β-Cryptoxanthin reduced lung tumor multiplicity and inhibited lung cancer cell motility by
down-regulating nicotinic acetylcholine receptor α7 signaling

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Abstract

Despite the consistent association between a higher intake of the provitamin A carotenoid β-cryptoxanthin (BCX) and a lower risk of lung cancer among smokers, potential mechanisms supporting BCX as a chemopreventive agent are needed. We first examined the effects of BCX on 4-[methyl nitrosamino]-1-[3-pyridyl]-1-butanone (NNK)-induced lung tumorigenesis in A/J mice. BCX supplementation was given daily to the mice starting two-weeks prior to the injection of NNK and continued 16 weeks post NNK injection. BCX supplementation resulted in a dose-dependent increase of BCX concentration in both serum and lungs of the mice without a significant alteration of vitamin A (retinol and retinyl palmitate) concentration. BCX significantly reduced the multiplicity of the NNK-induced lung tumor by 52-63% compared to the NNK-treated mice without BCX supplementation. The protective effect of BCX in the lungs was associated with reductions of both mRNA and protein of the homopentameric neuronal nicotinic acetylcholine receptor α7 (α7-nAChR), which has been implicated in lung tumorigenesis. We then conducted an \textit{in vitro} cell culture study, and found that BCX treatment suppressed α7-nAChR expression and inhibited the migration and invasion of α7-nAChR-positive lung cancer cells but not in cells lacking α7-nAChR. The activities of BCX were significantly attenuated by activators of α7-nAChR/PI3K signaling or by overexpression of constitutively active PI3K. Collectively, the results suggest that BCX, inhibits lung tumorigenesis and cancer cell motility through the down-regulation of α7-nAChR/PI3K signaling, independent of its provitamin A activity. Therefore, BCX can be used as a chemopreventive agent or a chemotherapeutic compound against lung cancer.
Introduction

β-Cryptoxanthin (BCX), abundant in red sweet peppers, tangerines, oranges, peaches, and pumpkins, is an oxygenated carotenoid (xanthophyll) with provitamin A activity (1). Previously, a pooled analysis of seven prospective cohort studies with 378,765 people and 3,155 lung cancer cases in North America and Europe found that among carotenoid intake (including β-carotene which proved ineffective in both the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) and the Carotene and Retinol Efficacy Trial (CARET)), only the intake of BCX was associated with a lower risk of lung cancer (2). When stratified based on smoking status (past-, never-, and current-smokers), a significant protective association between BCX intake and risk of lung cancer was found only among current-smokers (2). Recent data obtained from the Third Nutrition and Health Examination Survey (NHANES III) database and the NHANES III Linked Mortality File showed that high serum levels of BCX is associated with a lower risk of lung cancer death only in current smokers (3). In two studies among Asian populations, those who consumed approximately 0.7 mg/day of BCX also had a significantly lower risk of lung cancer (4, 5). Our previous in vitro studies have demonstrated that BCX decreased cell proliferation of immortalized bronchial epithelial and non-small cell lung cancer cells (6). In vivo, we have shown that BCX supplementation reduced cigarette smoke-induced lung inflammation in ferrets (7) and suppressed nicotine-promoted emphysema and lung tumorigenesis in A/J mouse model after the 4-[methyl nitrosamino]-1-[3-pyridyl]-1-butaneone (NNK)-initiation along with additional nicotine injection as tumor promoter (8). However, whether BCX treatment provides a protection prior to the NNK initiation remains unclear. Additionally, the potential mechanisms for the unique biological activity of BCX, e.g., independent its provitamin A activity, against lung tumorigenesis need further investigations.
NNK, derived from nicotine or cigarettes is a well-known lung carcinogen for rodents, independent of its route of administration (9). NNK and other cigarette-smoke components (e.g. nicotine and N’-nitrosonornicotine) have been shown to bind to ion channel neuronal nicotinic acetylcholine receptors (nAChRs) (10). This binding is thought to contribute to the promotion of tumorigenesis via the activation of nAChR-dependent signaling pathways (10). Because of its high calcium permeability which modulates intracellular signaling molecules (11), the homopentameric α7 type of nAChRs (α7-nAChR) has been implicated in lung tumorigenesis (12-14). A recent study has showed that the copy number variations (CNV-3956) which resulted in a duplicated α7-nAChR conferred to an increased risk of lung cancer and poor survival of lung cancer patients (15). Treatment with NNK, which binds with high affinity to α7-nAChR, increases the expression of α7-nAChR in papillomavirus-immortalized bronchial epithelial cells (13), as well as cell proliferation and cell motility (16, 17). Moreover, Plummer et al. revealed the ubiquitous expression of the α7-nAChR in both normal and cancer lung cells (squamous, carcinoid, adenocarcinoma, large cell carcinoma, and small cell lung cancer) that further confirmed its involvement in lung biology and lung cancer development (18). Interestingly, we have shown that the NNK induced-lung cancer in a non-rodent lung cancer model, ferret (Mustela putorius furo), was strongly associated with up-regulated lung α7-nAChR (19).

Phosphoinositide 3-kinase (PI3K)/Akt, Ras, and its downstream effector Erk1/2 are important downstream signaling molecules of α7-nAChR (17). Stimulation with an agonist of α7-nAChR (i.e., 3-[2,4]-Dimethoxybenzylidineanabseine) induces phosphorylation of AKT in lung cancer cell lines (20), whereas treatment with an antagonist of α7-nAChR (i.e., α-bungarotoxin) inhibits the NNK-induced AKT phosphorylation in a normal human bronchial epithelial cell line (21). AKT, one of the most frequently activated kinases in lung cancer (20),
increases phosphorylation of multiple downstream components that control cell cycle and protein translation, such as glycogen synthase kinase-3 (GSK-3), BAD and ribosomal kinase p70S6K (22). Rac family and ARF6 are other well-known downstream proteins of PI3K, which regulate actin cytoskeleton organization. Furthermore, Rac1 and ARF6 are two key molecules in regulation of actin remodeling, lamellipodia formation, and cell motility (23, 24). Therefore, α7-nAChR represents a valuable molecular target for prevention or therapy of tobacco-related lung cancers (12, 17). However, whether α7-nAChR is involved in the chemopreventive effect of BCX against lung tumorigenesis remains unknown.

In the present study, we investigated whether pre-treatment with BCX supplementation inhibits the initiation and suppresses the progression of NNK-induced lung tumorigenesis in a well-established lung cancer A/J mouse model, and whether BCX affects α7-nAChR expression and its downstream effectors for protection against lung tumorigenesis. Subsequently, we conducted an in vitro study to determine whether BCX treatment inhibits lung cancer cell motility by suppressing α7-nAChR expression and its mediated PI3K signaling pathways.

Materials and Methods

Animals, study groups, and treatment

Male A/J mice (6 weeks-old) were purchased from Jackson Labs (Bar Harbor, ME). The male A/J mice were used in this study to avoid the effects of estrogen (also known as 17-β estradiol) on the expression and activation of nAChRs (25). The tobacco-specific carcinogen (NNK) (>98% purity, Toronto Research Chemicals, Ontario, Canada) was injected once (intraperitoneal i.p. 100 mg/kg body weight) into mice to induce lung tumors, as previously described (26, 27). BCX (>99% purity, BASF, Ludwigshafen, Germany) was in a powder form
and directly mixed with AIN-93M semi-purified diet powder (Dyets, Bethlehem, PA) at concentration of 1 and 10 mg/kg diet, which is equivalent to daily human consumptions of approximately 0.087 mg and 0.87 mg of BCX, as described in our previous study (8). Mice were randomly divided into 4 groups with 16 mice per group that were fed the semi-purified diet powder AIN-93M: 1) Control Group received sham (normal saline, i.p.) injection; 2) NNK Group received a NNK injection; 3) NNK+BCX(1) Group received a NNK injection and supplementation of BCX at 1 mg/kg diet; and 4) NNK+BCX(10) group received a NNK injection and supplementation of BCX at 10 mg/kg diet. The supplementation of BCX at 1 and 10 mg/kg diet was given daily to the mice starting two-weeks prior to the NNK injection and continued for 16 weeks post the NNK injection. Body weights of the mice were recorded weekly. After 16 weeks post the NNK injection, the mice were terminally exsanguinated under deep isoflurane anesthesia. The study was conducted with the approval of the Animal Care and Use Committee at the Human Nutrition Research Center on Aging at Tufts University.

Quantification of lung tumor lesions

The incidences and multiplicity of the pulmonary surface tumors were used for the index of carcinogenicity in A/J mice, as previously described (26). A/J mice are very sensitive to the carcinogen NNK, and they develop lung tumors on the surface of lungs (an average of 8 tumors/per lung with high reproducibility). The tumors are easily recognized by eye, making A/J mice easily applicable for lung tumor incidence and multiplicity study (8). Lung tumor lesions were quantified by determining the incidences and multiplicity of the pulmonary surface tumors on the day of euthanasia by two independent researchers blinded to the treatment group, as previously described (26). Hematoxylin and Eosin (H&E)-stained lung sections were
microscopically examined to confirm the formation of lung tumors (adenoma and adenocarcinoma).

**Immunohistochemistry assays**

Four-micrometer sections of formalin-fixed, paraffin-embedded lung tissues were immunostained for α7-nAChR (AChRα7 (319): sc-58607, rat monoclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), using the standard avidin-biotin complex immunoperoxidase method (Vectastain ABC-Elite; Vector Laboratories, Burlingame, CA). Briefly, the tissue sections were incubated with a primary antibody at a dilution of 1:50. Subsequently, the sections were incubated with biotinylated anti-rat antibody (Vector Laboratories, Inc., Burlingame, CA), diluted 1:250, and further incubated with VECTASTAIN Elite ABC reagent. The sections were subsequently processed with peroxidase substrate solution. The sections were then counterstained with hematoxylin. The sections were examined under light microscopy.

**Protein isolation and Western blotting**

Protein isolation of the whole-cell lysates of the lung tissues and culture cells and Western blotting analysis were conducted as previously described (28). The following antibodies were used for Western blotting: α7-nAChR and MMP-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphorylated AKT (Ser473), total AKT, phosphorylated BAD, Akt (Ser473), p70S6K (Thr389), S6 (Ser235/236), BAD (Ser136), GSK-3β (Ser9), Erk1/2(Thr202/Tyr204) and actin (Cell Signaling, Danvers, MA), as well as GAPDH (Millipore, Billerica, MA). All of the antibodies were used according to the manufacturers’ protocols.
RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

An RNeasy kit (Qiagen, Valencia, CA) was used to extract RNA according to the manufacturer’s protocol and as previously described (27). cDNA was prepared from the RNA samples using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and an automated thermal cycler PTC-200 (MJ Research, Bio-Rad Laboratories, Hercules, CA). qRT-PCR was performed using Fast Start Universal SYBR Green Master (ROX) (Roche, Indianapolis, IN). The relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method.

High performance liquid chromatography (HPLC)

Serum, liver, and lung samples of the mice were prepared as previously described (28). Samples were reconstituted with 100 μL of an ethanol: methyl-tert butyl ether solution (2:1 ratio). A gradient reverse-phase HPLC system consisting of a Waters 2695 separation module and a Waters 2998 photodiode array detector was used for the detection of BCX, retinol, and retinyl palmitate. Briefly, BCX, retinol, and retinyl palmitate were analyzed on a reverse-phase C18 column (4.6×250 mm, 5 μm) (Vydac 201TP54, Grace Discovery Sciences, Inc., Bannockburn, IL) with a flow rate of 1.00 mL/min, and quantified relative to internal standards by determining the peak areas against known amounts of standards.

Cells, cell culture, and materials for the in vitro experiments

Human cancer or immortal cell lines (A549, BEAS-2B, U87MG, MCF-7, MDA-MB-231, 293T) were purchased from the ATCC, which characterizes them using cytogenetic analysis. We have not authenticated these cell lines. A549, U87MG, MCF-7, MDA-MB-231 and 293T cells were cultured in DMEM medium with 10% fetal bovine serum (FBS). BEAS-2B cells
were cultured in RPMI1640 medium with 10% FBS. All media were supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. All cells were cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. BCX treatments were carried out at a series of concentrations (0 to 4 μM) and incubated for different times, as specified. MG624 and PNU282987 were purchased from Tocris Bioscience. Rac1 vectors were purchased from GE Healthcare and ARF6 vectors were purchased from OriGene Technologies. PDGF, PMA, LY294002, tetramethyl rhodamine isothiocyanate (TRITC)-labeled phalloidin and all other reagents and chemicals were purchased from Sigma.

**Cell viability assay**

Cells were seeded into 96-well plates at the density of 5-10×10³ cells per well in 100 μl of the appropriate media. BCX was added at a series of concentrations and incubated for different time periods. A luminescence-based commercial kit (Cell Titer-Glo, Promega, Madison, WI) was used. In brief, 30 μl of the cell lysis/ATP detection reagent was added to each well, mixed for 10 min at room temperature, and the luminescence was measured using a Wallac Victor 3 plate reader (Perkin-Elmer, Wellesley, MA).

**Fluorescent immunocytochemistry**

Cells were seeded on coverslips, followed by transfection and/or treatments with compounds. Then cells were fixed with 4% polyformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 20 min, and incubated with 200 ng/ml TRITC-labeled phalloidin for 30 min. All images were obtained using Zeiss Axiovert 200 fluorescent microscope.
Cell invasion assay

Invasion of cells was evaluated using 24-transwell Boyden chamber (Costar, Bedford, MA) with a polystyrene membrane (6.5 mm diameter, 10 μm thickness, and 8 μm pore size). Cells were suspended in serum-free media and seeded in the upper compartment of each well (5×10^4 cells/well) with or without testing compounds. The lower compartment contained 600 μl of serum-free media supplemented with fibronectin. After a 12-hour incubation at 37°C, cells were fixed and stained with 0.1% crystal violet. The non-migrated cells on the upper surface of the filter were removed, and the migrated cells on the lower side were photographed using a microscope in five random fields. Then cells were lysed with 10% acetic acid, and colorimetric determination was made at 595 nm using Wallac Victor 3 plate reader.

Wound healing model

Cells were seeded into fibronectin-coated 96-well plates (2×10^4 cells/well). After reaching confluence, cell monolayers were scratched with a pipette tip to obtain a “wound”. The media and dislodged cells were aspirated, and replaced by fresh serum-free media with or without compounds. After a 12-hour incubation at 37°C, cells were photographed using a microscope in five random fields. The width of wounded cell monolayers in images was measured and the inhibition rates of migration were calculated.

Transient transfection

Cells were seeded on coverslips in 24-well plates at a density of 5×10^4 cells/well and incubated overnight. Then transient transfection was carried out using Fu GENE 6 Transfection
Reagent (Roche) following manufacturers' instructions. 24 hours after transfection, cells were subjected to migration or fluorescent immunocytochemistry analysis.

**Statistical analyses**

All the measurements are expressed as the mean ± the standard error of the mean (SEM) or as otherwise indicated. The comparisons across multiple groups were conducted by a one-way ANOVA with Tukey’s Honestly Significant Difference (HSD) post-hoc procedure. The differences between the two groups were analyzed by Student's t test. All analyses were performed using Statistical Analysis System (SAS®, version 9.2). Differences were considered significant if $P < 0.05$.

**Results**

**BCX supplementation restores the NNK-reduced body weights of mice and increases the concentrations of BCX but not vitamin A in the serum, lung, and liver**

There were no differences in the mean body weights of the mice across the groups at the beginning of the study (Table 1). At the end of the study, the mean body weight of the NNK alone group (25.6 ± 0.6 g) was significantly lower than the control group (28.0 ± 0.7 g) ($P = 0.05$). The mean body weights of the BCX-supplemented groups (26.5 ± 0.6 g for NNK+BCX(1), and 26.1 ± 0.5 g for NNK+BCX(10), respectively) were not significantly different from the control ($P = 0.48$).

BCX was not detected in the serum of the mice in the control and NNK groups as expected because the AIN-93M diet does not contain BCX. Despite limited absorption of intact carotenoids in mice compared to that in humans (29), the serum concentrations of BCX were
increased in a dose-dependent manner in the BCX-supplemented groups (Table 1). We also detected BCX in the lungs and livers of the mice supplemented with BCX at 10 mg/kg diet (Table 1). The concentrations of retinol did not differ among the groups in the serum, lungs, and livers of the mice. Moreover, the supplementation of BCX(10) increased both the lung and liver concentrations of retinyl palmitate (the major stored form of vitamin A) compared to the control group (Table 1).

**BCX supplementation reduces the multiplicity of the NNK-induced lung tumor**

The NNK group had a higher incidence of lung tumor (93%) when compared to the spontaneous lung tumor incidence in the control group (20%, \( P = 0.01 \)). There were no significant differences in the lung tumor incidences of the NNK+BCX(1) group (93%) and NNK+BCX(10) group (93%), as compared to the NNK group. Lung tumors (Fig. 1A) were microscopically examined by H&E-stained section to confirm the formation of both adenoma and adenocarcinoma (Fig. 1A). The mean of lung tumor multiplicity in the NNK group was 7-fold greater than that of spontaneous lung tumor multiplicity in the control group (\( P = 0.008 \)) (Fig. 1B). The means of lung tumor multiplicity in the NNK+BCX(1) group and the NNK+BCX(10) group were 52% and 63% lower, respectively than that of the NNK group (\( P = 0.04 \)). Due to the co-existence of lung adenoma and adenocarcinoma from all of the mice, we did not see any significant difference on tumor size and types between BCX treated and non-BCX treated mice.

**BCX supplementation is associated with reduction of \( \alpha7 \)-nAChR and phosphorylation of AKT and BAD in the NNK-treated mice**

Expression of \( \alpha7 \)-nAChR in lung was assessed by immunohistochemistry, which showed strong cytoplasmic immunoreactivity in airway epithelial cells, alveoli type II cells, and
pulmonary alveolar macrophages (Fig 1C). Strikingly, the cytoplasmic intensity of α7-nAChR immunoreactivity was markedly increased in lungs from NNK treated animals, and in areas of lung tumor, as compared with that of control mice (Fig. 1C). We found a non-significant increase of lung a7-nAChR mRNA in the NNK group as compared to the control group (P = 0.13) (Fig. 1D). The levels of lung a7-nAChR mRNA in the NNK+BCX(1) group was not statistically different than the NNK group. However, the level of a7-nAChR mRNA in the NNK+BCX(10) group were 42% lower than the NNK group (P = 0.01) (Fig. 1D). The NNK group had a 3-fold increase of α7-nAChR protein level compared to the control group (P = 0.04) (Fig. 1E). Although a reduced level of α7-nAChR in the NNK+BCX(1) group compared to the NNK group could not reach significant statistics, there was a significant 56% reduction of the α7-nAChR protein levels in the NNK+BCX(10) group, as compared to the NNK group (P = 0.03) (Fig 1E). Moreover, Pearson’s correlation coefficient test in the control, NNK, NNK+BCX(1), and NNK+BCX(10) groups showed a significant positive correlation between the levels of lung a7-nAChR mRNA, proteins and lung tumor multiplicity (P = 0.03, r = 0.394 for mRNA, P < 0.01, r = 0.355 for protein) (Supplementary Fig. 1S). Although several gene variants in the region of chromosome 15q24-25 containing the genes encoding α3, α5, and β4 subtypes of nAChRs (nachr-α3, -α5, and -β4, respectively) were associated with nicotine dependence and lung cancer risk, we did not find significant differences in the nachr-α3, nachr-α5, and nachr-β4 mRNA levels among all the groups (Supplementary Fig. 1S).

Since the promoter activity of α7-nAChR can be regulated by early growth response 1 (EGR1) (30), we examined whether the lung mRNA level of egr1 in NNK-induced lung tumorigenesis of A/J mice is associated with the chemopreventive effect of BCX (Supplemental Fig. 2). The lung levels of egr1 mRNA in the NNK-group was 2.5-fold greater than the control
group \((P = 0.03)\) (Supplemental Fig. 2). Although the decreased levels of \(egr1\) mRNA in the NNK + BCX(1) group did not reach a statistically significant difference compared to the NNK group \((P = 0.09)\), the levels in the NNK + BCX(10) group were lower than the NNK group \((P = 0.05)\) (Supplemental Fig. 2). Nevertheless, a linear test in the three groups, i.e., NNK, NNK + BCX(1), and NNK + BCX(10) groups, indicated a significant decreasing level of \(egr1\) mRNA in a BCX-dose dependent fashion \((P\text{-linear trend} = 0.04)\).

There was a 2-fold increase of level of lung AKT phosphorylation (Ser473) in the NNK group compared to the control group \((P = 0.01)\) (Fig. 1F). Although the levels of AKT phosphorylation between the NNK+BCX(1) and the NNK groups were not statistically different, the levels of AKT phosphorylation in the NNK+BCX(10) group were significantly 47% lower than the NNK group \((P = 0.01)\) (Fig. 1F). A linear test in the three groups, i.e., NNK, NNK+BCX(1), and NNK+BCX(10) groups, demonstrated significant decreasing levels of the phosphorylated AKT in a BCX-dose dependent manner \((P\text{-linear trend} = 0.04)\). The lung levels of BAD phosphorylation (Ser136) in the NNK group were not different from the control group (Fig. 1G), however, a linear test in the three groups, i.e., NNK, NNK+BCX(1), and NNK+BCX(10), demonstrated a significant decrease in the levels of the phosphorylated BAD in a BCX-dose dependent manner \((P\text{-linear trend} = 0.04)\).

**BCX suppresses \(\alpha7\)-nAChR expression and PI3K signaling pathways in lung cancer cells**

We first examined whether BCX incubation at concentrations ranging from 0.5-4.0 \(\mu\)M would suppress \(\alpha7\)-nAChR expression in human immortalized lung cells, BEAS-2B, and human lung cancer cell line, A549, both of which have a high level of \(\alpha7\)-nAChR \((22, 32)\). We found that BCX suppressed \(\alpha7\)-nAChR expression in both BEAS-2B and A549 cells (Fig. 2A). Interestingly, PNU282987 (PNU), a \(\alpha7\)-nAChR specific agonist (similar to NNK and nicotine),
increased the α7-nAChR expression in lung cells, which was decreased not only by BCX, but also by MG624 (MG), a α7-nAChR specific antagonist (Fig. 2B).

We next investigated the effects of BCX on PI3K signaling, an important downstream pathway of α7-nAChR (17). As shown in Fig. 2C, BCX significantly suppressed PI3K-mediated phosphorylation events in both cell lines. We also observed the effect of BCX on cell signaling triggered by PNU282987 (Fig. 2D). In addition, the α7-nAChR specific antagonist MG624 inhibited the same cellular phosphorylation events (Fig. 2D). Moreover, both BCX and MG624 inhibited phosphorylation of Erk1/2, an important direct downstream molecule of α7-nAChR, but not PI3K (Fig. 2C).

BCX inhibits lung cancer cell migration and invasion

Lung cancer cells have more active motility, which is considered a required process during cancer development (16). The movement of cancer cells into tissue surrounding the tumor and the vasculature is the first step in the spread of metastatic cancers. In particular, studies have shown that NNK enhances lung cancer cell motility and that cigarette smoking correlates with an increased metastasis of lung cancers (31, 32). Suppression of α7-nAChR/PI3K signaling by BCX led us to investigate the effects of BCX on cell migration. As shown in Fig. 3A-B, BCX substantially inhibited migration of both A549 and BEAS-2B cells in a concentration-dependent manner in a transwell assay. A similar inhibition was also observed in a wound healing assay (Fig. 3C-D). BCX also inhibited invasion of A549 and BEAS-2B cells through matrigel (Fig. 3E). By contrast, very weak inhibition was observed on the migration of MDA-MB-231 and 293T cells, two known α7-nAChR negative cell lines (33, 34) (Fig. 3A-E), suggesting the selective action mode of BCX. Importantly, BCX treatments had no significant
cytotoxic effects on the cells in parallel experiments at the same concentrations used in migration and invasion experiments (Fig. 3F).

**α7-nAChR/PI3K signaling contributes to cell motility inhibition by BCX**

We next characterized whether inhibition of α7-nAChR/PI3K signaling contributes to cell motility suppression by BCX. In both cell migration and invasion assays, BCX was effective at decreasing cell motility only in α7-nAChR positive cells, not in α7-nAChR negative cells, confirming the contribution of α7-nAChR to BCX-induced inhibition of cell migration and invasion. Similar α7-nAChR-dependent inhibition was also obtained using MG624, a α7-nAChR-specific antagonist (Fig. 3A-E). By contrast, LY294002, a PI3K inhibitor, suppressed cell migration in both α7-nAChR positive and negative cell lines (Fig. 3B and 3D). Furthermore, both BCX and MG624 efficiently attenuated nicotine and PNU282987-stimulated cell migration in A549 and BEAS-2B cells (Fig. 4A-B). Nicotine or PNU282987 treatment led to an increase in cell migration. Both BCX and MG624 inhibited migration of nicotine or PNU282987-stimulated cells more effectively than the control cells without stimulation (Fig. 4C-D), again confirming the involvement of α7-nAChR in attenuation of cell migration by BCX. We also found that inhibition of cell migration by BCX was attenuated in U87MG cells or MCF-7 cells expressing constitutively active PI3K (Fig. 4E). Next, we measured the expression of an important PI3K downstream molecule matrix metalloproteinase-2 (MMP-2), which is a representative member of the MMP family of proteins and plays crucial roles in cell invasion by degrading basement membranes. As shown in Fig. 4F and 4G, BCX decreased MMP-2 expression in the presence or absence of PNU282987 stimulation, and BCX reduced both pro- and activated-MMP-2 levels (Fig. 4).

**BCX inhibits actin remodeling and ruffling/lamellipodia formation in lung cells**
Cell motility is driven by actin rearrangement and lamellipodia formation at the leading edge of cells (22). The reduction of cell migration and invasion caused by BCX treatment led us to examine whether BCX can influence actin reorganization and reduce ruffling/lamellipodia formation. We used BEAS-2B cells in most of the experiments for actin cytoskeleton analysis. As shown in Fig. 5A, treatment with either BCX or MG624 significantly reduced the amount of F-actin in cells, including lamellipodia as the driving force for cell migration, at the leading edge and stress fibers within the cells, indicative of efficient inhibition of actin polymerization and remodeling. Pre-incubation with BCX efficiently inhibited nicotine or PNU282987-stimulated ruffles/lamellipodia formation. Similar α7-nAChR-dependent inhibition was obtained using MG624, confirming the involvement of α7-nAChR in these events. BCX pre-treatment also partially inhibited ruffles/lamellipodia formation induced by PDGF, a PI3K activator. Interestingly, BCX also suppressed ruffles/lamellipodia formation triggered by PMA, a PKC activator (Fig. 5A-B). Moreover, PDGF and PMA partially attenuated BCX-induced cell migration inhibition ($P = 0.04$, Fig. 5C).

**PI3K downstream Rac1 and ARF6 contribute to lamellipodia inhibition by BCX**

PI3K is a key modulator in actin remodeling and cell migration, and it regulates these processes through multiple downstream molecules (35). Based on the critical roles of Rac1 and ARF6 in PI3K-dependent regulation of lamellipodia formation (23, 36), we investigated the effects of BCX on lamellipodia formation in BEAS-2B cells overexpressing wild type or constitutively activated Rac1 or ARF6. We found that BCX efficiently decreased wild type Rac1 and ARF6-induced lamellipodia formation (Supplemental Fig. 3A and 3C). However, it failed to decrease lamellipodia formation induced by overexpression of constitutively activated Rac1 and ARF6 (Supplemental Fig. 3B and 3D), suggesting that inhibition of lamellipodia formation by
BCX is through blocking the upstream, but not the downstream stimulators of Rac1 and ARF6. A similar result was also obtained using MG624. We also measured the migration of cells overexpressing constitutively activated Rac1 and ARF6 in the presence of BCX or MG624. We found that overexpression of Rac1-Q61L or ARF6-Q67L significantly attenuated inhibition of cell migration by these molecules ($P = 0.05$, Supplemental Fig. 3E).

**Discussion**

The present study provided further experimental evidence and described potential mechanisms to explain the significant protective association between a high intake of BCX and the risk of lung cancer among current-smokers, reported previously in the analysis of seven prospective cohort studies (37). In the present study, the supplementation of BCX to the mice 2 weeks prior to the NNK injection was effective in reducing the lung tumor multiplicity by 52-63%, not the lung tumor incidences, indicating that the chemopreventive activity of BCX was able to suppress the tumor promotion, rather than to inhibit the initiation of lung tumorigenesis. This notion was supported the following: Firstly, our previous study has demonstrated that the BCX supplementation, given one week after injection of NNK, along with additional nicotine injection as tumor promoter, inhibited the lung tumor promoting effect of nicotine in the NNK-initiated A/J mouse model (8); Secondly, in the present in vivo study, the protective effect of BCX supplementation on the NNK-induced lung tumor multiplicity was associated with reduced levels of $\alpha$7-nAChR protein in the lungs of the mice. This is in agreement with previous studies in which $\alpha$7-nAChR particularly was up-regulated in male smokers and in smoking patients with lung cancer (38). Indeed, we observed that the overexpression of $\alpha$7-nAChR in both lung and lung tumor of NNK-treated animals by immunohistochemistry and western blotting analysis.
Thirdly, we found that BCX supplementation reduced the levels of AKT phosphorylation which was associated with that of BAD phosphorylation. Phosphorylation of BAD at Ser136 by AKT is suggested to be the principal induction for the interaction between BAD and 14-3-3 proteins which leads to apoptosis. Therefore, the decreasing levels of the phosphorylated BAD in a BCX-dose dependent manner indicated the role of BCX in inducing apoptosis to reduce tumor numbers in the mouse lungs; Fourthly, in the present in vitro study, BCX reduced levels of α7-nAChR expression and inhibited cancer cells’ motility including migration and invasion; and lastly, our results were aligned with previous human studies reporting that BCX is effective at decreasing risk of lung cancer among current-smokers, not among never and former smokers (37). In the present study, we did not see any significant induction of the genes of other nAChR sub-units that were associated with lung cancer risk (i.e. nachr-α3, -α5, and -β4) (39). Therefore, we propose that α7-nAChR represents a molecular target for BCX in the chemoprevention of tobacco-related lung cancers. Furthermore, we found that the decreasing lung mRNA level of egr1 in the NNK-induced lung tumorigenesis of A/J mice was associated with the chemopreventive effect of BCX in a dose dependent manner (Supplemental Fig. 2). The binding sites of EGR1 have been found in the promoter region of α7-nAChR (30); therefore, the suppression of egr1 mRNA expression may offer an explanation of how BCX influenced the levels of mRNA and protein of α7-nAChR. Whether BCX regulates the expression of α7-nAChR mRNA through EGR1 warrants further investigation.

Previous studies have reported that NNK can up-regulate the expression of α7-nAChR and enhance the proliferation and motility of lung cancer cells through stimulation of α7-nAChR (11, 40). Additionally, nicotine can activate PKC, Erk1/2, PI3K/Akt, and Rac1 in a α7-nAChR-dependent manner (17, 18). In this study, we provide multiple avenues of evidence from our in
*vitro* experiments which support the inhibition of α7-nAChR/PI3K pathways contributes to suppression of cancer cell motility by BCX: Firstly, MG624, a α7-nAChR-specific antagonist, which could suppress the expression of α7-nAChR, displayed very similar cellular activities with BCX in a wide range of assays. Secondly, BCX exerted strong cellular activities in α7-nAChR overexpressing lung cells and suppressed MMP-2 expression and activation, which validates the chemopreventive effect of BCX in α7-nAChR positive BEAS-2B cells (e.g., to mimic smokers), in addition to α7-nAChR positive A549 lung cancer cells (e.g., mimicking lung cancer patients) for chemotherapeutic function of BCX; Thirdly, BCX had little effect on α7-nAChR negative breast cancer cells and kidney cells, thus providing more supporting evidence that BCX targets α7-nAChR positive cells. Fourthly, BCX efficiently overcame or attenuated the PNU282987, a α7-nAChR-specific agonist, induced enhancement of α7-nAChR expression, phosphorylation of signaling molecules, lamellipodia formation, and cell migration. Lastly, the activities of BCX can be significantly attenuated by overexpression of constitutively active PI3K, activators of α7-nAChR/PI3K signaling, constitutively active Rac1 or ARF6, which contribute to inhibition of lamellipodia formation and lung cell motility. Therefore, BCX can be used not only as a chemopreventive agent but also as a chemotherapeutic compound against lung cancer.

It should be mentioned that our *in vivo* experiment demonstrated that the doses of BCX supplementation were within the physiological levels and could be translatable for human consumption. The doses of BCX at concentrations of 1 and 10 mg/kg diet were equivalent to daily human consumptions of approximately 87 μg and 870 μg of BCX, as described in our previous study (8). The median dietary intake of BCX for human is 88 μg daily with the reference range 24-319 μg from 10th-90th percentile (41). These doses of BCX can be easily achieved by daily consuming 3-4 ounces of a sweet red pepper (BCX 1300-2017 μg), a tangerine
(BCX 490-775 μg), or an orange (BCX 116-321 μg) (42). In addition, the tissue concentrations of BCX (0.16 nmol/g lung and 0.35 nmol/g liver) in the mice supplemented with 10 mg BCX/kg diet was similar to that reported in human tissues (0.1-0.2 nmol/g lung and 0.1-3.5 nmol/g liver) (43). Our study was also in agreement with previous reports that the people who consumed approximately 700 μg/day of BCX had a significantly lower risk of lung cancer (4, 5). The supplementation of BCX at 1 and 10 mg/kg diet in this mouse study resulted in much lower serum concentrations of BCX, with 4.3 nM and 17.4 nM (0.24 and 0.98 μg/L, respectively), as compared with that of humans (the median serum concentration of BCX in human is ~8 μg/L with the reference range 4-16.4 μg/L from 10th-90th percentile (41). This could be due to the fact that the absorption of carotenoids in rodents is lower than that of human absorption.

Finally, our data suggested that the preventive effect of BCX on the lung tumorigenesis was likely due to the activity of BCX itself rather than biologically generated vitamin A. There are several observations that support this notion: 1) While the AIN-93M semi-purified diet contained sufficient vitamin A, the supplementation of BCX at two doses (1 and 10 mg/kg body weight), without altering levels of retinol and retinyl ester in both serum and lung tissue, effectively reduced the multiplicity of NNK-induced lung tumors by 52-63%; 2) Previous studies using 13-cis retinoic acid (44) and 9-cis retinoic acid (at 1 mg/kg body weight) treatment (27) inhibited the lung tumor multiplicity with significant weight loss, while BCX restored the weight loss upon the treatment of NNK to the normal levels, in the control group, without any sign of toxicity; 3) The other carotenoids acting as vitamin A precursors, such as β-carotene, did not reduce the NNK-induced lung tumor multiplicity in the same A/J lung cancer mouse model (45), and we have previously shown that BCX (7), not β-carotene (46), inhibited smoke-induced precancerous lung lesions using the ferret as a human lung cancer model; and 4) It is unlikely
that the inhibition of BCX on the migration and invasion of α7-nAChR positive lung cancer cells was due to its conversion into vitamin A due to the low concentrations of BCX (0.5 to 4 μM) used in the present in vitro study. We have shown previously that the intracellular concentration of carotenoids were much lower than the concentrations of carotenoids in cell media (6). However, we cannot rule out the potential of BCX through retinoic acid receptors (RAR) or rexinoid-like compounds, engaged by retinoid X receptors (RXR), in cancer prevention and treatment which have been highlighted in a recent review (47). We and others have shown that BCX itself acts as a ligand for RAR transcription activation (6, 48) and induces CYP27A1 signaling pathway via RAR activation (49, 50). Further investigation of the protective effects of BCX against the lung tumorigenesis in the absence of carotenoid cleavage enzymes, including both vitamin A and other biological metabolites is currently underway in our laboratory.

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We thank Dr. Hansgeorg Ernst of the Fine Chemicals and Biocatalysis Research, BASF, Ludwigshafen, Germany, for providing β-cryptoxanthin. We also thank Dr. Ausman Lynne, Dr. Donald E. Smith, Dr. Xueyan Fu, Ms. Junrui Cheng, Ms. Rachel A. Chiaverelli and Ms. Jian Shi for their assistance.

Reference


Table 1. Body weight and concentrations of β-cryptoxanthin and retinoids in serum, lung, and liver of AJ mice after 18 weeks supplementation*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>NNK Group†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>BCX (0)</td>
<td>BCX (1)</td>
</tr>
<tr>
<td>Animal (n)</td>
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<td>13</td>
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</tbody>
</table>

*Body Weight (BW)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NNK Group†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW (g)</td>
<td>21.2 ± 0.4a</td>
<td>21.3 ± 0.4a</td>
</tr>
<tr>
<td>Final BW (g)</td>
<td>28.0 ± 0.7a</td>
<td>25.6 ± 0.6b</td>
</tr>
</tbody>
</table>

*Serum (nM)*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NNK Group†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCX</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Retinol</td>
<td>1051.6 ± 59.7a</td>
<td>1045.1 ± 17.0a</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Lung (nmol/g)*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NNK Group†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCX</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Retinol</td>
<td>4.93 ± 0.26a</td>
<td>6.30 ± 1.35a</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>462.05 ± 7.31a</td>
<td>757.73 ± 115.09ab</td>
</tr>
</tbody>
</table>
### Liver (nmol/g)

<table>
<thead>
<tr>
<th></th>
<th>BCX</th>
<th>Retinol</th>
<th>Retinyl palmitate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ND</td>
<td>14.09 ± 1.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1625.87 ± 111.79&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td></td>
<td>ND</td>
<td>13.95 ± 2.67&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>18.06 ± 1.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2767.29 ± 175.95&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.35 ± 0.03</td>
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</table>

*Values are expressed as mean ± SEM, n=13-15, statistical analysis by one-way ANOVA followed by post-hoc testing using Tukey’s HSD. Means sharing the same superscript were not significantly different from each other.

† NNK: 4-(N-Methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone; BCX (β-cryptoxanthin) concentration in mg/kg diet, indicated in parenthesis. ND: not detected.
Figure legends

Figure 1. BCX suppresses lung tumor, α7-nAChR expression and α7-nAChR signaling in the NNK-treated mice.

(A) Lung surface tumors (indicated by arrows) in the NNK-treated mice (left). Hematoxylin and eosin (H&E) staining of the lung tissues with tumor regions shown under low and high magnification, middle and right, respectively. (B) Lung tumor multiplicity with different treatment. (C) Expression of α7-nAChR in lung tissue of mice without NNK treatment (left) and non-cancerous lung tissue (middle) and lung tumor (right) of NNK-treated mice. Immunohistochemical localization of α7-nAChR (arrows) in lung showing brownish cytoplasmic staining in airway epithelial cells, alveoli type II cells and pulmonary alveolar macrophage, but intense staining in non-cancerous lung tissue (middle) and lung tumor (right) of NNK-treated mice. (D) Levels of α7-nAChR mRNA with different treatment. (E) Levels of α7-nAChR protein with different treatment. (F) Quantification of the phosphorylated AKT (pAKT) on Ser473. (G) The quantification of the phosphorylated BAD (pBAD) on Ser136. Group denotation: 1: Control; 2: NNK; 3: NNK+BCX(1); 4: NNK+BCX(10). Abbreviation: BCX(1): β-cryptoxanthin 1 mg/kg diet; BCX(10): β-cryptoxanthin 10 mg/kg diet; nachr: nicotinic acetylcholine receptor; NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

Figure 2. BCX suppresses α7-nAChR expression and α7-nAChR/PI3K signaling in lung cells.

BEAS-2B and A549 cells were treated with BCX (0.5-4.0 μM) or MG624 (1.0 μM) for 48 hr. The α7-nAChR protein level was evaluated in the absence (A) or presence (B) of PNU282987.
The phosphorylation levels of the proteins in α7-nAChR/PI3K pathways were evaluated without PNU282987 (C) or with 1 μM PNU282987 for 1 hr (D).

Figure 3. BCX inhibits migration and invasion of lung cells.
BEAS-2B, A549, MDA-MB-231 and 293T cells were treated with BCX (0.5-4.0 μM) or MG624 (1.0 μM) or LY294002 (1.0 μM) for 12 hr. Cell migration was determined by a transwell assay (A) or a wound healing assay (C). Quantitation of the inhibition from transwell assay (A) and wound healing assay (C) was displayed in (B) and (D), respectively. The inhibition on cell invasion is shown in (E). Cell viability was evaluated using ATP assay under the same condition (F).

Figure 4. Inhibition of α7-nAChR/PI3K signaling contributes to cell migration suppression by BCX.
BEAS-2B, A549, MDA-MB-231 and 293T cells were treated with BCX (0.5-4.0 μM) or MG624 (1.0 μM) in the presence of nicotine (1.0 μM) or PNU282987 (1.0 μM) for 12 hr. The migrated cells were quantified with nicotine (A) or PNU282987 (B) treatment. The inhibition of α7-nAChR agonists-induced cell migration by BCX in BEAS-2B (C) or A549 cells (D) was calculated respectively and compared with blank without stimulation. BEAS-2B, U87MG or MCF-7 cells were treated with BCX (0.5-4.0 μM) or MG624 (1.0 μM) for 12 hr, Cell migration was analyzed treated with overexpression of constitutively active PI3K or mutant of PTEN (E). BEAS-2B and A549 cells were treated with BCX (0.5-4.0 μM) or MG624 (1.0 μM) for 48 hr, then the MMP2 level was measured without PNU282987 treatment (F) or treated with 1.0 μM PNU282987 for 1 hr (G).
Figure 5. BCX inhibits lamellipodia formation in lung cells.

BEAS-2B cells were incubated with BCX (1.0, 2.0, 4.0 μM) or MG624 (1.0 μM) for 12 hr, or cells were serum starved and treated with compounds for 12 hr, followed by nicotine (1.0 μM) or PNU282987 (1.0 μM) stimulation for 1 hr, or PDGF (100 ng/ml) or PMA (250 ng/ml) stimulation for 30 min. Then cells were stained with TRITC-labeled phalloidin and analyzed using fluorescent microscopy. The representative fluorescent images are shown (A, the lamellipodia are indicated by arrowheads). The number of cells with significant lamellipodia was counted randomly in five fields (B). Scratch wound was generated in BEAS-2B cell monolayer, followed by treatment with BCX (0.5-4.0 μM) or MG624 (1.0 μM) in the presence or absence of PDGF or PMA stimulation for 12 hr. The width of wounded cell monolayer was measured in five random fields, and the inhibition was calculated respectively and compared with blank without stimulation (C).
Fig. 1

A. Images showing control, NNK treated (non-tumor), and NNK treated (tumor) tissues.

B. Bar graph showing tumor number with control and NNK treated groups.

C. Images showing α7-nAChR expression in control and NNK treated (tumor) tissues.

D. Graph showing mRNA levels of α7-nAChR with control and NNK treated groups.

E. Western blot showing NACHR-α7 and pAKT(Ser473) levels.

F. Western blot showing AKT and pBAD (Ser136) levels.

G. Graph showing fold change in pBAD (Ser136) levels with control and NNK treated groups.

Control NNK treated (non-tumor) NNK treated (tumor)

a7-nAChR

Tumor number

p = 0.13

* indicates statistical significance.

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Fig. 3

Control    MG624 (1.0 µM)    BCX (0.5 µM)     BCX (1.0 µM)      BCX (2.0 µM)     BCX (4.0 µM)

BEAS-2B
A549
MDA-MB-231

Control    MG 624 (1.0 µM)    BCX (0.5 µM)     BCX (1.0 µM)      BCX (2.0 µM)     BCX (4.0 µM)

BEAS-2B
A549
MDA-MB-231

E

BEAS-2B    A549
MDA-MB-231

F

BEAS-2B    A549
MDA-MB-231 293T
Fig. 4

A, B, C, D, E, F, G: Various bar charts and graphs showing the inhibition of migrated cells and the expression of MMP2 in BEAS-2B and A549 cell lines treated with different concentrations of BCX and MG624.

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Fig. 5

(A) Control, BCX (1.0 μM), BCX (2.0 μM), BCX (4.0 μM), MG624 (1.0 μM)

(B) Cells with lamellipodia (%)

(C) Inhibition (%)
β-Cryptoxanthin reduced lung tumor multiplicity and inhibited lung cancer cells motility by down-regulating nicotinic acetylcholine receptor α7 expression

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