-related end-points. The reported references are just examples drawn from a very broad literature. In the list reported in Table I, we chose to ignore the distinction between mechanisms from effects, since most of the reported end-points are strictly interconnected and it is virtually impossible to distinguish whether a given end-point reflects a primary mechanism or is rather a secondary mechanism, i.e. the epiphenomenon of mechanisms working upstream in an intricate and feedback-controlled network of events. For instance, it is likely that nucleophilicity and scavenging of ROS are genuine mechanisms related to the intrinsic characteristics of the NAC molecule and its intracellular derivatives. In contrast, inhibition of genotoxicity and attenuation of carcinogenicity are the consequences of NAC mechanisms. In other cases it is hard to discriminate primary and secondary mechanisms. For instance, is modulation of signal transduction pathways governed by specific NAC mechanisms or is it the consequence of the ability of this thiol to regulate the redox state? Similarly, stimulation of apoptosis, as comparatively shown in cancer cells but not in non-cancer cells (103), is likely to represent a protective mechanism of NAC in cancer cells. On the other hand, inhibition of apoptosis by NAC, as observed in the large majority of in vitro and in vivo studies (see the relevant sections dealing with CS-related apoptosis), is presumably the
result of the ability of NAC to attenuate oxidative stress, DNA damage and other signals which ultimately trigger apoptosis (2). However, this is not a general rule, as exemplified by the demonstration that in the alveolar epithelium NAC favours Bcl-2, an anti-apoptotic gene, at the expense of Bax, a gene promoting apoptosis (105). Inhibition of apoptosis may be particularly useful in post-mitotic tissues, such as brain, skeletal muscle and heart, in which it is important to protect perennial cells from death.

It is important that all the effects and mechanisms shown in Table I were produced by NAC under a range of conditions due to a variety of in vitro and in vivo treatments or imbalances of homeostasis. Even inhibition of ‘spontaneous’ mutations, which was detected in oxidant-sensitive bacteria, could be ascribed to deficiencies in DNA repair mechanisms (49). Our preliminary results show that, per se, the oral administration of NAC to A/J mice does not appreciably change the expression of 140 genes in the lung, as assessed by multiple cDNA array technology (unpublished data).

Smoking- and oxidative stress-related end-points evaluated in in vitro test systems

The protective effects of NAC towards genotoxicity of CS and its constituents were evaluated in a variety of in vitro experimental models using either acellular systems, bacteria or cultured mammalian cells. Early data (up to 1995) were previously analysed in order to create antimutagenicity profiles with this chemopreventive agent (106).

DNA binding and damage in acellular systems

Incubation of plasmid DNA with aqueous extracts of CS tar and a nitric oxide (NO)-releasing compound, which results in the formation of potent reactive species such as peroxynitrite, caused synergistic induction of DNA single-strand breakage. This effect, which may play an important role in smoking-related diseases including lung cancer, was inhibited by NAC (107). N-Acetylcysteine inhibited oxidative DNA fragmentation, as assessed by agarose gel electrophoresis, and formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG), as assessed by a 32P-post-labelling procedure, consequent to exposure of calf thymus DNA to either H2O2 or a mixture of H2O2 with CuSO4, which generates hydroxyl radicals (•OH) in a Fenton-type reaction (108). Another study evaluated by 32P-post-labelling the formation of 8-oxo-2′-deoxyguanosine (8-oxo-dG), a tautomeric of 8-OH-dG, following exposure of calf thymus DNA to a mixture of H2O2, CuSO4, nitrilotrifluoroacetic acid and ascorbic acid. Addition of NAC to the •OH-generating test system inhibited formation of this purine adduct in calf thymus DNA (109). N-Acetylcysteine inhibited the in vitro formation of adducts to calf thymus DNA of metabolically activated N-nitrosopyrrolidine, an N-nitrosamine present in CS (110). Conversely, NAC failed to inhibit the formation of 32P-post-labelled DNA adducts of metabolically activated benzo[a]pyrene (111) and dibenz[a,l]pyrene (112). This conclusion is consistent with the results obtained in bacterial mutagenicity test systems when using liver preparations from rats treated with enzyme inducers. A technical comment is given in the next section.

Genotoxicity in bacteria

A number of studies have used the Ames reversion test with Salmonella typhimurium his strains of the TA series and, in some cases, a DNA repair test measuring the differential lethality of test compounds in Escherichia coli strain WP2 (wild-type) and its DNA repair-deficient counterpart CM871 (uvrA recA lexA). Direct-acting mutagens

N-Acetylcysteine was consistently effective in decreasing the potency of some direct-acting mutagens which have been reported to be detectable in CS, such as formaldehyde (both in liquid form and vapour form) (113), the urethane metabolite vinyl carbamate epoxide (114), the metal sodium dichromate (8) and the agricultural chemical captan (4).

Reactive oxygen species

Similarly, NAC was quite effective in inhibiting the direct mutagenicity of ROS, e.g. by attenuating the genotoxicity of peroxides, including cumene hydroperoxide (4) and hydrogen peroxide (8,115). Protective effects were also observed by testing NAC towards the genotoxicity of freshly generated ROS, for instance towards •OH generated by incubating human phagocytic leukocytes with bacteria (116), and towards superoxide anion (O2−) generated by mono-electronic reduction in the reaction between xanthine oxidase and hypoxanthine, and further converted into H2O2 in the presence of superoxide dismutase (117). It is noteworthy that NAC also reduced the ‘spontaneous’ mutagenicity in TA104 (49,117), a S.typhimurium strain which is particularly sensitive to oxidative mutagens, and counteracted an opposite effect produced by ascorbic acid in the same system (98). Moreover, NAC attenuated the differential lethality produced in E.coli by mixed volatile ROS generated by illuminating the chromophore rose bengal, which results both in electron-transfer reactions (O2, H2O2 and •OH) and energy transfer reactions (O2, singlet oxygen) (118).

Promutagens

Evaluation of the effects of NAC on promutagens, which require the presence of post-mitochondrial (S9) fractions or microsomal fractions from rat liver in order to yield mutagenic metabolites, is of more complex interpretation. The tested agents included mainstream CS, a CSC and promutagens contained in CS, such as the polycyclic aromatic hydrocarbon benzo(a)pyrene, the volatile N-nitrosamine N-nitrosodimethylamine, the heterocyclic amine 3-amino-1-methyl-5H-pyrrole(4,3-b)indole (Trp-P-2) and the three aromatic amines 2-aminoanthracene, 2-aminofluorene and 2-acrylaminofluorene. The effect of NAC often depended on its dose and on pre-treatment with enzyme inducers of the rats used for preparing S9 fractions. Sometimes NAC was found to exert frank protective effects (33,119,120). In other studies, NAC was ineffective (119,121) or was only effective at the highest tested dose (119). Another frequent finding of these studies was that NAC inhibited the mutagenicity of promutagens when tested at high doses and, in contrast, enhanced their mutagenicity when tested at medium doses (8,33). This type of result was recorded when the liver preparations used in vitro were obtained from rats pre-treated with enzyme inducers capable of specifically stimulating the metabolism of the test promutagens.

The enhancement of mutagenicity observed at medium doses of NAC required the presence of the thiol during the metabolic activation step, whereas the inhibition observed at high doses also occurred when the thiol was added at the end of the metabolic activation step (33). These findings suggest that, at least under certain conditions, NAC does not inhibit or even stimulate metabolic activation, but the resulting reactive
metabolites are detoxified by their coordinate blocking, due to the nucleophility of NAC, and by concomitant stimulation of phase II enzymes. This two-stage mechanism is likely to provide the maximum efficiency in detoxification, because it avoids the accumulation of unmetabolized precursors in the organism and favours the excretion of conjugated metabolites (24).

Genotoxicity, disruption of intercellular communications, and cell transformation in cultured mammalian cells

N-Acetylcysteine significantly reduced the DNA damage produced by water-soluble CS in human lymphoid cells containing Epstein–Barr virus episomes, as detected by alkaline single-cell electrophoresis (COMET assay) (122). In addition, NAC inhibited the genotoxicity of acrolein, a direct-acting CS constituent which was positive in the same test system (122). N-Acetylcysteine exerted protective effects towards the induction of sister chromatid exchanges in Chinese hamster ovary cells co-cultivated with phagocytic leukocytes, which are genotoxic through generation of ROS (123). Cultured endothelial cells of human origin were protected by NAC against cytogenetic damage produced by the oxidizing agent paraquat (124). N-Acetylcysteine counteracted the tumour promoting activity of H₂O₂ in rat liver epithelial oval cells by inhibiting phosphorylation of connexin 43 and disruption of gap junctional intercellular communications (73). By using an assay in cultured rat tracheal epithelial cells, NAC was shown to inhibit cell transformation following treatment with benzo[a]pyrene (52).

Gene expression in cultured mammalian cells

In cultured mammalian cells, NAC inhibited the CS-induced expression of early response genes, such as c-fos, which is involved in cell proliferation and apoptosis. This effect was ascribed to peroxynitrite, resulting from the reaction of NO with O₂⁻, and to depletion of cellular GSH due to the aldehyde fraction of CS (125). It is noteworthy that water-soluble components of CS targeted free sulfhydryl groups and decreased free GSH levels in the same cells (126). Tobacco-specific N-nitrosamine 4-(methyl-nitrosamo)-1-(3-pyridyl)-1-butanone (NNK), which is formed by N-nitrosation of nicotine during tobacco smoking, can be activated to electrophilic mutagenic intermediates in a human macrophages cell line via cyclooxygenase (COX) metabolism. NNK bioactivation leads to the production of ROS, which are known to activate the nuclear factor-xB (NF-xB) acting as a regulatory element of COX-2 expression. N-Acetylcysteine was found to be a strong inhibitor of NNK-induced prostaglandin E₂ (PGE₂) synthesis by inhibiting COX-1 expression in these cells (27). N-Acetylcysteine was found to preclude c-Ha-ras expression in vascular smooth muscle cells treated with benzo[a]pyrene (67). Increased expression of COX-2 has been detected both in atherosclerotic lesions and in epithelial cancer. Treatment of human and rat arterial smooth muscle cells with benzo[a]pyrene increased levels of COX-2 proteins and mRNA, and enhanced prostaglandin synthesis. At least in part, this process is mediated by NF-xB and involves an increase in phosphorylation of extracellular signal-regulated kinase (ERK). Exposure of cells to NAC suppressed the induction of COX-2 by benzo[a]pyrene (29).

Signal transduction in cultured mammalian cells

Several other studies available in the literature are consistent with the ability of NAC to modulate signal transduction pathways altered by various agents (Table I). It is noteworthy that the activator protein-1 (AP-1), a heterodimeric complex formed from c-jun and c-fos gene products, can bind to the promoter region of intermediate genes required for cell division and other cell functions, also including transcription of type IV collagenases, which are responsible for the degradation of vascular basal membranes triggering invasion of malignant cells and subsequent spread of metastases. Interestingly, NAC is an efficient inhibitor of type IV collagenases and prevents invasion and metastasis in murine models, either alone (93) or in synergism with the cytotoxic drug doxorubicin (94,96).

Antiproliferative effects in cultured mammalian cells

The antiproliferative activity of NAC was demonstrated by evaluating cell numbers and DNA synthesis in cultured human brain tumour cells exposed to oxidants (88). Moreover, NAC induced p16 (INK4a) and p21 (WAF/CIP1) gene expression and prolonged cell-cycle transition through G₁ phase in various types of cultured mammalian cells. The proportion of cells in G₁ arrest following NAC treatment was governed by p16 (INK4a) and was independent of p53 expression. These results suggest a potentially novel molecular basis for chemoprevention by NAC, also because increase of intracellular GSH was not required for G₁ arrest, and other antioxidants whose action is limited to scavenging radicals did not induce G₁ arrest (63). In addition, NAC slowed down cell cycle progression by inhibiting topoiseromase-IIα activity. This effect was more pronounced with l-NAC (GSH precursor) but was also detected with d-NAC (non-precursor of GSH) (127).

Anticytotoxic effects in cultured mammalian cells

N-Acetylcysteine increased survival in cultured human bronchial cells and counteracted the toxicity of CSCs and their non-volatile and semi-volatile fractions in rat hepatocytes and lung cells (128). Moreover, NAC protected the isolated perfused rat lung against the GSH-depleting effect of CS (129), and prevented a variety of cytotoxic effects, mainly ROS-mediated, induced by CS (130–132) and its constituents (133–136). The N-hydroxylamino metabolites of the heterocyclic amines 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-2-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), which are formed in the combustion process and have also been detected in CSC (137), were toxic to isolated rat cardiomyocytes. N-Acetylcysteine was by far the most protective of the agents investigated (138). N-Acetylcysteine protected endothelial cells from injury following exposure to CS gas-phase (139). Moreover, NAC attenuated the endothelium-dependent relaxation of isolated rabbit aortas incubated with the water-soluble component of CS extract (140). NAC increased intracellular GSH and protected cultured pulmonary endothelial cells from injury produced by hyperoxia (141), and exerted a dose-dependent inhibition of toxicity in primary cultures of porcine aortic endothelial cells incubated in the presence of the hypoxanthine-xanthine oxidase system (142).

Modulation of apoptosis in cultured mammalian cells

The ability of NAC to modulate the apoptotic process is well established (see also the section on intermediate biomarkers in experimental animals). One study addressed this problem by exposing normal human monocytes to CS, a treatment which induced the expression of stress proteins, mitochondrial depolarization and apoptosis. N-Acetylcysteine prevented CS-induced deltamapsis disruption and apoptosis (39). As many as
105 data were selected from MEDLINE (as to November 2000) concerning the effects of NAC on apoptosis induced in cultured mammalian cells by ROS or imbalances of redox potential. These effects were investigated with a variety of inducers, including peroxides, ROS donors, NO and NOx donors, inducers of arachidonic acid metabolism, other modulators of metabolism, GSH depletors, signal transduction modulators, a variety of chemical agents, complex mixtures other than CS, mitogenic stimuli, toxins, hormones, viral infections and physical agents. Of these 105 data, 91 (86.7%) were consistent with the ability of NAC to inhibit apoptosis, 10 (9.5%) showed no effect of NAC and 4 (3.8%) showed an enhancement of apoptosis by NAC. Interestingly, NAC was capable of inducing apoptosis in several transformed cell lines and transformed primary cultures but not in normal cells (53), which clearly highlights a protective mechanism of NAC in cancer cells. Moreover, NAC induced apoptosis in rat and human smooth muscle cells, which may help prevent their proliferation in atherosclerotic lesions (143).

Smoking- and oxidative stress-related end-points evaluated in experimental animals

A number of studies evaluated the ability of NAC to modulate intermediate biomarkers involved in the CS-related carcinogenesis process and occurrence of lung tumours induced by CS or its constituents in animal models.

Intermediate biomarkers of lung carcinogenesis

Metabolic parameters

Several studies showed that NAC poorly influences phase I enzymes involved in the metabolic activation of carcinogens, while it stimulates detoxifying phase II enzymes to some extent (32,33,144–146). Such a modulation is likely to contribute to the protective effects of NAC but does not appear to be a major mechanism in NAC anticarcinogenicity (4).

Adducts to nuclear DNA

Several studies evaluated the protective effects of NAC towards the formation of DNA adducts in Sprague–Dawley rats exposed to CS, either mainstream or environmental, or CS constituents. Whole-body exposure of rats to mainstream CS failed to induce formation of DNA adducts, as detected by synchronous fluorescence spectrophotometry (SFS), which specifically detects adducts to DNA of polycyclic aromatic hydrocarbons, in liver (42), oesophagus (4), brain or testis (43), whereas adducts were detected in lung and heart (42), aorta (43) and kidney (4). In all cases oral NAC significantly decreased the level of DNA adducts (4,42,43). In two further experiments, rats were exposed whole-body to mainstream CS for either 40 or 100 consecutive days, and DNA adducts were detected by 32P-post-labelling in testis (44) and dissected tracheal epithelium (44). Note that the histological structure of rat trachea resembles that of the human bronchus, the major site of smoking-related cancers in humans (147), and tracheal epithelial cells of rats exposed to CS undergo pre-neoplastic changes (148). In both experiments exposure of rats to CS resulted in the formation of DNA adducts in tracheal DNA, and oral NAC exerted significant protective effects both in testis (4) and tracheal epithelium (44). Interestingly, similar protective effects of NAC were observed in the lung of rats receiving intra-tracheal instillations of polluted air particle extracts (45).

Other studies on DNA adducts were performed by exposing rats either to sidestream CS or to environmental CS. The latter consists of a mixture of sidestream CS and mainstream CS. In one study, dietary NAC did not significantly influence the levels of most major and minor lipophilic DNA adducts formed in lung, heart, whole trachea or bladder of rats exposed whole-body to sidestream cigarette smoke (149). In contrast, in our laboratory, NAC given in the drinking water was successful in decreasing the level of DNA adducts in bronchoalveolar lavage cells, dissected tracheal epithelium, lung, heart (47) and aorta (Izzotti, A., Camoirano, A., Cartiglia, C., Tampa, E., and De Flora, S., in preparation) of rats exposed whole-body to environmental CS. Further comparative experiments provided evidence that the contrasting conclusions generated in these studies are methodological in nature, and namely depend on the different chromatographic conditions used for the separation of 32P-post-labelled DNA adducts (47). Indeed, the chromatographic system used by Arif et al. (149) yields much lower amounts of DNA adducts and fails to detect the massive diagonal radioactive zone, which is the expression of the multitude of DNA-binding agents present in cigarette smoke (47). Interestingly, NAC interacted synergistically with the other chemopreventive agent oltipraz in decreasing CS-induced DNA adducts in the lung (47).

Intra-tracheal instillations of benzo[a]pyrene, which in the long-term are known to cause the formation of squamous cell carcinomas of the lung in the same rat strain (150), produced the appearance of DNA adducts, measured by SFS in both liver and lung. Administration of NAC by gavage totally eliminated adducts to liver DNA and significantly decreased these molecular lesions in the lung (41). These results were confirmed in a further study, in which NAC significantly decreased benzo[a]pyrene-induced SFS-positive DNA adducts in lung, liver, heart and testis (43). N-Acetylcysteine inhibited DNA fragmentation, DNA–protein cross-links, 32P-post-labelled DNA modifications and 8-OH-dG in the lung of Sprague–Dawley rats receiving intra-tracheal instillations of chromium(VI), a metal contained in cigarette smoke (46). Incidentally, chromium(VI) and CS have less than additive clastogenic effects in rodents (151).

Adducts to mitochondrial DNA (mtDNA)

Mitochondrial DNA is a particularly important target since, mainly as a result of defects in oxidative phosphorylation, its alterations are associated with ageing processes as well as with a variety of chronic degenerative diseases affecting postmitotic tissues that have a high energy requirement, including some forms of ischemic heart disease, cardiomyopathies, adult-onset diabetes, Parkinson’s disease, Huntington’s disease and several other neurological disorders (152). Whole-body exposure of Sprague–Dawley rats to mainstream CS resulted in the formation of mtDNA adducts, evaluated by 32P-post-labelling, in the lung and, to a lesser extent, in the liver. Levels of adducts to mtDNA were consistently higher than levels of adducts to nuclear DNA in the same cells, a conclusion which was confirmed by measuring DNA adducts in the liver of rats treated with benzo[a]pyrene intraperitoneally (i.p.) or 2-acetylaminoflourene by gavage (38). The oral administration of NAC to rats resulted in a significant decrease of mtDNA adduct levels in the liver of 2-acetylaminoflourene-treated rats and in both lung and liver of CS-exposed rats (38).

Oxidative damage to lung DNA

8-OH-dG formation was measured in rats exposed whole-body to environmental CS. Administration of NAC in the drinking
water to CS-exposed rats decreased 8-OH-dG to the same levels as those observed in CS-free controls (47).

Adducts to haemoglobin
In the same animals, NAC significantly reduced the levels of adducts to haemoglobin of two typical constituents of CS, i.e. 4-aminobiphenyl and benzo[a]pyrene-7,8-diol-9,10-epoxide, which were considerably increased following exposure to CS. This effect was potentiated by the combined administration of NAC in the drinking water and oltipraz in the diet (47).

Cytogenetic damage
Several studies addressed the issue regarding the ability of NAC to inhibit the cytogenetic damage produced by CS, polycyclic aromatic hydrocarbons, aromatic amines or urethane in different rodent cells, such as pulmonary alveolar macrophages (PAM) recovered by bronchoalveolar lavage, polychromatic erythrocytes (PCE) in bone marrow and normochromatic erythrocytes (NCE) in peripheral blood.

A detailed study on the time-course induction, persistence and modulation by NAC of cytogenetic alterations was performed in BDF1 mice exposed whole-body to mainstream CS for up to 3 weeks (72). Administration of NAC, throughout the duration of the experiment, strongly inhibited the smoke-induced formation of micronuclei in PAM and had some transiently significant effect on the induction of binucleated PAM. NAC tended to decrease the smoke-induced formation of micronuclei in bone marrow cells, but not to a significant extent, and significantly attenuated the formation of micronuclei in peripheral blood NCE (72). When given after discontinuation of exposure to CS, NAC did not affect the cytogenetic alterations but normalized the altered bronchoalveolar lavage cellularity, which 11 weeks after withdrawal of exposure to CS was still altered, with an almost 10-fold increase of polymorphonucleates and a parallel decrease of PAM (72). The accumulation of polymorphonucleate leukocytes has also been reported to occur in smoking humans (153) and is considered to be a useful parameter for evaluating the pulmonary inflammatory response (154). Once these cells accumulate in the lung, it is possible that they may actually interfere with PAM functions (153). The protective effect of NAC towards this end-point may tentatively be related to the proposed role of this thiol in modulating the production and release of cytokines and ROS (70), and in defending bronchoalveolar lavage cells from toxic products such as ROS generated during phagocytosis (155).

A recently completed study evaluated the induction of micronuclei in the liver of Swiss mouse fetuses whose mothers had been exposed to environmental CS during pregnancy. The fetal liver, which works in the fetus as a hematopoietic organ, was examined on day 18 of pregnancy. The frequency of micronucleated PCE in the liver was significantly enhanced in fetuses from CS-exposed mothers, and NAC, given during pregnancy, exerted a significant protective effect (R.Balansky, F.D’Agostini, A.Izzotti and S.De Flora, unpublished data).

In addition, NAC attenuated cytogenetic alterations in Sprague–Dawley rats exposed whole-body to CS, either mainstream (77) or environmental (47). In particular, NAC administration by gavage produced a significant and considerable protective effect towards mainstream CS-induced alterations of bronchoalveolar lavage cellularity, increase of micronucleated PAM and bone marrow cytotoxicity, although it did not attenuate the induction of micronuclei in PCE (77). Exposure of Sprague–Dawley rats to environmental CS resulted in significant increases of micronucleated and binucleated PAM and of micronucleated PCE in bone marrow. Administration of NAC in the drinking water and its combination with dietary oltipraz significantly inhibited all these cytogenetic alterations (47).

In Sprague–Dawley rats receiving intra-tracheal instillations of benzo[a]pyrene, NAC inhibited the increase of both micronucleated and binucleated PAM, and prevented cytogenetic alterations in bronchoalveolar lavage cells, including a relative increase in polymorphonucleates over PAM. Benzo[a]pyrene did not significantly enhance the number of micronucleated PCE in bone marrow (41). N-Acetylcysteine failed to inhibit the induction of micronucleated PCE in bone marrow of C57BL/6 mice treated by gavage with 7,12-dimethylbenz[a]anthracene (156). In BDF1 mice receiving a single i.p. injection of the aromatic amine 2-acetylaminofluorene, the frequency of micronucleated PCE in bone marrow was enhanced 5.5-fold as compared with controls. Pre-treatment of mice with NAC at two dose levels, in drinking water, resulted in a significant and dose-related protective effect (R.Balansky and S.De Flora, unpublished data). In the same mouse strain, oral NAC significantly inhibited the increase of micronucleated PCE produced by an i.p. injection of potassium dichromate (R.Balansky and S.De Flora, unpublished data). In BALB/c mice treated with i.p. injections of urethane, the time-course frequency of micronucleated NCE was monitored by periodically collecting the blood from the lateral tail vein. Administration of NAC in the drinking water resulted in a dose-related decrease of clastogenicity, which predicted the subsequent inhibition of lung tumours (157). Another study showed that, 24 h after a single i.p. injection of urethane, the frequency of micronucleated PCE in bone marrow was enhanced 22.5-fold as compared with controls. Pre-treatment of mice with NAC in drinking water resulted in a significant protective effect (R.Balansky and S.De Flora, unpublished data).

Apoptosis
N-Acetylcysteine protected the respiratory tract of Sprague–Dawley rats from CS-induced apoptosis. In particular, in a first study exposure of rats to mainstream CS for either 28 or 100 consecutive days produced a significant and time-dependent increase in the proportion of apoptotic cells in the bronchial and bronchoalveolar epithelium. In a second study, exposure of rats to environmental CS for 28 consecutive days resulted in a >10-fold increase in the frequency of PAM undergoing apoptosis. The strong induction of apoptosis by CS may explain, at least in part, the difficulties in reproducing the lung tumorigenicity of CS in animal models (see below). In both studies, administration of NAC in the drinking water significantly inhibited induction of apoptosis by CS (158). N-Acetylcysteine also prevented hair follicle cell apoptosis and alopecia in C57BL/6 mice exposed to environmental CS (68). Interestingly, in the same mouse strain oral NAC also prevented the alopecia induced by doxorubicin, which typically induces oxidative DNA damage (96).

The protective effect of NAC towards smoke-induced apoptosis in PAM and in the bronchial/bronchiolar epithelium may be relevant as a defence mechanism not only in smoking-related lung carcinogenesis but also in the pulmonary inflammatory response as well as in other pulmonary diseases (159–1005).
In addition, three studies demonstrated the ability of NAC to inhibit ROS-mediated apoptosis under situations which may play a role in different pathological conditions. In fact, NAC down-regulated the apoptotic process induced by contralateral tectal lesion in the eye retinal ganglion of chicken embryos (162), experimental diabetes in pancreatic β-cells of mice (163) and balloon-catheter injury in carotid artery of rabbits (164).

Morphological and functional alterations of the respiratory tract

Either alone or in combination with other agents, oral NAC increased survival in rats treated with acrolein, formaldehyde and acetaldehyde, which is a toxicant common to cigarette smoke and alcohol consumption (165). Unless in combination with the seleno-organic agent Ebselen, NAC did not inhibit the development of Sephadex-induced lung oedema and cell infiltration (73). Studies in Wistar rats, rendered ‘bronchitic’ by whole-body exposure to mainstream CS, showed that administration of NAC in the drinking water significantly prevents epithelial secretory cell hyperplasia, especially in the smallest bronchioli, as well as the smoke-induced hypersecretion of mucus in the larynx and trachea (75,76). In another study, NAC, either alone or in combination with CS, increased both the volume and albumin content of respiratory tract fluid in bronchitic rats, and did not reduce the CS-induced increase in epithelial secretory cell number (166). In Sprague–Dawley rats exposed whole-body to CS for 40 days, severe histopathological changes were detected in terminal airways, including an intense inflammation of bronchial and bronchiolar mucosae, with multiple hyperplastic and metaplastic lesion foci of micropapillomatous growth. A severe emphysema, with extensive disruption of alveolar walls, was also observed. All these changes were efficiently prevented in rats treated by gavage with NAC (77). Oral NAC also prevented alterations in morphometry consisting of airway wall thickening of small, medium and large bronchi, and in ventilation distribution after exposure to CS for 10 weeks (78).

Lung tumours

In spite of the predominant role played by CS in the epidemiology of human lung cancer and cancers at other sites, it is extremely difficult to reproduce the carcinogenicity of this complex mixture in animal models (16,167). Recently, Witschi et al. (168) were successful in inducing lung tumours in A/J mice exposed whole-body to environmental CS for 5 months and kept for an additional 4 months in filtered air. This effect was mainly ascribed to the gas phase of environmental CS (169). Pilot studies performed in our laboratory supported these results, and confirmed that continued exposure of A/J mice to environmental CS for 5–9 months fails to increase the lung tumour yield over the background levels observed in sham-exposed mice (170). Using this model, NAC did not affect the weak increase of lung tumours in mice exposed to environmental CS (171). It is noteworthy that a chemopreventive effect in this system was only observed with myo-inositol-desamethasone (172,173), while other well-known chemopreventive agents, including phenethyl isothiocyanate (171) and its combination with benzyl isothiocyanate (173), decaffeinated green tea (171), acetylsalicylic acid (172), β-limonene and 1,4-phenlenbis(methylene)selenoisocyanate (173) were all unsuccessful in attenuating the environmental CS-related increase in lung tumours in this experimental model.

Recently, a small pilot study performed in our laboratory demonstrated that the whole-body exposure of outbred Swiss albino mice to environmental CS during pregnancy resulted, 8.5 months later, in a significant increase of lung tumour incidence, multiplicity and size, which was prevented by oral NAC. More extensive studies are now in progress in order to confirm these data and in particular to clarify the importance of the mouse strain (Swiss versus A/J), the role of exposure during pregnancy as compared with non-pregnant mice, and the protective effect of NAC.

Four studies evaluated modulation by NAC of lung tumour formation in urethane-treated mice. In Swiss albino mice treated with a single i.p. injection of urethane (1 g/kg body weight) administration of 0.2% NAC in the diet significantly decreased both tumour incidence and multiplicity (146). In BALB/c mice treated with 10 daily i.p. injections of urethane (each of 0.4 g/kg body weight) administration of NAC in the drinking water (0.1 or 0.5 g/kg body weight) produced a significant and dose-dependent decrease of tumour multiplicity (157). In A/J mice treated with a single i.p. injection of urethane (0.25 g/kg body weight), administration of 0.2% NAC in the diet significantly decreased tumour multiplicity. However, no such protective effect of NAC was observed when urethane was dosed at either 1 or 0.1 g/kg body weight (171). In a further study in A/J mice receiving an i.p. injection of urethane (1 g/kg body weight), administration of NAC in the drinking water (1 g/kg body weight) significantly decreased tumour multiplicity (98). Ascorbic acid (AsA), given in drinking water (1 g/kg body weight) did not significantly decrease tumour multiplicity when given alone, but its combination with NAC further decreased this parameter. Moreover, both NAC alone and in combination with AsA reduced tumour size. In the same study, NAC and AsA behaved in an additive fashion in inhibiting the in vitro mutagenicity of chromium(VI) and, at the same time, NAC prevented an adverse effect of AsA, which alone enhanced the ‘spontaneous’ mutagenicity in S.typhimurium TA104, a strain sensitive to oxidative mechanisms (98).

Other studies evaluated the effect of dietary NAC on the tumour yield in rodents treated with the tobacco-specific nitrosamine NNK. Fischer rats received subcutaneous (s.c.) injections of NNK (1.5 mg/kg body weight) three times a week for 21 weeks. In NNK-treated animals, the lung tumour incidence was 67% (22/36). The determination of lung tumour multiplicity was not possible in this study (174). Dietary administration of NAC, at either 40 or 80 mmol/kg diet, did not affect lung tumour incidence. The incidence of nasal cavity tumours in NNK-treated rats was decreased from 78 to 61 and 47% in rats receiving NAC at 40 and 80 mmol/kg diet, respectively. In the same study, no spontaneous pancreatic tumours were detected in control rats receiving a diet supplemented with 80 mmol NAC/kg. Similarly, the incidence of Leydig cell tumours of the testis in NNK plus NAC-treated rats was even lower than that recorded in NNK-free rats (174). In a further study by the same group, NNK was given in a single i.p. injection (10 µmol) to A/J mice. NAC, administered in the diet at either 80 or 160 mmol/kg diet, failed to affect the yield of lung tumours 16 weeks after injection of the carcinogen. After 52 weeks, however, the incidence of NNK-induced adenocarcinomas was significantly decreased by 160 mmol NAC/kg diet, which was accompanied by a corresponding increase in adenomas. Therefore, this study provided evidence that NAC significantly retards malignant progression in the lung of NNK-treated A/J mice (86).
Most isothiocyanates (ITCs) administered to humans are excreted as NAC conjugates, which are degradation products of ITC–GSH via the mercapturic acid pathway (175). Inhibition of lung tumours in NNK-treated mice by ITCs, such as phenethyl isothiocyanate (PEITC), 6-phenylhexyl isothiocyanate (PHITC) and benzyl isothiocyanate (BITC), was not potentiated following conjugation of these compounds with NAC. However, Jiao et al. (99) concluded that use of ITC–NAC conjugates compares favourably to ITCs alone because (i) the conjugates are less toxic than ITCs, yet they maintain a similar chemopreventive efficacy towards NNK-induced lung tumours; (ii) the higher lipophilicity of the ITC–NAC conjugate may facilitate its absorption, making it a more effective inhibitor; and it can gradually release ITC and NAC via a dissociation mechanism; and (iii) these conjugates can be viewed as hybrids of chemopreventive agents (ITC and NAC) with distinct modes of action (99). Furthermore, it was preliminarily reported that BITC–NAC and PEITC–NAC conjugates significantly inhibited NNK-induced lung tumours in A/J mice also at post-initiation stages, i.e. when they were administered 2 days after NNK dosing (176).

Out of the other CS constituents, it is suspected that aldehydes may increase the genotoxicity of N-nitroso compounds both by causing DNA damage and by inhibiting the repair of O^6^-methylguanine via DNA methyltransferases, as shown by testing in vitro formaldehyde in N-methyl-N-nitrosourea-exposed cells (177). In connection with this problem, it is noteworthy that NAC significantly inhibited squamous carcinomas in the trachea of Syrian golden hamsters receiving a local application of 5% N-methyl-N-nitrosourea once a week for 15 weeks (178).

**Smoking- and oxidative stress-related end-points evaluated in humans**

Several studies evaluating the ability of NAC to modulate smoking-related cancer biomarkers in humans have been completed in recent years. Oral NAC, given in three daily doses of 600 mg for up to 142 days, significantly lowered the levels of 4-aminobiphenyl–haemoglobin adducts (48). Although this finding was generated in non-smokers, this biomarker is typically enhanced following exposure to cigarette smoke, as also shown by our studies in rats, in which NAC inhibited the smoke-related formation of 4-aminobiphenyl–haemoglobin adducts (47). Mechanistically, it should be taken into account that NAC is a good precursor of GSH in red blood cells (33), and GSH and haemoglobin have been shown to compete for reaction with nitrosobiphenyl, a reactive metabolite of 4-aminobiphenyl (179).

Although colorectal cancer is not one of the major tobacco-related cancers, smoking has been associated with an increased risk of colorectal adenomas and hyperplastic polyps (180), and an increased risk of colorectal cancer has been reported in studies with at least 20 years of follow-up (181). It had been predicted that agents such as NAC which have antimutagenic activity may protect against the numerous mutagenic events occurring throughout colon carcinogenesis (182), and preliminary data (183) suggested that NAC (600 mg/day) can decrease the recurrence rate of adenomatous polyps. Moreover, in 34 patients with previous adenomatous colonic polyps, a significant decrease in the proliferation index of colonic crypts occurred after treatment for 12 months with oral NAC (800 mg/day), whereas no variation was detected in 30 subjects receiving a placebo (90).

We made a time-course evaluation of the urinary excretion of mutagens in 10 smokers receiving oral NAC at 600–800 mg/day. A significant decrease of mutagenicity in strain YG1024 of *Styphnum* was observed in six subjects, and in another subject NAC administration almost totally prevented the ability of urine to induce a differential lethality in *Escherichia coli* strains having distinctive DNA repair capacities. The decrease of urinary mutagenicity commenced on the first day of NAC administration and was reversed when the treatment was withdrawn (35).

A recently completed randomized double blind phase II chemoprevention trial (van Schooten,F.J., Nia,A.B., De Flora,S. et al., in preparation) evaluated healthy smoking volunteers a battery of biomarkers at time 0 and after 6 months of treatment either with NAC, at a daily regimen of 2×600 mg oral tables (20 subjects), or with placebo (21 subjects). In the placebo group there was no significant variation in any of the investigated biomarkers. In the NAC group a significant decrease occurred in some of the investigated biomarkers, including the levels of lipophilic DNA adducts and 8-OH-DG in bronchoalveolar lavage cells as well as the frequency of micronuclei in mouth floor and soft palate cells (van Schooten,F.J., Nia,A.B., De Flora,S. et al., in preparation).

The attenuation of these biomarkers is predictive of the potential ability of NAC to decrease the risk of smoke-related aerodigestive tract cancer in humans, since DNA damage is an essential step in the carcinogenesis process.

A multicenter intervention study (EUROSCAN) was performed in 2592 patients who had previously been treated for head and neck cancer or lung cancer (184). The patients were randomly assigned to four groups, receiving either no treatment, retinyl palmitate, NAC (600 mg/day) or both drugs for 2 years. After a median follow-up of 49 months, 916 patients underwent an event (local/regional recurrence, second primary, distant metastases or death), without any significant difference among the four intervention arms. Therefore, either alone or in combination with retinyl palmitate, treatment with NAC had no benefit for patients with head and neck cancer or with lung cancer, most of whom were previous or current smokers (184). Clearly, the target of this phase III trial was different from that of the previously reported phase II study (van Schooten,F.J., Nia,A.B., De Flora,S. et al., in preparation), which evaluated the effect of NAC in healthy smokers and accordingly reproduced a primary prevention setting.

**Concluding remarks**

The prominent role of CS in the epidemiology of lung cancer, cancers at other sites, cardiovascular diseases, chronic obstructive lung diseases and several other chronic degenerative conditions is well documented. Collectively, CS-related diseases represent a major cause of premature death in the population, the average loss of life in smokers having been estimated to be 8 years (185). CS is responsible for 24% of all male deaths and 7% of all female deaths in developed countries (185), but even greater is the concern for future epidemiological scenarios in developing countries, where there are 800 million smokers in the context of a worldwide figure of about 1100 million smokers (186). DNA damage and oxidative stress are key mechanisms in carcinogenesis and are possibly involved in the pathogenesis of other CS-related diseases, such as atherosclerosis and heart disease (187,188).
Smoking cessation is the primary goal for the control of CS-related diseases (189), as also shown by the evidence that the age-standardized mortality for cancer in general and specifically for lung cancer tends to decline in the male population of those countries in which CS consumption has decreased in recent years (190). While dietary and pharmacological interventions are well established preventive measures in cardiovascular diseases, cancer chemoprevention is a relatively young discipline which is gaining more and more credibility in the scientific community. This strategy is particularly important in individuals who are at high risk either because of occurrence of genetic susceptibility factors or of heavy exposures, both present and past. For instance, this is typical for current smokers and ex-smokers, respectively.

All pharmacological agents proposed for any therapeutic or prophylactic use in humans, including prevention of cancer and other mutation-related diseases, should possess some general requisites, regarding cost, practicality of use, safety and efficacy (2,191). In this domain, it is noteworthy that NAC preparations have a low cost and are of practical use, being stable for years in dry form. Moreover, oral administration is compatible with long-term use. At variance with other novel molecules that are candidate chemopreventive agents, for which phase I studies are needed in order to evaluate pharmacokinetics and tolerability, the pharmacokinetics and bioavailability of NAC have been extensively investigated (see, for example, refs 192 and 193). The safety of NAC has been well established after almost 40 years of clinical experience. Doses of NAC as high as 500 mg/kg body weight per os. (194) or 300 mg/kg intravenously (195) have been used in cases of acute intoxications. A phase I clinical trial showed that doses of NAC up to 6.4 g/m² skin surface/day just yielded minor gastro-intestinal disturbance in the treated subjects (192). N-Acetylcysteine also has a low toxicity in experimental animals, its oral LD₅₀ being >10 g/kg body weight in both rats and mice, and the LD₅₀ after intravenous administration being 4.6 g/kg in mice and 2.8 g/kg in rats (196). A daily dose of 1 g/kg, given per os. for 18–24 months, was devoid of detrimental effects in both rats (196) and mice (R.Balansky, G.Gancher and S.De Flora, unpublished data).

The vast majority of the reported studies in in vitro models, experimental animals and human trials point to the conclusion that NAC has the capability to modulate in a protective sense a broad variety of mechanisms involved in DNA damage and carcinogenesis, and therefore has the potential to prevent cancer and other mutation-related diseases. Certainly, more studies are desirable in order to evaluate efficacy in humans, a drawback which is common to virtually all putative cancer chemopreventive agents. The ability of NAC to prevent cancer should be assessed in further clinical trials evaluating the effects of this drug either on intermediate biomarkers (phase II trials) or tumours (phase III trials). In addition, based on the outcome of experimental studies (94,96), it would be of interest to design studies aimed at evaluating the association of NAC with cytostatic drugs, such as doxorubicin, in advanced stages of the carcinogenesis process. Interindividual variability is likely to occur in response to NAC, as shown by the assessment of biomarkers in NAC-treated smokers (35; van Schooten,F.J., Nia,A.B., De Flora,S. et al., in preparation). This prompted us to implement studies, which are now in progress, aimed at evaluating whether responsiveness to NAC and other chemopreventive agents may be influenced by pharmacogenetic factors, such as metabolic polymorphisms. At the molecular level, multiple cDNA array technology will provide an important tool for assessing both safety and efficacy of chemopreventive agents. In fact, in our opinion, an optimal agent should be able to protect against alterations produced by CS or any other carcinogen but, per se, should not disturb the normal homeostasis of gene expression. Besides the preliminary results reported in the Introduction, we have planned ad hoc studies in both experimental animals and humans treated with NAC.

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References


Chemopreventive mechanisms of N-acetylcysteine


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