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Effects of 1,4-phenylenebis(methylene)selenocyanate on mutagenesis and p53 protein expression in the tongue of *lacI* rats treated with 4-nitroquinoline-N-oxide

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Abstract

Previously we showed that the organoselenium compound, 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC)1 inhibits 4-nitroquinoline-N-oxide (4-NQO)-induced tongue tumorigenesis in Fisher rats. Here we investigate possible mechanisms of this inhibition by monitoring mutagenesis and p53 protein levels in *lacI* and conventional Fisher rats treated with: 1) a carcinogenic dose of 4-NQO for 10 weeks in drinking water, 2) 4-NQO + *p*-XSC (15 ppm as selenium), and 3) 4-NQO followed by *p*-XSC. For mutagenesis studies, rats were euthanized at 7, 12 or 23 wks after the start of 4-NQO. For studies on p53 levels, rats were euthanized at 11, 15 and 23 wks. Appropriate controls were also monitored. In the 4-NQO-alone groups, the mutant fraction (MF) in the *cII* gene in tongue increased at least 50 × background level. The MF (in units of mutants/10⁵ plaque forming units) for the 7, 12 and 23 week 4-NQO groups were respectively, 184 ± 88, 237 ± 105, 329 ± 110. Thus, mutagenesis increased with length of exposure and post-treatment time. *p*-XSC modestly (ca. 15 - 30%) inhibited mutagenesis under all conditions. The inhibition reached significance at the last time point. When *p*-XSC was administered after 4-NQO, the MF was also modestly reduced. In 4-NQO-alone animals, levels of p53 in tongue (determined by Western blotting) were 1, 1.5 and 2.4 control levels at 10, 15 and 23 weeks respectively. In the *p*-XSC + 4-NQO group, the enhancement in p53 levels by 4-NQO treatment was decreased about 90% at 15 weeks and 45% ($P < 0.05$) at 23 weeks, and by slightly smaller percentages in corresponding post-treatment groups. *p*-XSC alone did not alter p53 levels. As p53 levels generally increase in response to DNA damage, these results suggest that *p*-XSC reduces 4-NQO-induced DNA damage, resulting in reduced 4-NQO-induced mutagenesis and carcinogenesis. However, the fact that *p*-XSC is also effective when administered after 4-NQO, suggests additional mechanisms of inhibition exist.

¹*p*-XSC, phenylenebis(methylene)selenocyanate; MF, mutant fraction; 4-NQO, 4-nitroquinoline-N-oxide

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Keywords

oral; chemoprevention; cII; mutagenesis; 4-NQO; selenium; p-XSC

Introduction

In the USA, over 30,000 cases and about 8,000 deaths from oral cancer occur annually [1]. Oral cancer is the most common neoplasm of the head and neck squamous cell carcinoma (HNSCC) [2]. Worldwide, the annual incidence of new cases is about 390,000 [3]. Developing countries, especially in southern Asia (e.g. India), as well as in China and certain regions of Brazil, are known to have the highest rates of oral cancer [3-7] it constitutes up to 25 % of all types of cancers [7]. The oral cancer incidence and mortality rates have been increasing in the USA, Japan, Germany and Scotland, especially among young males [8] [6]. Histologically, the most frequent cancers of the oral cavity are squamous cell carcinomas (SCC). Oral cancer progresses from hyperplastic lesions, through dysplasia and carcinoma *in situ* to invasive carcinoma [9-11]. Patients with oral cancers have an increased incidence of second primary tumors of the oral cavity [12] [13]. Although patients with early lesions have a high cure rate of their primary tumors; continued exposure to carcinogenic insult (e.g. tobacco and alcohol) and potential promoters will accelerate the development of a second primary [13,14]. Currently, oral cancer is generally treated with surgery although there has been some success with retinoids [15]. Surgery is only partially successful and can lead to disfigurement. Prevention is obviously preferential to treatment after the fact, and preventive strategies can be tailored to the status of the individuals. An obvious means of preventing the initiation and progression of the disease would be to remove the etiological factors; however, this strategy is very difficult to implement due to the addictive power of etiological agents (tobacco products and alcohol). These agents are believed responsible for about 75% of oral cancers in the US [16]. The use of naturally-occurring or synthetic agents that can inhibit the appearance of clinically detectable tumors is an attractive and reasonable approach to the prevention of cancer [17]. Studies on chemoprevention of oral cancer have generally focused on two carcinogens, namely 4-nitroquinoline-N-oxide (4-NQO) [11] and 7,12-dimethylbenz[a]anthracene [18].

Previously, we showed that the organoselenium compound, 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC) administered in the diet during either the initiation or post-initiation phases of carcinogenesis, significantly reduced the incidence of carcinoma of the tongues of rats treated with 4-NQO [19]. A reasonable hypothesis is that selenium compounds act as suppressing agents, possibly by inducing apoptosis and inhibiting cell proliferation [20]. Consistent with this hypothesis, we showed that *p*-XSC induces Fas-L/JNK-dependent apoptosis in oral cancer cells [21]. In addition, *p*-XSC was found to enhance the endogenous antioxidant and glutathione (GSH)-related antioxidant levels in the lung of A/J mice [22] and caused significant reduction of NNK-induced 8-oxo-dG formation in A/J mice [23].

As mutations in proto-oncogenes and tumor suppressor genes are generally only detectable in tumor tissue (where the mutants outgrow the normal cells), if mutations at earlier stages of carcinogenesis are to be investigated, a surrogate gene must be employed. The *lacZ* and *lacI* transgenic rodent systems have proved to be appropriate models for *in vivo* mutagenesis before tumors develop [24-26].

4-NQO is a DNA damaging agent [27] and if that damage is not repaired, it may result induction of mutations. In the paradigm of the multi-step model of carcinogenesis, the accumulation of multiple alterations in critical genes is essential for the development of cancer. An example of one such gene is *p53*, where characteristic mutations (signature mutations) in human cancers have the potential to serve as molecular markers of past exposure to specific carcinogens

[28]. Inactivation and/or mutation of p53 protein now represents the best described and a most common genetic change in all human cancers [29,30]. About 50 % of all primary HNSCC harbor *p53* mutations in the conserved region (exons 5-9)[31,32]. The expression and accumulation of p53 phenotype is associated with histologic progression of the oral squamous cell carcinoma and acquisition of invasive potential [33,34]. As a “guardian of the genome” p53 protein is activated and its levels are generally increased in response to cellular stress, including that resulting from DNA damaging agents [35]. In the present study we have investigated the possible mechanisms by which *p*-XSC inhibits 4-NQO-induced tongue carcinogenesis [19] by monitoring mutagenesis and p53 protein levels in tongues of *lacI* F344 rats.

Methods

Rats and treatment

Male Big Blue™ transgenic Fisher 344 rats (BBR) were purchased from Stratagene (La Jolla, CA) For studies not requiring the *lacI* gene, conventional male F344 (Charles River, Kingston, NY) were used, as these are less costly. Rats were housed in an animal facility maintained on a 12-h light/dark cycle, at a constant temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 15\%$) controlled room. The rats were fed a Purina Chow 5001 diet *ad libitum*.

The experimental protocol is shown in Fig. 1 and is similar to that previously described [19]. It was used for BB and conventional F344 rats. The bioassays for both mutagenesis and p53 protein expression were performed in parallel. Animals in group 1 received 10 ppm 4-NQO in their drinking water for 10 weeks. Group 2 received 4-NQO, and a diet containing 30 ppm *p*-XSC (15 ppm as selenium) starting one week before 4-NQO treatment and continuing for 12 weeks. Group 3 received 4-NQO for 10 weeks and 30 ppm *p*-XSC starting one week after removing 4-NQO from drinking water and continuing for another 12 weeks. Group 4 received 30 ppm *p*-XSC in the diet for 24 weeks and group 5 received regular tap water and diet only.

Mutagenesis assay

The effects of *p*-XSC on mutagenesis were determined at different time points during the progress of the bioassay. Eight rats from group 1 and group 2 were euthanized at 7 weeks or 12 weeks respectively, after the start of 4-NQO treatment. In addition, another 8 rats from all groups were euthanized 23 weeks after the start of 4-NQO treatment. The tongues were excised and stored at -80°C until isolation of DNA.

DNA isolation

A portion of the tongue tissue was gently homogenized by hand in a ground glass homogenizer using three volumes of 10mM Tris-HCl (pH, 8.0), 10mM EDTA, 150mM NaCl per part tissue weight (w/v). To this mix, SDS 1% and protease K, one mg/ml, were added. The mixture was incubated overnight at 37°C or 2-3 hr at 50°C , and then for 30 min at 37°C with 0.1mg/ml RNaseA. After this, one third volume of 6M, pH 7.4 ammonium acetate was added; the mixture was gently mixed and then centrifuged at 14,000 rpm in an Eppendorf microfuge. The supernatant was carefully removed, leaving a small volume behind, so as not to transfer any of the precipitate. To the supernatant an equal volume of isopropyl alcohol was added at room temperature to precipitate DNA. The supernatant was removed, the DNA was washed once with 70% ethanol, suspended in 10mM Tris-HCl (pH, 8.0), 1mM EDTA, and left at room temperature overnight to dissolve.

cII assay

The BB rat contains a lambda shuttle vector that includes the bacterial *lacI* locus and also the *cII* gene, which is the target for the mutagenesis studies. This assay detects mutations at the *cII* locus and possibly the regulator *cI* locus [36,37]. The *cII* protein is a positive regulator of gene transcription that controls the decision between lytic or lysogenic development pathways in phage-infected cells. In appropriate *E.coli* (*E.coli* 1250) host cells, under specified conditions (25°C) only mutants give rise to phage plaques, whereas at 37°C all infected cells give rise to plaques, providing a phage titer [37]. The ratio of mutant to non-mutant plaques is the measure of mutagenesis, the mutant fraction. Phage packaging was carried out using a Transpack packaging mix (Stratagene, La Jolla, CA), and the *cII* mutation assay was performed according to published techniques [37]. At least 20 mutants were scored for each sample.

Amplification and sequencing

Mutants were cored from petri dishes and the agar plug was mixed with 100 µl phage buffer. 10 µl of the buffer was then spread on a selective plate to confirm mutant phenotype and purify mutant phages. Mutant plaques were randomly selected. The purified mutant plaques were the subjected to amplification and sequencing of the *cII* gene by PCR. Sequencing was performed by Roderick Haesevoets, University of Victoria, B.C., Canada.

Amplification: Primer sequences, forward 5'-AAAAAGGGCATCAAATTAAC-3', reverse: 5'-CCGAAGTTGAGTATTTTGCTGT-3' Reaction Mixture (100 µl reaction) H₂O, 59.1 µl; 100mM dNTP mix, 1.0 µl; 10× buffer, 10 µl; *cII* forward primer (10 µM) 2.0 µl; *cII* reverse primer (10.0 µM) 2.0 µl; 50mM MgCl₂, 3.5 µl; Taq, 2.4 µl; sample, 20 µl; 10× buffer: 100 mM Tris HCl pH 9.0, 500 mM KCl, 1.0% Triton×100.

PCR conditions: 94.0°, 4.0 min; 30 cycles: 95.0°, 30 sec; 55.0°, 30 sec; 72.0°, 2.5 min; 4.0°, hold.

Purification: PCR Product Pre-Sequencing Kit (USB); as per kit instructions.

Sequencing: Primer sequences, *cII* forward: 5'-ACCACACCTATGGTGTATG-3', *cII* reverse, 5'-GTCATAATGACTCCTGTTGA-3' (only used to confirm a mutation if the sequence from *cII* forward primer was not clear).

Reaction Mixture: CEQ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman-Coulter).

PCR conditions: 96.0°, 5.0 min, 30 cycles: 96.0°, 20 sec; 52.0°, 25 sec; 60.0°, 4.0 min; 4.0°, hold.

Electrophoresis/Base Calling/Trace Generation: Sequencing was done on a CEQ8000 Capillary Electrophoresis DNA Sequencer (Beckman-Coulter).

Analysis software: SeqMan II v6.1 (DNASTAR).

p53 protein expression using western blotting

The effects of 4-NQO and *p*-XSC individually and in combination on the expression of p53 protein were determined at different time points during the progress of the assay using the tongue tissues of F344 rats. Three rats from each of the five groups were euthanized 11, 15 and 23 week after the start of treatment with 4-NQO. The tongues were excised and stored at -80°C until isolation of protein.

The inside muscle of the tongue was removed, and the outside layer was used. The tongue tissues were homogenized by hand in a ground glass homogenizer using 3 volume of ice-cold RIPA buffer with protease inhibitor cocktail (Calbiochem, Darmstadt, Germany) and 1mM phenylmethylsulfonylfluoride. The resulting homogenate was then centrifuged at 14000 rpm for 10 min at 4°C. The supernatants were collected and assayed for protein concentration by D_c Protein assay (BioRad, Hercules, CA). Each protein sample, 25 µg was taken and mixed 1:1 with sample buffer, boiled for 5 min, electrophoresed on a 10% acrylamide-bis-acrylamide gel at 100 V for 110 min, then electro-transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk in phosphate-buffered solution with 1% Tween-20 (TBS-T) for one hour, and then incubated with primary antibody to mouse monoclonal anti-p53 (Upstate, Lake Placid, New York) or β -actin (as a reference standard, Sigma, St. Louis, Mo.) for another hour followed by incubation with their corresponding secondary antibody for another hour. After extensive washing, specific bands were detected using immobilon western chemiluminescent substrates (Millipore, MA.). Each blot compared 5 samples, they are untreated control, *p*-XSC treated, *p*-XSC → 4-NQO (*p*-XSC given post 4-NQO), *p*-XSC + 4-NQO (*p*-XSC given with 4-NQO), 4-NQO alone. All the bands were normalized to 1.0 against the untreated control's band in their own blot.

A two tailed t-test was used to compare the differences between bands.

Immunohistochemistry (IHC)

Bouin's fixed, paraffin-embedded tissue blocks were sectioned at 5 microns. Slides were baked for 1 h at 50°C, deparaffinized in three changes of xylene and hydrated through a series of ethanol solutions (100%, 100%, 95%). Slides were then cleared in lithium carbonate saturated 70% ethanol solution for 4 minutes. Slides were heated in a household vegetable steamer in 1 mM EDTA buffer (pH 8.0) for 20 minutes for antigen retrieval and allowed to cool at room temperature for 20 minutes. Subsequently, the slides were treated with 0.05% Tween 20 in TBS for 5 min to permeabilize tissues and washed twice in TBS. The slides were treated with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. After washing, the slides were incubated with the primary antibody, p53 ([EP155Y] (phospho S392) (Abcam) diluted to 1:50), for 30 min at room temperature, incubated with Dako Envision ⁺™ anti-rabbit labeled polymer conjugated with HRP for 20 min at room temperature, developed using Dako DAB⁺™, and counter-stained with Meyer's modified hematoxylin. All immunohistochemical staining procedures were performed on a Dako Autostainer Plus.

Results

Mutagenesis Assay

The results of mutagenesis assay are summarized in Table 1. The total treatment period with 4-NQO was 10 weeks. After seven weeks the mutant fraction was 184 ± 88 mutants/ 10^5 plaque forming units, or about 50 times the background level. The mutant fraction further increased to 237 ± 10^5 at 12 weeks after the start of 4-NQO treatment and even further (329 ± 110) 23 weeks after the start of treatment. Since the 4-NQO-treatment only lasted ten weeks, the complete fixation of mutations occurred well after the termination of treatment.

In all cases, inhibition of mutagenesis by *p*-XSC was observed. When co-administered with 4-NQO, *p*-XSC inhibited mutagenesis by 15 – 30%. The inhibition was statistically significant at the last time point (23 weeks). When *p*-XSC was administered after 4-NQO, the observed mutant fraction was reduced compared to the 4-NQO-alone group, but the decrease in mutagenesis did not quite reach significance ($P = 0.06$).

In order to rule out the possibility that clonal expansion was contributing to mutagenesis, mutant plaques from DNA from each of four rats were sequenced (Table 2). All rats were euthanized at 23 weeks. This relatively long time period should provide the greatest opportunity for clonal mutants to accumulate. A total of ten mutant plaques were randomly chosen from rat DNA, and PCR-amplified before sequencing. One plaque from each DNA failed to amplify, possibly due to a mutation that modified a primer binding site, or a large insertion or deletion in the *cII* sequence. In three of four rats (all exhibiting very high MF's) only one clonal mutation was detected. In the fourth (which had a lower mutant fraction), 1/3 of the mutants were identical. Hence, it is very unlikely clonal mutants contribute significantly to the higher MF's at the last time point.

p53 protein expression using western blotting

The results of the Western blotting analysis are summarized in Table 3 and images of the blots are displayed in Figure 2. In addition a figure providing an overview of the effects of *p*-XSC on mutagenesis and p53 levels at 23 weeks is included (Fig. 3). These results are consistent with the Western blotting results, but are not quantitative. After 10-weeks of treatment with 4-NQO and one week post-treatment, p53 levels were similar to control levels. However, by 15 weeks (five weeks post-treatment) the level of p53 protein was elevated 1.5-fold above control levels, and by 23 weeks post-treatment, p53 levels increased 2.4-fold relative to the control.

When *p*-XSC was co-administered with 4-NQO, the enhancement in p53 level was inhibited by about 90% at 15 weeks, and 45% by *p*-XSC at 23 weeks. The increase in p53 levels was also inhibited by *p*-XSC treatment during the post-treatment phase at 15 and 23 weeks. A graphical comparison of the effects of *p*-XSC on mutagenesis and p53 protein levels is shown in Fig. 4.

Discussion

After 7 weeks of exposure to 4-NQO, the mutant fraction in the tongues of 4-NQO treated rats was nearly 50 times greater than background. Similar results were previously observed in the *lacZ* gene of *lacZ* mice [38,39]. As mutagenesis generally requires an expression period during which DNA damage is converted to mutations [40], the 7 week treatment period presumably encompasses sufficient time for 4-NQO-induced damage to be converted to mutations. Twelve weeks after the start of 4-NQO exposure (including two weeks post-exposure) the mutant fraction further increased. An additional 12 weeks post exposure resulted in a further substantial increase in the mutant fraction. This increase may reflect replication across unrepaired DNA damage, and/or error-prone DNA repair. It has been reported that 4-NQO treatment leads to enhanced rates of cell proliferation in rat tongue relative to controls, and that co- and post-treatment with *p*-XSC reduces this effect [19]. It should also be noted that the treatment with 4-NQO continued for 10 weeks and typically four weeks post-treatment are needed for the fixation of most mutants [40] (although this is organ and mutagen specific). It is then possible that all of the mutations were fixed by 14 weeks and at that point mutagenesis was complete. This could not be ascertained from this study, since 14 weeks did not coincide with the time points sampled. Also, as the 4-NQO treatment protocol here eventually leads to a high fraction of tongue tumors [19] there were dysplastic and neoplastic cells in the tongues, and genetic instability in such cells could also be driving mutagenesis at longer time periods. At all time points, mutagenesis was lower in *p*-XSC + 4-NQO treated rats than 4-NQO-treated only rats. The difference in mutant fraction was statistically significant at the last time point.

p-XSC was also an effective inhibitor of mutagenesis when administered shortly after 4-NQO. As a substantial portion of mutations are fixed following cessation of 4-NQO administration, certain events during this time period must be susceptible to modification by *p*-XSC. Since mutagenesis is highly dependent upon post-exposure period [37] a delay in replication by *p*-

XSC during this period, represents one potential mechanism of inhibition of mutagenesis. Indeed, inhibition of cellular proliferation in rat tongue weeks after co-administration of 4-NQO and *p*-XSC has been reported [19]. A decreased rate of cell proliferation allows more time for DNA repair. Removal of severely damaged cells via *p*-XSC-induced apoptosis represents another potential mechanism of protection. In addition to its effects on the cellular response to 4-NQO-induced damage, (which could occur during and after 4-NQO treatment) *p*-XSC could lead to enhanced detoxification of 4-NQO and/or its metabolites during 4-NQO treatment. For instance, it has been reported that administration of *p*-XSC enhances GST levels in rat tongue [19]. Transgenic GST expression was found to provide protection against genotoxicity induced (i.e. DNA adduct formation) by 4-NQO by catalyzing the nucleophilic substitution of the nitro group of 4-NQO with thiols leading to deactivation of 4-NQO [41, 42].

Similar to the results in shown in Table 3, the elevation of p53 levels in 4-NQO-treated rats has been previously reported [43,44]. *p*-XSC reduced the 4-NQO-induced elevation in p53 protein levels. Since p53 levels increase in response to many types of cellular stress, including DNA damage [35,45] these results also suggest that *p*-XSC reduces DNA damage. The observation that *p*-XSC inhibits the 4NQO-induced increase in p53 levels is consistent with the inhibition of 4-NQO-induced mutagenesis and carcinogenesis by *p*-XSC [19].

The fact that the percentage inhibition by *p*-XSC of p53 induction was greater at 15 weeks than 23 weeks might indicate that *p*-XSC delays the induction of p53 protein. However, the increase in p53 levels relative to background levels, was much smaller at 15 weeks than 23 weeks, and the higher ratio of variance in determination of p53 to the increase in p53 level at 15 weeks (i.e., lower signal to noise ratio at 15 weeks) could also account for the apparent large inhibition in the increase in p53 level at this time point.

In summary, the results of this study together with those reported in the literature by us and by others [21,23,46], suggest several potential mechanisms (Fig. 5), by which *p*-XSC can inhibit 4-NQO-induced carcinogenesis in the tongue of rats [19]. The mechanisms are based on the ability of the synthetic organoselenium compound, *p*-XSC, to inhibit DNA damage, mutagenesis, and cell proliferation [23,47], and to induce apoptosis [21].

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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106–130. [PubMed: 16514137]
2. K, P.; Stewart, BW., editors. *World Cancer Report*. 2003.
3. Parkin DM, Laara E, Muir CS. Estimates of the worldwide frequency of sixteen major cancers in 1980. *Int J Cancer* 1988;41:184–197. [PubMed: 3338870]
4. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. *CA Cancer J Clin* 1999;49:33–64. 31. [PubMed: 10200776]
5. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999;80:827–841. [PubMed: 10074914]
6. Mackenzie J, Ah-See K, Thakker N, Sloan P, Maran AG, Birch J, Macfarlane GJ. Increasing incidence of oral cancer amongst young persons: what is the aetiology? *Oral Oncol* 2000;36:387–389. [PubMed: 10899679]
7. Magrath I, Litvak J. Cancer in developing countries: opportunity and challenge. *J Natl Cancer Inst* 1993;85:862–874. [PubMed: 8492315]

8. Davis S, Severson RK. Increasing incidence of cancer of the tongue in the United States among young adults. *Lancet* 1987;2:910–911. [PubMed: 2889100]
9. Suzui M, Yoshimi N, Tanaka T, Mori H. Infrequent Ha-ras mutations and absence of Ki-ras, N-ras, and p53 mutations in 4-nitroquinoline 1-oxide-induced rat oral lesions. *Mol Carcinog* 1995;14:294–298. [PubMed: 8519419]
10. Kramer IR, Lucas RB, Pindborg JJ, Sobin LH. Definition of leukoplakia and related lesions: an aid to studies on oral precancer. *Oral Surg Oral Med Oral Pathol* 1978;46:518–539. [PubMed: 280847]
11. Tanaka T, Kojima T, Okumura A, Yoshimi N, Mori H. Alterations of the nucleolar organizer regions during 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rats. *Carcinogenesis* 1991;12:329–333. [PubMed: 1704823]
12. Jovanovic A, van der Tol IG, Schulten EA, Kostense PJ, de Vries N, Snow GB, van der Waal I. Risk of multiple primary tumors following oral squamous-cell carcinoma. *Int J Cancer* 1994;56:320–323. [PubMed: 8314317]
13. Licciardello JT, Spitz MR, Hong WK. Multiple primary cancer in patients with cancer of the head and neck: second cancer of the head and neck, esophagus, and lung. *Int J Radiat Oncol Biol Phys* 1989;17:467–476. [PubMed: 2674075]
14. Franco EL, Kowalski LP, Kanda JL. Risk factors for second cancers of the upper respiratory and digestive systems: a case-control study. *J Clin Epidemiol* 1991;44:615–625. [PubMed: 2066741]
15. Lippman SM, Sudbo J, Hong WK. Oral cancer prevention and the evolution of molecular-targeted drug development. *J Clin Oncol* 2005;23:346–356. [PubMed: 15637397]
16. Canto MT, Devesa SS. Oral cavity and pharynx cancer incidence rates in the United States, 1975–1998. *Oral Oncology* 2002;38:610–617. [PubMed: 12167440]
17. El-Bayoumy K, Sinha R. Molecular chemoprevention by selenium: a genomic approach. *Mutat Res* 2005;591:224–236. [PubMed: 16099479]
18. Schwartz JL, Gu X, Kittles RA, Baptiste A, Shklar G. Experimental oral carcinoma of the tongue and buccal mucosa: possible biologic markers linked to cancers at two anatomic sites. *Oral Oncol* 2000;36:225–235. [PubMed: 10745177]
19. Tanaka T, Makita H, Kawabata K, Mori H, El-Bayoumy K. 1,4-phenylenebis(methylene)selenocyanate exerts exceptional chemopreventive activity in rat tongue carcinogenesis. *Cancer Res* 1997;57:3644–3648. [PubMed: 9288763]
20. Sinha R, El-Bayoumy K. Apoptosis is a critical cellular event in cancer chemoprevention and chemotherapy by selenium compounds. *Curr Cancer Drug Targets* 2004;4:13–28. [PubMed: 14965264]
21. Ghose A, Fleming J, El-Bayoumy K, Harrison PR. Enhanced sensitivity of human oral carcinomas to induction of apoptosis by selenium compounds: involvement of mitogen-activated protein kinase and Fas pathways. *Cancer Research* 2001;61:7479–7487. [PubMed: 11606383]
22. Richie JP Jr, Kleinman W, Desai DH, Das A, Amin SG, Pinto JT, El-Bayoumy K. The organoselenium compound 1,4-phenylenebis(methylene)selenocyanate inhibits 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced tumorigenesis and enhances glutathione-related antioxidant levels in A/J mouse lung. *Chem Biol Interact* 2006;161:93–103. [PubMed: 16620795]
23. Rosa JG, Prokopczyk B, Desai DH, Amin SG, El-Bayoumy K. Elevated 8-deoxyguanosine levels in lung DNA of A/J mice and F344 rat treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and inhibition by dietary 1,4-phenylenebis(methylene)selenocyanate. *Carcinogenesis* 1998;19:1783–1788. [PubMed: 9806159]
24. Hakura A, Tsutsui Y, Sonoda J, Kai J, Imade T, Shimada M, Sugihara Y, Mikami T. Comparison between in vivo mutagenicity and carcinogenicity in multiple organs by benzo[a]pyrene in the lacZ transgenic mouse (Muta Mouse). *Mutat Res* 1998;398:123–130. [PubMed: 9626972]
25. Lambert IB, Singer TM, Boucher SE, Douglas GR. Detailed review of transgenic rodent mutation assays. *Mutat Res* 2005;590:1–280. [PubMed: 16081315]
26. van Delft JH, Bergmans A, van Dam FJ, Bates AD, Howard L, Winton DJ, Baan RA. Gene-mutation assays in lambda lacZ transgenic mice: comparison of lacZ with endogenous genes in splenocytes and small intestinal epithelium. *Mutat Res* 1998;415:85–96. [PubMed: 9711265]

27. Jones CJ, Edwards SM, Waters R. The repair of identified large DNA adducts induced by 4-nitroquinoline-1-oxide in normal or xeroderma pigmentosum group A human fibroblasts, and the role of DNA polymerases alpha or delta. *Carcinogenesis* 1989;10:1197–1201. [PubMed: 2500268]
28. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994;54:4855–4878. [PubMed: 8069852]
29. Hollstein MC, Sidransky D, Vogelstein B, Harris CC. p53 Mutations in Human Cancer. *Science* 1991;253:49–53. [PubMed: 1905840]
30. Hussain SP, Harris CC. Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. *Cancer Res* 1998;58:4023–4037. [PubMed: 9751603]
31. Boyle JO, Hakim J, Koch W, van der Riet P, Hruban RH, Roa RA, Correo R, Eby YJ, Ruppert JM, Sidransky D. The incidence of p53 mutations increases with progression of head and neck cancer. *Cancer Res* 1993;53:4477–4480. [PubMed: 8402617]
32. Brennan JA, Boyle JO, Koch WM, Goodman SN, Hruban RH, Eby YJ, Couch MJ, Forastiere AA, Sidransky D. Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. *N Engl J Med* 1995;332:712–717. [PubMed: 7854378]
33. Soni S, Kaur J, Kumar A, Chakravarti N, Mathur M, Bahadur S, Shukla NK, Deo SV, Ralhan R. Alterations of rb pathway components are frequent events in patients with oral epithelial dysplasia and predict clinical outcome in patients with squamous cell carcinoma. *Oncology* 2005;68:314–325. [PubMed: 16020958]
34. Raju B, Mehrotra R, Oijordsbakken G, Al-Sharabi AK, Vasstrand EN, Ibrahim SO. Expression of p53, cyclin D1 and Ki-67 in pre-malignant and malignant oral lesions: association with clinicopathological parameters. *Anticancer Res* 2005;25:4699–4706. [PubMed: 16334163]
35. Hussain SP, Harris CC. p53 biological network: at the crossroads of the cellular-stress response pathway and molecular carcinogenesis. *Journal of Nippon Medical School = Nihon Ika Daigaku Zasshi* 2006;73:54–64.
36. Zimmer DM, Harbach PR, Mattes WB, Aaron CS. Comparison of Mutant Frequencies at the Transgenic Lambda LacI and cII/cI Loci in Control and ENU-treated Big Blue Mice. *Environ Mol Mutag* 1999;33:249–256.
37. Jakubczak JL, Merlino G, French JE, Muller WJ, Paul B, Adhya S, Garg S. Analysis of Genetic Instability During Mammary Tumor Progression Using a Novel Selection-Based Assay for In Vivo Mutations in a Bacteriophage Transgene Target. *Proc Natl Acad Sci, USA* 1996;93:9073–9078. [PubMed: 8799156]
38. Guttenplan JB, Kosinska W, von Pressentin MM, Rosa J, El-Bayoumy K. Effects of 1,4-phenylenebis (methylene)selenocyanate (p-XSC) and vitamin E on 4-nitroquinoline-N-oxide (4-NQO)-induced mutagenesis in lacZ mouse upper aerodigestive tissue. *Mutat Res* 2002;518:85–93. [PubMed: 12063070]
39. von Pressentin MM, Kosinska W, Guttenplan JB. Mutagenesis induced by oral carcinogens in lacZ mouse (MutaMouse) tongue and other oral tissues. *Carcinogenesis* 1999;20:2167–2170. [PubMed: 10545421]
40. Suzuki T. Target organ and time-course in the mutagenicity of five carcinogens in MutaMouse: a summary report of the second collaborative study of the transgenic mouse mutation assay by JEMS/MMS. *Mutation Research* 1999;444:259–268. [PubMed: 10521667]
41. Osugi Y. p53 expression in various stages of 4-nitroquinoline 1-oxide induced carcinoma in the rat tongue. *J Osaka Dent Univ* 1996;30:29–35. [PubMed: 9485768]
42. Li T. [Expression of p53 protein during 4NQO-induced lingual carcinogenesis in two strains of rats]. *Zhonghua Kou Qiang Yi Xue Za Zhi* 2000;35:126–128. [PubMed: 11780483]
43. Fields WR, Li Y, Townsend AJ. Protection by transfected glutathione S-transferase isozymes against carcinogen-induced alkylation of cellular macromolecules in human MCF-7 cells. *Carcinogenesis* 1994;15:1155–1160. [PubMed: 8020149]
44. Varnes ME, Biaglow JE. Interactions of the carcinogen 4-nitroquinoline 1-oxide with the non-protein thiols of mammalian cells. *Cancer Res* 1979;39:2960–2965. [PubMed: 110443]

45. Lloyd DR, Hanawalt PC. p53 controls global nucleotide excision repair of low levels of structurally diverse benzo(g)chrysene-DNA adducts in human fibroblasts. *Cancer Res* 2002;62:5288–5294. [PubMed: 12234998]
46. El-Bayoumy K, Chae YH, Upadhyaya P, Meschter C, Cohen LA, Reddy BS. Inhibition of 7,12-dimethylbenz(a)anthracene-induced tumors and DNA adduct formation in the mammary glands of female Sprague-Dawley rats by the synthetic organoselenium compound 1,4-phenylenebis (methylene) selenocyanate. *Cancer Res* 1992;52:2402–2407. [PubMed: 1568209]
47. el-Bayoumy K. Environmental carcinogens that may be involved in human breast cancer etiology. 1992[Review] [80 refs]

Fig. 1.

Schematic representation of the animal protocol. Animals in groups 1-3 received 10 ppm 4-NQO in their drinking water for 10 weeks. Group 2 received a diet containing 30 ppm *p*-XSC (15 ppm as selenium) one week before 4-NQO treatment and continuing for 12 weeks. Group 3 received the diet containing *p*-XSC one week after removing 4-NQO from drinking water and continuing for another 12 weeks. Group 4 received 30 ppm *p*-XSC in the diet for 24 weeks and group 5 received regular tap water and diet only. For the mutagenesis study, 8 rats / group were used, and for the P53 study, 5 rats / group were used.

Fig. 2.
Representative images showing the effects of *p*-XSC on p53 expression in 4-NQO treated rat tongue tissues, using western blot analysis.

Gr. 1
4-NQO alone
 Invasive well-differentiated
 squamous cell carcinoma.
 Strong p53 expression in
 invasive and in-situ components.

H and E- 2x, invasive SCC

P53- 10x

P53- 20x

Gr. 2
4-NQO + p-XSC (ini.)
 Invasive well-differentiated
 squamous cell carcinoma.
 Weak p53 expression in invasive
 and in-situ components.

H and E- 2x, invasive SCC

P53- 10x

P53- 20x

Gr. 3
4-NQO + p-XSC (post)
 Mild dysplasia of squamous
 epithelium.
 Small foci of p53
 staining in basal cells.

H and E- 2x, dysplasia

P53- 10x

P53- 20x

Gr. 4
p-XSC alone
 Mild dysplasia of squamous
 epithelium.
 No p53 expression.

H and E- 2x, dysplasia

P53- 10x

P53- 20x

Gr. 5
no treatment
 Normal squamous epithelium.
 No p53 expression.

H and E 2x

H and E 20x

P53- 10x

P53- 20x

Fig. 3.
 Photos of Immunohistochemical slides showing the effects of 4-NQO and *p*-XSC treatments
 on p53 levels in rat tongue tissues at 23 weeks.

Fig. 4.

Comparison of results from mutagenesis and p53 expression studies. * $p = 0.032$ for 4-NQO + pXSC (initiation) vs. 4-NQO alone at 23 weeks in mutagenesis assay. ***,*** $p \leq 0.05$ for 4-NQO + pXSC (initiation) vs. 4-NQO alone at 15 and 23 weeks in p53 protein expression using western blotting.

Fig. 5. Potential mechanisms by which *p*-XSC can inhibit 4-NQO-induced mutagenesis and carcinogenesis. The figure is based on the results of this study combined with those reported previously [19,21,46].
 →, indicates induction or increase
 ┘, indicates inhibition

Table 1
Effects of treatment time and *p*-XSC on mutagenesis induced in tongue by 4-NQO in the *chl* gene of *lacI* rats ^a

	untreated	<i>p</i> -XSC	Mutant Fraction (in units of mutants/10 ⁵ plaque forming units)		
			4-NQO	4-NQO + <i>p</i> -XSC (initiation)	4-NQO + <i>p</i> -XSC (post-initiation)
7 weeks ^b	ND	ND	184 ± 88	141 ± 56	
12 weeks	ND	ND	237 ± 105	202 ± 117	
23 weeks	4.2 ± 1.3	3.1 ± 1.7	329 ± 110	237 ± 117 ^d	260 ± 73

Initiation, *p*-XSC given with 4-NQO; post-initiation, *p*-XSC given after 4-NQO.

^aEight rats/group

^bWeeks represent time after the start of 4-NQO administration. 10 weeks total administration.

^c± refers to the SD.

^d*p* = 0.032 vs. 4-NQO alone in 2-tailed Mann-Whitney test.

Table 2
Mutational distribution in the *cII* gene in tongues from 4-NQO-treated rats

Rat ¹	Group ²	plaque #	Position ^{3,4}	mutation	Context ⁵
1	1	1	-14	g>a	cta agg aaa
		2	103	G>C	GGC GTT GAT
		3	117	G>T	TCG CAG ATC
		4	125	G>A	AGC AGG TGG
		5	175	G>T	CTT GAA TGG
		6	190	G>A	GTT GAC GAC
		7	193	G>T	GAC GAC GAC
		8	212	C>T	TTG GCG CGA
		9	226	G>C	GCT GCG ATT
2	1	1	57	C>A	CTT AAC AAA
		2	135-136	GG>TT	AAG AAG GAC TAG
		3	140	G>A	GAC TGG ATT
		4	141	G>A	GAC TGG ATT
		5	163	C>A	CTG CTT GCT
		6	190	G>T	GTT GAC GAC
		7	193	G>T	GAC GAC GAC
		8	212	C>A	TTG GCG CGA
		9	214	C>T	GCG CGA CAA
		1	73	G>C	CTT GGA ACT
		2	89	C>T	ACA GCG GAA
		3	100	G>T	GTG GGC GTT
		4	103	G>T	GGC GTT GAT
		5	103	G>T	GGC GTT GAT
		6	160	C>A	ATG CTG CTT
		7	163	C>A	CTG CTT GCT
		8	205	C>T	GCT CGA TTG
		9	212	C>A	TTG GCG CGA
		1	58	A>C	AAC AAA ATC
		1	64	G>T	ATC GCA ATG
1	3	2	58	A>C	ACC AAA ATC

Rat ¹	Group ²	plaque #	Position ^{3,4}	mutation	Context ⁵
		2	64	G>T	ATC <u>G</u>CA ATG
		3	73	G>C	CTT <u>G</u> GA ACT
		4	73	G>C	CTT <u>G</u> GA ACT
		5	73	G>A	CTT <u>G</u> GA ACT
		6	111	G>C	GAT AAG TCG
		7	123	C>A	ATC AG <u>C</u> AGG
		8	179	G>T	GAA TGG GGG
		9	196	G>T	GAC <u>G</u> AC ATG

¹ Each group of 9 plaques was obtained from tongue DNA from a single rat, belonging to the indicated group.

² Group 1, 4-NQO alone; group 2, 4-NQO + *p*XSC (init); group 3, 4-NQO → *p*XSC (post).

³ Negative numbers represent upstream (from the *cII* gene) positions in the sequence.

⁴ Lower case letters represent upstream (from the *cII* gene) positions in the sequence.

The mutated position is underlined.

Identical mutants are bolded. In two cases in the group 3 sample, there were two mutations detected in the same mutant plaque.

Table 3
Effects of treatment time and *p*-XSC on p53 levels in tongue in the presence and absence of 4-NQO determined by Western blotting^a

Treatment and relative levels of p53				
	Untreated Control	<i>p</i> -XSC	4-NQO	4-NQO + <i>p</i> -XSC (init.)
				4-NQO + <i>p</i> -XSC (post.)
11-weeks ^b	1.00	1.20 ± 0.24	1.07 ± 0.09	1.00 ± 0.19
15-weeks	1.00	0.83 ± 0.25	1.45 ± 0.08	1.04 ± 0.23 ^d
23-weeks	1.00	0.99 ± 0.32	2.38 ± 0.70	1.87 ± 0.48

Init., *p*-XSC given with 4-NQO; post, *p*-XSC given after 4-NQO.

^aThree rats/group

^bWeeks represent time after the start of 4-NQO administration. 10 weeks total administration

^c*p* ≤ 0.05 vs. 4-NQO alone (a two tailed t-test was used to compare the differences between bands). ± refers to SD.