Nanoparticles increase the efficacy of cancer chemopreventive agents in cells exposed to cigarette smoke condensate

Alessandra Pulliero1, Yun Wu2, Daniela Fenoglio3,4, Alessia Parodi3, Massimo Romani5, Christiane P.Soares6, Gilberto Filaci3,4, James L. Lee2,7, Patrick N.Sinkam8 and Alberto Izzotti1,5,*

1Department of Health Sciences, University of Genoa, 16132 Genoa, Italy, 2Nanoscale Science and Engineering Center for Affordable Nano-engineering of Polymeric Biomedical Devices, The Ohio State University, Columbus, OH 43210, USA, 3Centre of Excellence for Biomedical Research and 4Department of Internal Medicine, University of Genoa, 16132 Genoa, Italy, 5Mutagenesis Unit, IRCCS AOU (Institute for Hospitalization and Cure with Scientific Character) San Martino–IST Genoa, 16132 Genoa, Italy, 6Department of Clinical Analysis, UNESP The Universidade Estadual Paulista is already written hereinafter–University Estadual Paulista, Araraquara, School of Pharmaceutical Sciences, Rua Expedicionários do Brasil, 1621 Araraquara, SP, Brazil, 7William G. Lowrie Department of Chemical and Bimolecular Engineering, 125A Koffolt Labs and 8Division of Pulmonary Allergy, Critical Care and Sleep Medicine, The Ohio State University, Columbus, OH 43210, USA

*To whom correspondence should be addressed. Department of Health Sciences, University of Genoa, Via A. Pastore 1, Mutagenesis Unit, IRCCS AOU San Martino-IST, I-16132 Genoa, Italy. Tel:+39 010 3538522; Fax: +39 010 3538504; Email: izzotti@unige.it

Abstract

Lung cancer is a leading cause of death in developed countries. Although smoking cessation is a primary strategy for preventing lung cancer, former smokers remain at high risk of cancer. Accordingly, there is a need to increase the efficacy of lung cancer prevention. Poor bioavailability is the main factor limiting the efficacy of chemopreventive agents. The aim of this study was to increase the efficacy of cancer chemopreventive agents by using lipid nanoparticles (NPs) as a carrier. This study evaluated the ability of lipid NPs to modify the pharmacodynamics of chemopreventive agents including N-acetyl-L-cysteine, phenethyl isothiocyanate and resveratrol (RES). The chemopreventive efficacy of these drugs was determined by evaluating their abilities to counteract cytotoxic damage (DNA fragmentation) induced by cigarette smoke condensate (CSC) and to activate protective apoptosis (annexin-V cytofluorimetric staining) in bronchial epithelial cells both in vitro and in ex vivo experiment in mice. NPs decreased the toxicity of RES and increased its ability to counteract CSC cytotoxicity. NPs significantly increased the ability of phenethyl isothiocyanate to attenuate CSC-induced DNA fragmentation at the highest tested dose. In contrast, this potentiating effect was observed at all tested doses of RES, NPs dramatically increasing RES-induced apoptosis in CSC-treated cells. These results provide evidence that NPs are highly effective at increasing the efficacy of lipophilic drugs (RES) but are not effective towards hydrophilic agents (N-acetyl-L-cysteine), which already possess remarkable bioavailability. Intermediate effects were observed for phenethyl isothiocyanate. These findings are relevant to the identification of cancer chemopreventive agents that would benefit from lipid NP delivery.

Introduction

Lung cancer is the leading cause of cancer death worldwide. The US National Cancer Institute has estimated that 160 340 deaths were due to lung cancer in the USA in 2012 (1,2). Oxidative stress has been implicated as an important mechanism of the pulmonary inflammation that occurs in cigarette smokers and thus a potential target for chemoprevention. In vitro experiments using...
alveolar-type-II-like A549 cells have demonstrated that platinum nanoparticles (NPs) stabilized with polyacrylate to form a stable colloid solution (PAA-Pt) are a new class of antioxidants that efficiently quench reactive oxygen species. In addition, PAA-Pt inhibits cell death after exposure to cigarette smoke (CS) extract (3). Because CS contains and generates a large amount of reactive oxygen species, which elicit pulmonary inflammation, antioxidants may be effective therapeutic agents for CS-related inflammatory lung diseases, such as chronic obstructive pulmonary disease (4). Although limiting exposure to CS is undoubtedly the most important strategy for preventing lung cancer, there is a need to develop effective chemopreventive strategies for addicted and ex-smokers. The administration of N-acetylcysteine (NAC) to smokers for 6 months reduced oxidative DNA damage in blood lymphocytes and micronuclei formation in oral epithelial cells (5), but the efficacy of similar approaches has yet to be confirmed for clinical application. This lack of efficacy is related to a variety of problems, including the limited bioavailability of cancer chemopreventive compounds.

The chemopreventive drugs NAC, myoinositol, indole-3-carnbol and 3,3’-diindolylmethane were recently used alone or in combination to assess the proliferation of human bronchial epithelial cells and human lung adenocarcinoma cells pre-treated with cigarette smoke condensate (CSC) (6). NAC, phenylethyl isothiocyanate (PEITC) and 5,6-benzoflavone exerted a significant protective effect on DNA adducts in bronchoalveolar lavage–derived cells, tracheal epithelium, lung and heart as well as against oxidative damage to pulmonary DNA (7). Importantly, the protective effects of NAC have been observed under a range of conditions produced by a variety of treatments or homeostatic imbalances. However, we recently demonstrated that under physiological conditions, NAC does not alter the expression of multiple genes in the mouse lung, as detected by complementary DNA array technology. Despite this finding, there is overwhelming evidence that NAC has the ability to modulate a variety of DNA damage and cancer-related end-points (8).

PEITC is a naturally occurring isothiocyanate produced by Brassicaceae that has entered phase 1 clinical trials (National Institute of Cancer) as a preventive agent against lung cancer (9). The main mechanism of action of PEITC is the stimulation of enzymatic activities involved in the detoxification of carcinogens, i.e. phase II metabolic reactions. Furthermore, PEITC possesses complementary pharmacodynamic mechanisms, including inhibition of angiogenesis and hypoxia-inducible factor, a transcription factor that plays an important role in the expression of pro-angiogenic factors. PEITC has antitumor activity in various in vitro and in vivo models (10). PEITC is insoluble in water and is rapidly catabolized by glucuronidation and extruded outside of cells by efflux proteins. Accordingly, high doses of PEITC are required for chemopreventive effects (11). These limitations have posed major challenges to the development of an efficient delivery system for PEITC and achievement of its therapeutic concentrations in target cells.

Similar problems limit the chemopreventive efficacy of resveratrol (RES). RES is a naturally occurring polyphenol contained in the skin of red grapes. RES possesses a broad spectrum of mechanisms of action, including stimulating enzymatic activities involved in the detoxification of carcinogens (12) and inducing apoptosis in DNA damage-bearing cells (13). The potential modulation of HSP27 levels using natural alternative agents such as RES might be an effective adjuvant in breast cancer therapy (14). Because of these properties, RES has been proposed as a putative chemopreventive agent for various diseases, including lung cancer, colon cancer, breast cancer, cardiovascular disease and glaucoma (14–18). However, RES is insoluble in water and is rapidly catabolized by glucuronidation and extruded outside the cell by efflux proteins. These mechanisms greatly limit its bioavailability, which has been estimated to be <1% when administered by the oral route (19).

The use of nanotechnologies in medicine to selectively transport drugs into cancer cells or target tissues is growing exponentially. Liposomes are spherical bilayer vesicles with an aqueous interior that are formed by the self-association of amphiphilic phospholipids. This self-associating behavior of phospholipids originates from their tendency to shield hydrophobic groups from an aqueous environment while interacting with the aqueous phase of their hydrophilic groups. These lipid-based carriers could, in principle, significantly enhance the solubility of poorly water-soluble drugs, such as cancer chemopreventive agents. Depending on their lipophilic character, drugs can distribute inside lipid NPs in the phospholipid bilayer, the interior aqueous phase, or the lipid bilayer-water interface. The lipophilic nature of many chemopreventives, including PEITC and RES, makes them suitable candidates for liposomal drug delivery because the lipophilic core of these liposomes provides an optimum environment for the entrapment of a lipophilic drug (20,21).

Administration via liposomal NPs could significantly enhance the efficacy of poorly water-soluble agents such as PEITC and RES. NP delivery has also been proposed for highly water-soluble agents such as NAC (22), despite its prompt bioavailability and efficacy when administered alone both in vitro and in vivo (8). Liposomal NPs have been used for microRNA delivery and show great potential for the development of microRNA-based therapeutics for cancer treatment and prevention. Recently, cationic lipids, as those used in this study, have been used both in vitro and in vivo to condense microRNAs to form lipoplexes to enhance the cellular uptake and pharmacological effectiveness of short oligonucleotides (23). This study provided evidence that lipid NP are able to reach high tissue concentration in various organs including lung (23).

The aim of this study was to increase the efficacy of cancer chemopreventive agents by using liposomal NPs as a carrier. The liposomal NPs used in this study had the following characteristics: (1) cationic head groups on their surfaces to efficiently bind to negatively charged cell membrane and (2) the ability to contain hydrophobic drugs on the lipid membrane or hydrophilic drugs inside the hollow space in the interior. Because of these characteristics, these NPs appear to have potential as carriers for chemopreventive drugs to increase their efficacy in counteracting DNA damage induced by carcinogens. This study evaluates the effects of liposomal NPs on the pharmacodynamic properties of chemopreventive agents, including NAC, PEITC and RES. The chemopreventive efficacy of these drugs was determined by evaluating their ability to counteract cytotoxic and molecular damage induced by CSC in epithelial bronchial...
cells. In vitro analyses in continuous cell line were used to set up the conditions to be applied in ex vivo experiment. The ex vivo experiment was performed in healthy epithelial cells collected from mouse trachea immediately after killing. Obtained results provided evidence that lipid NPs increase the efficacy of chemopreventive agents tested in protecting the respiratory system from CSC.

Materials and methods

Cell line
Human H727 bronchial carcinoma cells were obtained from the Cell Factory IST (Genoa, Italy). H727 cells were cultured in RPMI 1640 in the presence of 10% fetal calf serum and 2 mM l-glutamine in a humidified environment at 37°C with 5% CO2. H727 is the best available differentiated bronchial carcinoid cell line. The cells express easily detectable levels of p53 messenger RNA compared with levels found in normal lung.

Chemicals
NAC, PEITC and RES were purchased from Sigma-Aldrich® (St Louis, MO). To perform the experiments, the compounds were solubilized in phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO) to obtain stock solutions: NAC was diluted in PBS (Sigma-Aldrich) to a final concentration of 1.2 M; PEITC was diluted in DMSO to a final concentration of 10 mM and RES was diluted in DMSO (Sigma–Aldrich®) to a final concentration of 0.45 M.

CSC was obtained in our laboratory by bubbling CS into DMSO. A CSC stock solution was obtained by diluting CSC in DMSO to a final concentration of 120 mg/ml.

In all cell treatments, the final DMSO concentration in culture flasks was maintained below 1% to avoid any toxic effect.

NP preparation
Chemopreventive agents (NAC, PEITC and RES) were diluted in OptiMEM (Life Technologies Europe BV) containing NPs at 8 µg/ml. Empty liposomes were prepared by injecting 100 µl lipid mixture (1,2-di-O-octadecenyl-3trimethylammonium propane (chloride salt):cholesterol:α-Alpha-tocopheryl polyethylene glycol 1000 succinate = 49.5:49.5:1 molar ratio) in ethanol to 900 µl N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer (20mM, pH = 7.4). The solution was then mixed, vortexed for 5 s, incubated at room temperature for 15 min with chemopreventive agents (NAC, PEITC and RES) and added to the cells. The mean diameter of the obtained liposomal NPs was 70.0 ± 5.8 nm. Empty liposomes processed in the same manner but in absence of chemopreventive agents were used as controls.

Treatment with chemopreventive agents in vitro
H727 cells were pre-treated for 24 h with chemopreventive agents alone or inserted into NPs. After pre-treatment, cells were exposed to CSC (100 µg/ml) for 48 h. The following control conditions were tested without exposure to either chemopreventive agents or CSC: empty NPs (8 µg/ml), vehicle control (DMSO 0.5% or PBS 0.5% vol/vol in cell culture medium) and a negative control (cells maintained with cell culture medium only).

Dosing procedures were based on the literature, available data and preliminary experiments evaluating dose-response toxicity. All compounds were first tested alone to establish the IC20, i.e. the dose inhibiting cell viability by 20% as evaluated by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The following concentration ranges were tested: NPs (0.3–625 µg/ml), CSC (7.81–500 µg/ml), NAC (0.19–12 mM), PEITC (3.1–100 µM) and RES (0.01–0.45 µM). A concentration-response curve was plotted to obtain the IC20 using the software OriginPro 8.0. The following doses were then used: NAC (0.75, 1.5, 3.0 mM), PEITC (6.3, 12.5, 25.0 µM), RES (0.05, 0.10, 0.23 µM) and NPs (8.14 µg/ml). For CSC,

Figure 1. Microscopical appearance of H727 bronchial epithelial cells treated with chemopreventive agents (NAC, PEITC, RES) and NPs. Photographs on the left show that no difference from Sham can be observed for non-toxic treatments (NP, NAC). Conversely, toxic treatments (PEITC, RES) decrease cell count, results in loss of intercellular adherence, change cell shape compared with Sham. Histograms on the left report cell viability at various treatments doses. Statistical significant differences between chemopreventive compound + NPs (white columns) versus chemopreventive compound alone (dark gray columns) are indicated by asterisks (*P < 0.05, **P < 0.01).
an IC₅₀ of 88.00 µg/ml was determined. CSC was used at 100 µg/ml, corresponding to the IC₃₀.

Each experiment was performed as two independent experiments in triplicate. All treatments were performed using cells seeded in 96-well plates at 1 × 10⁴ cells per well. Treatments and MTT assays were performed 24 h after seeding, when the cells reached semi-confluence in all wells.

MTT cell viability assay

The MTT assay was performed to assess cell viability. After treatment with chemopreventive agents and/or CSC, the medium was removed, and the cells were incubated with 10 µl of MTT solution (5 mg/ml) for 3 h at 37°C. After incubation, DMSO was added to dilute the formazan salts, and the absorbance was read at 570 nm, with a subtraction background read at 660 nm. The percentage of viability was calculated by considering the untreated control as 100% growth using the following formula: viability (%) = (absorbance of treatment × 100)/(absorbance of negative control).

Comet assay

After treatment with chemopreventive agents either in the absence or presence of CSC and NPs for 24 h, the cells were trypsinized, re-suspended, homogenized in low-melting-point agarose, spread on a microscope slide pre-coated with normal-melting-point agarose and covered with a cover slip. The alkaline version of the comet assay (single-cell gel electrophoresis) was performed as described by Singh et al. (24). Duplicate slides were prepared and stained with ethidium bromide. For each sample, 50 cells were screened using a fluorescence microscope (Leica®, Wetzlar, Germany) equipped with an excitation filter at 515–560 nm and a barrier emission filter at 590 nm at ×400 magnification. The level of DNA damage was assessed by an image analysis system (TriTek CometScore® 1.5, 2006, 5363 Stewart Road Sumerduck VA, USA; http://AutoComet.com) to calculate the DNA per cent in the comet tail for each treatment.

Statistical comparison of the results of different treatments was performed using the non-parametric Kruskal–Wallis test associated with the Dunn post-test using statistical software (GraphPad Prism 5 software).

Annexin-V apoptosis assay

After treatments, cells were washed twice with PBS and detached by scraper. The collected cells were centrifuged at 1200g for 2 min, washed twice with PBS, incubated with 2 µM PBS-ethylenediaminetetraacetic acid at room temperature for 10 min to avoid the formation of aggregates and centrifuged. To evaluate the apoptosis level, annexin-binding buffer (100 µl) was added to pellet, followed by 5 µl of allylalcohol-cyanin-Annexin-V (BD Biosciences Pharmingen 2350 Qume Drive San Jose, CA). Then, 5 µl of 7-amino-actinomycin D (7-AAD; BD Biosciences) was added to exclude non-viable cells in the flow cytometric assay. The sample was incubated at room temperature for 15 min. Next, 400 µl of binding buffer was added, and the cell death profile was acquired and analyzed in a FACS Canto II cytometer (Becton Dickinson BD) by FACS Diva software (BD). Statistical analysis was performed by comparing the cytometric data of the vehicle control (0.5% DMSO or 0.5% PBS in cell culture medium) and the negative control (untreated cells) with the results of the treatments by one-way analysis of variance and Dunnett’s post-test. All results are expressed as mean ± SD.

DNA adduct analysis by ³²P postlabeling

An established ‘chemoprevention paradigm’ to evaluate the efficacy of chemopreventive compounds is represented by the inhibition of DNA adduct formation. Accordingly, we evaluated bulky DNA adducts in our experimental conditions by ³²P postlabeling after butanol enrichment as previously reported (7). Each sample was tested in triplicate and results expressed as adduct/10⁸ normal nucleotides (mean ± SD).

Figure 2. Microscopical appearance of H727 bronchial epithelial cells treated with CSC and chemopreventive agents (NAC, PEITC, RES) either in absence or presence of NPs. Photographs on the left show that CSC and CSC + NP results in decreased cell count, dramatic decrease of cell size, lost of intercellular adherence and formation of floating dead cells. These alterations are attenuated by NAC, PEITC + NP and RES + NP. Histograms on the left report cell viability at various treatments doses. Statistical significant differences between chemopreventive compound + NPs (white columns) versus chemopreventive compound alone (dark gray columns) are indicated by asterisks (*P < 0.05, **P < 0.01).
Treatment with chemopreventive agents in ex vivo experiment

Tracheas were collected from AJ mice and cleaned from surrounding muscle/connective tissue.

To evaluate the chemopreventive effect of NAC, PEITC and RES, the tracheas were maintained in basic medium added with the above-mentioned chemopreventive agents for 1 h and then treated with CSC for 2 h at 37°C and 5% CO₂. The protocol adopted the following eight experimental groups each composed of four mice accounting for a total of two mice: (1) Sham; (2) CSC (100 μg/ml); (3) CSC + NAC 3 mM; (4) CSC + NAC 3 mM + NP; (5) CSC + PEITC 25 μmol; (6) CSC + PEITC 25 μmol + NP; (7) CSC + RES 0.1 μmol; (8) CSC + RES 0.1 μmol + NP.

Glucose (8.14 mM) was added to each experimental group to maintain cell viability during the 3 h treatment.

At the end of the treatments, tracheas were incubated at 37°C for 30 min with collagenase IV at 1 mg/ml instilled in the lumen by incannulation. Tracheal epithelial cells were repeatedly washed out from lumen by isotonic phosphate buffer, pH 7.4. Obtained cell suspension was filtered through nylon strainers with 70 μm diameter pores and cells collected by centrifugation at 1000 r.p.m. for 10 min at 4°C. For each experimental group, collected cells were tested by cytofluorimetry to evaluate apoptosis by annexin-V staining utilizing a Muse Cell Analyzer (Millipore-Merck, Vimodrone MI, Italy). Cells were re-suspended in the Cell Dispersal Reagent (cat. MCH100107) to obtain a single-cell suspension. Cell viability and count were determined with the dedicated Muse kit (cat. MCH100102). Annexin-V staining was determined with the Muse Annexin V and Dead Cell Assay Kit (cat. MCH100105) following the manufacturer’s instructions. Gating was standardized utilizing the parameters obtained from three independent control samples.

Housing, breeding and treatment of mice were in accordance with National Institutes of Health and European (86/609/EEC Directive) guidelines.

Results

The NPs modified the pharmacodynamic properties of the tested chemopreventive agents.

Figure 1 (left panels) indicates cell morphology as an index of toxicity of chemopreventive agents when used alone. Spheric shape, assumed as indicator of cell suffering, was moderately increased by PEITC and RESV but not by NAC.

Figure 1 (right panels) reports the dose-response viability of H727 cells treated with NPs and/or chemopreventive agents. For NPs, the highest non-toxic dose that decreased viability by 20% (IC₂₀) was 8.14 µg/ml. An IC₂₀ of 3 mM was established for both NAC and NAC + NPs.

The IC₂₀ of PEITC was 12.5 mM both alone and in combination with NPs. The effects of PEITC were dose dependent in the presence and absence of NPs. In the absence of NPs, the lowest PEITC concentration (6.3 mM) resulted in 100% cell viability, whereas the highest concentration (25 mM) resulted in 10% cell viability. When PEITC was used with NPs, the lowest PEITC concentration (6.3 mM + NPs) resulted in 90% cell viability, whereas the highest PEITC concentration (25 mM + NPs) resulted in 5% cell viability.

For RES, the highest concentration (0.23 μM) resulted in 50% viability, whereas the lowest concentration (0.05 μM) resulted in 78% viability. When RES was administered with NPs (RES + NPs), the highest RES concentration (0.23 μM) resulted in 93% viability, whereas the lowest concentration (0.05 μM) resulted in 99% viability. This finding indicates that RES decreases NPs toxicity. This effect could be related to the decrease of intra-cellular membrane damage related to NP phagocytosis, RES being an established preservative of lipid membrane integrity (25).

Figure 2 shows the viability of H727 cells when treated with CSC alone (12.5 μg/ml) or in combination with either NPs or chemopreventive agents. CSC (12.5 μg/ml) decreased cell viability from 100% (Sham) to 69%. NPs increased CSC toxicity, decreasing cell viability to 47%. The increased CSC toxicity is likely related to the increased bioavailability of the lipophilic components of CSC induced by the NPs.

NAC alone counteracted CSC toxicity effectively at all tested doses. NAC efficacy was decreased in the presence of NPs but remained dose dependent; cell viabilities of 46% and 71% were observed at NAC concentrations of 0.75 and 3 mM, respectively.

Figure 3. Analysis by flow cytometry of live (dark gray columns), apoptotic (black columns) and necrotic (light gray columns) percentages in H727 cells treated with three different concentrations of chemopreventive agents (NAC, PEITC, RES) in presence or absence of NPs. Statistical significant differences between chemopreventive compound + NPs versus chemopreventive compound without NP are indicated by asterisks (* P < 0.05, ** P < 0.01).
The decrease in NAC efficacy in the presence of NPs may be due to the entrapment of lipophilic CSC components by the NPs, preventing their direct de-activation by NAC in the extracellular compartment.

PEITC treatment resulted in a moderate inhibition of CSC toxicity when this agent was used at 6.3 or 12.5 mM. When PEITC was used at the highest dose (25 mM), no decrease in CSC toxicity was observed, most likely due to the toxicity of PEITC at this high concentration. NPs significantly decreased the chemopreventive effects of PEITC, as evidenced by a decrease in cell viability. The decreased chemopreventive effect of PEITC in the presence of the NPs may be due to the increase in CSC bioavailability or PEITC bioavailability induced by the NPs.

RES alone was not effective for decreasing CSC toxicity. Conversely, when carried by NPs, RES was highly effective in counteracting the decreased cell viability induced by CSC. Accordingly, NPs dramatically and significantly increased the chemopreventive efficacy of RES against CSC, particularly when used at low, non-toxic doses. The NPs likely increase the cell penetration of RES, thereby increasing its typically low bioavailability. Indeed, only 1% of RES consumed with food intake penetrates inside cells (26).

The safety of the NPs was evaluated by assessing their ability to modulate DNA fragmentation induced by chemopreventive agents alone. The efficacy of the NPs was evaluated by assessing their ability to modulate the effects of chemopreventive agents on CSC genotoxicity. The antigenotoxicity of the chemopreventive agents was evaluated using a comet assay after treatment with CSC in the presence of NAC, PEITC or RES at different doses in either the absence or the presence of NPs.

When tested alone, NAC was safer than PEITC or RES. At the highest tested dose, the per cent DNA in the tail after NAC was 1.6%, compared with 8.0% for PEITC and 14.0% for RES. The use of NPs did not modify NAC-induced DNA damage at low or intermediate doses, although a further decrease was detected at the highest tested dose. The NPs did not significantly modify the amount of DNA damage detected in the presence of PEITC at any tested dose. Conversely, NPs remarkably and significantly decreased DNA fragmentation induced by RES at all tested doses. These findings indicate that NPs are effective at decreasing RES toxicity.

NAC was effective at counteracting DNA fragmentation induced by CSC at all tested doses, regardless of its association with NPs. At all tested doses, PEITC significantly decreased CSC-induced DNA damage, and this protective effect was significantly increased by NPs only at the highest tested dose (5 µM). RES significantly decreased CSC genotoxicity at all tested doses. NPs significantly increased the protective effects of intermediate and high doses of RES (0.1 and 0.23 µM). NP effect on RESV was significant at both doses (0.1 and 0.23 µM) without any significant difference between the doses.

Figure 4. Analysis by flow cytometry of live (dark gray columns), apoptotic (black columns) and necrotic (light gray columns) percentages in H727 cells exposed to CSC and treated with three different concentrations of chemopreventive agents (NAC, PEITC, RES) either in presence or absence of NPs. Statistical significant differences between chemopreventive compound plus NPs versus chemopreventive compound without NP are indicated by asterisks (*P < 0.05, **P < 0.01).
Apoptosis was detected using annexin-V-fluorescein isothiocyanate and 7-AAD to distinguish apoptotic cells from necrotic cells (Figure 3). An example of the flow cytometry analysis is shown in the lower right panel of Figure 3. In this panel, the upper-right quadrant (Q2) contains necrotic cells, which were positive for both annexin-V and 7-AAD. The lower-left quadrant (Q3) contains live cells, which were negative for both annexin-V and 7-AAD, and the lower right quadrant (Q4) contains apoptotic cells, which were positive for annexin-V but negative for 7-AAD.

NAC did not induce any significant variation in necrosis or apoptosis compared with the control when used alone or in combination with NPs (Figure 3, upper-left panel). PEITC increased apoptosis at all tested doses, without a clear dose-response relationship. PEITC increased necrosis only at the highest tested dose. NPs increased PEITC-induced apoptosis at all tested doses while decreasing necrosis only at the lowest tested (6.3 μM) dose (Figure 3, upper-right panel).

RES increased apoptosis only when used at the highest dose. Necrosis was increased at all tested doses, but a clear dose-response relationship was not observed. NPs significantly increased RESV-induced apoptosis while decreasing necrosis at the intermediate (0.1 μM) dose (Figure 3, lower-left panel).

Apoptosis and necrosis were assessed in cells exposed to CSC and treated with chemopreventive agents in the presence or absence of NPs (Figure 4). CSC dramatically increased necrosis, but apoptosis was significantly decreased (*P < 0.05). The effects of NAC on apoptosis and necrosis were not altered by either alone or in combination with NP, significantly increased RESV-induced apoptosis while decreasing necrosis at the intermediate (0.1 μM) dose (Figure 3, lower-left panel).

The ability of NP in increasing efficacy of RES and PEITC, but not of NAC was confirmed by DNA adduct analysis. Indeed, as reported in Table 1, NAC significantly decreased DNA adducts induced by CSC independently of NP presence. NAC used at high dose (3.0 mM) without NP counteracted CSC-induced adduct formation to such an extent that no significant difference with Sham occurred (CSC + NAC). PEITC displayed an intermediate protective effect significantly decreasing DNA adducts compared with CSC but still maintaining their levels higher than Sham. NP increased by 44% the efficacy of PEITC 6.3 μM in attenuating CSC-induced DNA adducts.

RES at intermediate and high doses (0.10 and 0.23 μM), either alone or in combination with NP, significantly increased DNA adduct level compared with Sham. However, this adverse effect was not detected at the low RES dose (0.05 μM). RES alone displayed only minimal effect on CSC-induced DNA adducts, slightly attenuating their formation at intermediate and at high doses. NP dramatically increased the chemopreventive efficacy of RES. Indeed, when used at high dose (0.23 mM) with NP, RES fully counteracted CSC-induced adduct formation to such an extent that no significant difference with Sham occurred.

### Table 1. Bulky lipophilic DNA adducts as evaluated in variously treated H727 cells by 32P postlabeling

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adducts/10⁶ nucleotides</th>
<th>Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.06 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>CSC</td>
<td>11.84 ± 2.46</td>
<td></td>
</tr>
<tr>
<td>NAC (0.75 mM)</td>
<td>1.45 ± 0.20</td>
<td>↑ versus Sham***</td>
</tr>
<tr>
<td>NAC (1.50 mM)</td>
<td>1.09 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>NAC (3.00 mM)</td>
<td>1.17 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>NAC (0.75 mM) + NP</td>
<td>1.02 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>NAC (1.50 mM) + NP</td>
<td>1.45 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>NAC (3.00 mM) + NP</td>
<td>1.12 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>CSC + NAC (0.75 mM)</td>
<td>3.04 ± 1.08</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + NAC (1.50 mM)</td>
<td>2.44 ± 0.92</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + NAC (3.00 mM)</td>
<td>2.03 ± 0.58</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + NAC (0.75 mM) + NP</td>
<td>5.24 ± 0.74</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + NAC (1.50 mM) + NP</td>
<td>3.28 ± 0.66</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + NAC (3.00 mM) + NP</td>
<td>3.1 ± 0.75</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>PEITC (6.3 μM)</td>
<td>2.02 ± 0.34</td>
<td></td>
</tr>
<tr>
<td>CSC + PEITC (6.3 μM)</td>
<td>5.30 ± 1.62</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + PEITC (6.3 μM) + NP</td>
<td>3.67 ± 1.02</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + PEITC (12.5 μM) + NP</td>
<td>3.31 ± 0.87</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + PEITC (25.0 μM) + NP</td>
<td>2.77 ± 0.56</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>RES (0.05 μM)</td>
<td>2.75 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>RES (0.10 μM)</td>
<td>3.21 ± 0.50</td>
<td>↑ versus Sham***</td>
</tr>
<tr>
<td>RES (0.23 μM)</td>
<td>4.18 ± 0.78</td>
<td>↑ versus Sham***</td>
</tr>
<tr>
<td>RES (0.05 μM) + NP</td>
<td>2.94 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>RES (0.10 μM) + NP</td>
<td>2.99 ± 0.57</td>
<td>↑ versus Sham***</td>
</tr>
<tr>
<td>RES (0.23 μM) + NP</td>
<td>3.56 ± 0.74</td>
<td>↑ versus Sham***</td>
</tr>
<tr>
<td>CSC + RES (0.05 μM)</td>
<td>10.39 ± 3.21</td>
<td>↑ versus Sham***</td>
</tr>
<tr>
<td>CSC + RES (0.10 μM)</td>
<td>7.74 ± 2.35</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + RES (0.23 μM)</td>
<td>8.59 ± 2.50</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + RES (0.05 μM) + NP</td>
<td>5.47 ± 1.94</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + RES (0.10 μM) + NP</td>
<td>4.00 ± 0.88</td>
<td>↑ versus Sham***; ↑ versus CSC***</td>
</tr>
<tr>
<td>CSC + RES (0.23 μM) + NP</td>
<td>1.56 ± 0.43</td>
<td>↑ versus Sham***</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001.
The *ex vivo* treatment of tracheal epithelial cells from mice confirms that NPs are effective in significantly increasing protecting efficacy, in terms of apoptosis induction, of RESV while being not effective with NAC and PEITC. Indeed, as reported in Figure 5, total apoptotic cells in each experimental group were 17.3% (control), 27.8% (CSC), 35.5% (CSC + NAC), 40.4% (CSC + NAC + NP), 59.0% (CSC + PEITC), 62.7% (CSC + PEITC + NP), 25.9% (CSC + RESV), 48.1% (CSC + RESV + NP). NP effects on RESV efficacy were more pronounced on early (2.3-fold increase) than late (1.5-fold increase) apoptosis.

**Discussion**

These results provide experimental evidence that liposomal NPs can modify the pharmacodynamics of chemopreventive agents and that this modulation is strictly dependent on the characteristics of the specific chemopreventive agent. Highly lipophilic agents, which have poor bioavailability, such as RES, were dramatically influenced by NPs, which increased RES bioavailability, decreased its toxicity and increased its efficacy in protecting against CSC-induced damage. An intermediate effect was observed for PEITC, and NPs did not exert any influence on hydrophilic chemopreventive agents with high bioavailability, such as NAC. Many studies have focused on NAC, which increases survival in cultured human bronchial cells and counteracts the toxicity of CSCs and their non-volatile and semi-volatile fractions in rat hepatocytes and lung cells. NAC has protective effects against DNA damage and carcinogenesis. We previously detailed these mechanisms in the context of smoking-related end-points, as evaluated in *in vitro* test systems, experimental animals and clinical trials (8). We have also shown that in mouse lung under physiological conditions, NAC does not alter the expression of multiple genes as detected by complementary DNA array technology. In this study, we evaluated the effect of NPs on the chemopreventive efficacy of NAC against exposure to CSC. The results obtained using our model confirmed the antigenotoxic effects of NAC against CSC. However, NPs did not exert any influence on NAC efficacy due to the high inherent bioavailability of NAC, which allows it to fully exert its effects in both extra- and intra-cellular compartments in the absence of NPs.

An intermediate result was obtained for the effect of NPs on PEITC. PEITC is an established chemopreventive agent that is able to attenuate the *in vivo* molecular alterations induced by exposure to CS, including DNA adduct formation, gene expression modifications and microRNA alterations in the lung (30–32). PEITC acted as an antigenotoxic compound protecting mammalian cells from CSC only at high concentrations (6.3 µM) and induced a dose-dependent decrease in cell viability. PEITC was barely effective in protecting cells from a CSC-induced decrease in viability but was more effective at decreasing CSC-induced DNA fragmentation and increasing apoptosis. The *in vitro* experiment confirmed the effect of PEITC on the induction of apoptosis in HT-29 cells in a time- and dose-dependent manner via the mitochondrial caspase cascade, which is critical for the initiation of apoptotic processes. This mechanism may play an important role in the killing of cancerous cells and offers a potential explanation for its anticancer action. PEITC has demonstrated effectiveness against metastatic breast cancer and small cell lung cancer (33,34). Although PEITC is a potent anticancer agent, its usage is limited due to its low water
solubility and high susceptibility to efflux-transporters in tumor cells. Because PEITC-induced cell cycle arrest and apoptosis are time-dependent processes, prolonged exposure of PEITC exerts more significant effects than multiple PEITC doses in Calu-3 cells (35). We demonstrated that PEITC increases apoptosis in the presence of NPs at 8 µg/ml, in agreement with the results of the previously cited study.

In this study, NPs significantly increased the efficacy of PEITC for counteracting CSC-induced DNA fragmentation only when PEITC was used at the highest tested dose (6.3 µM), likely due to the intermediate bioavailability of PEITC. This result is in agreement with a previous study of the efficacy of chitosan-solid lipid NP-microparticles as carriers for PEITC in which the release profile of PEITC loaded in these microparticles and its cytotoxicity in the presence or absence of efflux-transporter inhibitors were evaluated (21).

NPs had the greatest effect on the activity of RES, particularly when this compound was used at higher concentrations (1.2 µg/ml). Many studies have demonstrated that RES is slightly cytotoxic. This cytotoxicity can be prevented by loading it into solid lipid NPs, which preserve cell morphology (36). The cytotoxic effect of solid lipid NPs and RES was much higher than that of RES in solution. Delivery of RES by solid lipid NPs enhanced the effectiveness of RES for decreasing cell proliferation, with potential benefits for the prevention of cancer (37).

The percentage of encapsulation of both solid unloaded and RES-loaded NPs indicated a homogenous size distribution with no statistically significant differences, suggesting that RES incorporation does not influence the size of the NPs (38). In vitro release studies under storage conditions revealed negligible RES release over several hours in both nanosystems and in an in vitro simulation of gastrointestinal transit. Both of these approaches demonstrated that RES remained mostly associated with the lipid NPs, even after their incubation in digestive fluids (38).

On the whole, obtained results indicate that (1) NP does not have any effect on NAC, (2) PEITC reduces cell viability, as reported in Figure 2, and (3) NPs increase RES-induced apoptosis. Accordingly, the results obtained here provide evidence that liposomal NPs are quite effective in increasing chemopreventive efficacy, particularly for lipophilic chemopreventive agents characterized by poor bioavailability, such as RES (39). These findings are in line with the established efficacy of RES in counteracting CS toxicity in epithelial cells as recently reported for keratinocytes (40). Liposomal NPs have been reported to possess a full range of positive effects as controlled nano-delivery systems. For instance, lipopexes achieve much higher lung accumulation (30%) (23) without inducing adverse effects at variance with solid metal-based NPs (41). Hematoxylin and eosin staining of lung tissues indicated that the lipopexes did not affect inflammation, suggesting a lack of immediate lung toxicity (23).

In conclusion, this study demonstrates that NP has the potential to improve drug delivery to the periphery of CS-damaged lungs (42). NAC, PEITC and RES were significantly effective at reducing the levels of oxidative stress and genotoxicity induced by CSC in epithelial bronchial cells.

Acknowledgement
Conflict of Interest Statement: None declared.

References


