

Gamma-aminobutyric acid, a potential tumor suppressor for small airway-derived lung adenocarcinoma

Hildegard M.Schuller^{1,*}, Hussein A.N.Al-Wadei^{1,2} and Mourad Majidi¹

¹Experimental Oncology Laboratory, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA and ²Department of Preventive Medicine, Sana'a University, Sana'a, Yemen

*To whom correspondence should be addressed. Tel: +1 865 974 8217; Fax: +1 865 974 5616; Email: hmsch@utk.edu

Pulmonary adenocarcinoma (PAC) is the leading type of lung cancer in smokers and non-smokers that arises in most cases from small airway epithelial cells. PAC has a high mortality due to its aggressive behavior and resistance to cancer therapeutics. We have shown previously that the proliferation of human PAC cells NCI-H322 and immortalized human small airway epithelial cells HPL1D is stimulated by cyclic adenosine monophosphate (cAMP)/protein kinase A-dependent phosphorylation of cyclic adenosine monophosphate response element-binding (CREB) protein and transactivation of the epidermal growth factor receptor and that this pathway is activated by beta-1-adrenoreceptors (β_1 -ARs) and the non-genomic estrogen receptor beta. Our current *in vitro* studies with HPL1D and NCI-H322 cells showed that signaling via the gamma-amino butyric acid receptor (GABA_BR) strongly inhibited base level and isoproterenol-induced cAMP, p-CREB, cyclic adenosine monophosphate response element-luciferase activity and p-extracellular regulated kinase-1 (ERK1)/2 and effectively blocked DNA synthesis and cell migration. The inhibitory effects of gamma-amino butyric acid (GABA) were disinhibited by the GABA_BR antagonist CGP-35348 or GABA_BR knockdown. Immunohistochemical investigation of hamster lungs showed significant underexpression of GABA in animals with small airway-derived PACs induced by the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). These findings suggest that GABA may have tumor suppressor function in small airway epithelia and the PACs derived from them and that downregulation of GABA by NNK may contribute to the development of this cancer in smokers. Our findings suggest that marker-guided treatment with GABA or a GABA_BR agonist of individuals with downregulated pulmonary GABA may provide a novel targeted approach for the prevention of PAC in smokers.

Introduction

Pulmonary adenocarcinoma (PAC) is an aggressive cancer with a mortality near 100% within 5 years of diagnosis. The majority of PACs are thought to arise from small airway epithelial cells while alveolar type

Abbreviations: ANOVA, analysis of variance; β -AR, beta-adrenoreceptor; BrdU, Bromodeoxyuridine; cAMP, cyclic adenosine monophosphate; CRE, cyclic adenosine monophosphate response element; CREB, cyclic adenosine monophosphate response element binding; EGFR, epidermal growth factor receptor; ERK1, extracellular regulated kinase-1; GABA, gamma-amino butyric acid; GABA_BR, gamma-amino butyric acid receptor; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PAC, pulmonary adenocarcinoma; PBS, phosphate buffered saline; PDAC, pancreatic ductal adenocarcinoma; PKA, protein kinase A.

II cells and mucin-producing cells may be the origin of small subsets of PAC (1,2). The incidence of PAC continues to rise in smokers and non-smokers (3) and this malignancy predominates in women.

The epidermal growth factor receptor (EGFR) is frequently over-expressed in PAC and interference with its signaling pathway by small molecule tyrosine kinase inhibitors has been the focus of intense research. While these agents have shown great promise in preclinical studies, they have disappointed in clinical trials (4–6). Studies by our laboratory have identified an important role of cyclic adenosine monophosphate (cAMP) signaling in the growth regulation of human small airway epithelial cell line HPL1D and in the human PAC cell line NCI-H322 (7), both of which express the bronchiolar Clara cell-specific CC10 antigen. Classic agonists for beta-1-adrenoreceptors (β_1 -ARs) or the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a high affinity agonist for this receptor (8), stimulated DNA synthesis in both cell lines via cAMP-dependent signaling. The resulting activation of protein kinase A (PKA) transactivated the EGFR and its downstream effectors while additionally phosphorylating the transcription factor cyclic adenosine monophosphate response element-binding (CREB) protein (7). Estrogen enhanced the responses to β_1 -AR signaling by non-genomic cAMP signaling via the estrogen receptor beta (9). This cooperation between β_1 -AR and estrogen receptor beta signaling may contribute to the prevalence of PAC in women. Small airway-derived PACs induced in Syrian golden hamsters by NNK overexpressed β -ARs and their downstream effectors, PKA, p-CREB, p-EGFR, raf-1 and p-extracellular regulated kinase-1 (ERK1)/2 (10,11). A beta-blocker had significant cancer preventive effects in this animal model (12). On the other hand, treatment of the animals with the stress hormone epinephrine, a physiological β -AR agonist, had significant tumor-promoting effects on NNK-induced PAC in hamsters (12). Epinephrine or its precursor norepinephrine has also been shown to stimulate the migration of adenocarcinoma of the prostate (13), colon (14), mammary gland (15), stomach (16) and ovary (17). It hence appears that hyperstimulation of cAMP-mediated signaling in response to β -AR stimulation by stress hormones or NNK contribute to the aggressive behavior of some of the most common solid human cancers, all of them are adenocarcinomas. While antagonists for β -ARs (beta-blockers) have shown significant antitumorigenic effects in preclinical studies of adenocarcinoma of the lungs (12), prostate (13), colon (14) and breast (15), their clinical use for the prevention and treatment of human adenocarcinomas is problematic. Beta-blockers have significant cardiovascular effects and their chronic use results in the sensitization of β -ARs, rendering these receptors more sensitive to agonists. Accordingly, long-term treatment with such agents may promote the development and progression of cancers under positive growth control by cAMP signaling.

In search for a more suitable agent capable of counteracting hyperactive cAMP signaling, our current study has focused on the neurotransmitter gamma-amino butyric acid (GABA). GABA is the major inhibitory neurotransmitter in the central nervous system and controls the excitatory effects of cAMP signaling by inhibiting adenylyl cyclase via activation of the inhibitory G-protein (G_{α_i})-coupled gamma-amino butyric acid receptor (GABA_BR) (18). GABA and its receptors are also expressed in most non-neuronal tissues, including the lungs (19). Our data provide evidence, for the first time, that GABA is underexpressed in NNK-induced small airway-derived PACs. In addition, our *in vitro* data show that GABA_BR signaling strongly inhibits cAMP-dependent signaling in human small airway epithelial and PAC

cells in the presence or absence of β -AR stimulation, thereby effectively blocking DNA synthesis and cell migration while the inhibitory effects of GABA are disinhibited by GABA_BR knockdown.

Materials and methods

Immunohistochemical assessment of GABA in hamster lung tissues

Archived tissue blocks of lungs from 10 control hamsters and from 10 hamsters with NNK-induced PACs (2.5 mg per 100 g three times per week for 20 weeks by subcutaneous injection) that were reported previously to overexpress the beta-adrenergic signaling pathway were used. This dosing regimen of NNK reproducibly results in the development of PAC in this animal species at an incidence of 80–100%. PACs were diagnosed by histopathology and are in accordance with the lung tumor classification (20) recommended as criteria for lung adenocarcinomas in the mouse (lesions comprised cuboidal to columnar cells, >5 mm in diameter, locally invasive). The sections were deparaffinized in xylene, dehydrated with graded ethanols, washed with phosphate buffered saline (PBS, pH 7.4) and incubated with 0.3% hydrogen peroxide in 50% methanol for 20 min at room temperature. Immunostains were conducted using the Vectastain Universal kit (Vector Laboratories, Burlingame, CA) according to the vendor's instructions. Incubations with primary antibody (polyclonal anti-GABA, Sigma, St Louis, MS; 1:1000 dilutions) were performed in a humid chamber at 4°C overnight. Sections exposed to diluent alone without primary antibody served as negative controls. The specificity of the GABA antibody was verified by pre-incubation of the primary antibody with 200 μ M GABA. This pre-incubation completely abolished the positive immunoreactivity to GABA (Figure 1A). Diaminobenzidine served as substrate and Mayer's hematoxylin as counterstain. The sections were photographed with a digital camera and analyzed densitometrically using National Institutes of Health (NIH) Scion image analysis software. Briefly, each photograph was zoomed to 200%, yielding a magnification of $\times 400$. Densitometric analysis in a rectangular unit area of 0.5×0.3 cm was performed in the cytoplasm of small airway epithelial cells, alveolar cells or tumor cells with 100 measurements per cell type per hamster. To ensure randomization of the measured areas, a clear plastic overlay with a grid was taped over the computer screen and meeting points of horizontal with vertical grid lines were selected for the measurements. Data were analyzed by one-way analysis of variance (ANOVA) and Tukey–Kramer multiple comparison test.

Cell culture

The immortalized human small airway epithelial cell line HPL1D was a kind gift from Dr Takahashi (21) and was maintained at 37°C in Ham's F12 medium supplemented with 1% fetal calf serum, 5 μ g/ml insulin, 5 μ g/ml human transferrin, 50 nM hydrocortisone hemisuccinate, 4.75 pM 3,3',5'-triiodo-L-thyronine and 50 nM Na-selenite. The human PAC cell line NCI-H322 was purchased from European Collection of Cell Cultures (Health Protection Agency, Porton Down, Salisbury, Wiltshire, UK) and maintained in RPMI-1640 culture medium (Gibco, Frederick, MD) supplemented with fetal bovine serum (10% vol/vol) at 37°C in an atmosphere of 5% CO₂. All assays were conducted in basal media without supplements following three washes with PBS.

Assessment of DNA synthesis by BrdU incorporation assays

Bromodeoxyuridine (BrdU) incorporation assays were conducted with a kit (Roche Applied Science, Indianapolis, IN) according to the vendor's instructions as described previously (9). Cells were cultured in 96-well plates (1×10^4 per well) deprived of serum and supplements for 24 h and then treated with isoproterenol (10 nM) with and without pre-incubation (4 h) with GABA or the selective GABA_BR agonist baclofen (30 μ M; Sigma) for 72 h or they were exposed to each of the inhibitors alone for the duration of the assay. Cells were labeled with 10 μ l per well BrdU and re-incubated at 37°C for 4 h. After removal of the labeling medium, cells were fixed and probed with anti-BrdU monoclonal antibody and its substrate, tetramethylbenzidine for 1 h. The absorbance of each sample was measured in an enzyme-linked immunosorbent assay reader at 370 nm. A blank was run in each experiment to provide information about BrdU and anti-BrdU non-specific binding. The non-specific binding was subtracted from all other values. Each experiment was conducted twice with five replicates per data point. Statistical analysis of data was by one-way ANOVA and Tukey–Kramer multiple comparison tests.

Determination of cell migration

The metastasis of cancer cells is facilitated by their ability to migrate. Measurement of cell migration is therefore frequently used as a tool to assess the metastatic potential of cancer cells. A colorimetric cell migration assay kit (Cell Biolabs, San Diego, CA) consisting of 24-well plates that contain polycarbonate membrane filter inserts (8 μ m pore size) was used according to the vendors's instructions. Cells (0.5×10^6 cells/ml of basal medium) were seeded onto the top chamber above the filter insert and pretreated for 4 h with

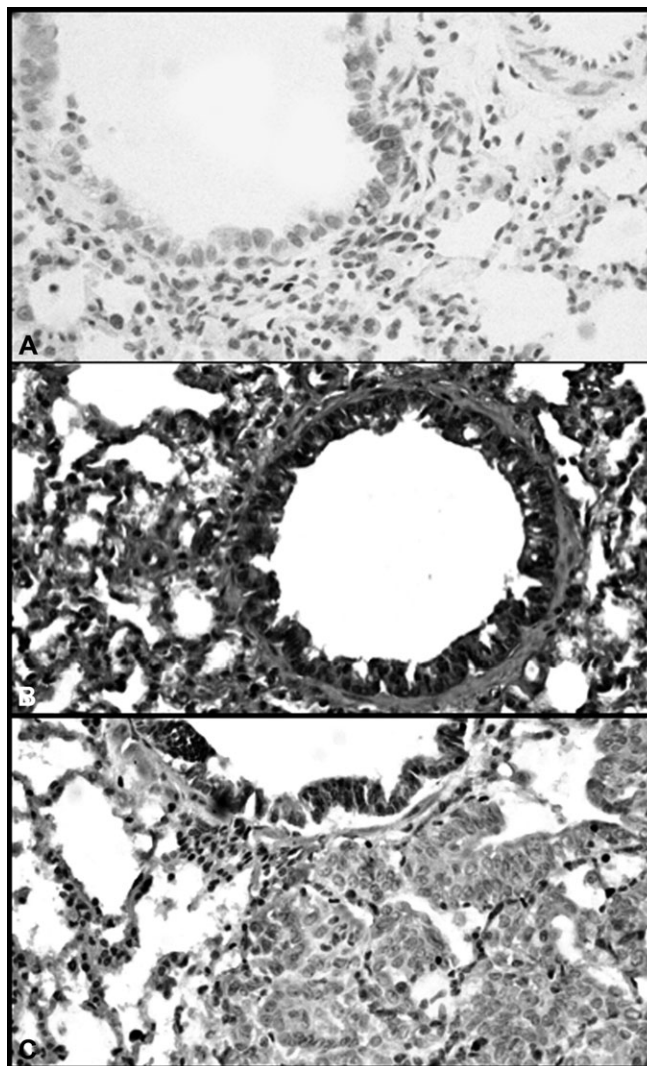


Fig. 1. Photomicrographs (magnification: $\times 200$) of hamster lung tissues showing positive immunoreactivity to anti-mammalian GABA (brown stain) in a control animal (**B**) and a hamster (**C**) treated for 20 weeks with NNK. Figure (**A**) demonstrates the specificity of the primary antibody by lack of immunoreactivity on a section from a control animal incubated with primary anti-GABA antibody after preabsorption with GABA (200 μ M).

GABA (30 μ M) or baclofen (30 μ M). Isoproterenol (10 nM) was then added. Following a 24 h incubation period, non-migratory cells were removed from the top of the filters by cotton swab. The filter with cells that had migrated to its bottom surface were incubated with staining solution for 10 min, washed three times with tap water, air-dried and photographed. Following extraction of each filter, optical density at 560 nm was read with a plate reader. Each assay was conducted in triplicate. Statistical analysis of data was by ANOVA and Tukey–Kramer multiple comparison tests.

Determination of intracellular cAMP by immunoassays

Cells were plated in complete medium at 4×10^5 cells per six-well plate and grown until 65–70% confluence. The cells were washed with PBS and maintained in basal medium without additives for 24 h. Following two washes with PBS, the cells were then pre-incubated for 30 min with 1 mM isobutylmethylxanthine (IBMX) (Sigma) and then exposed to isoproterenol (10 nM) in the presence and absence of pre-incubation with GABA (30 μ M) or baclofen (30 μ M) in PBS in fresh basal medium containing 1 mM IBMX for 30 min. After three washes with distilled water, cells were treated with 0.1 M HCl for 20 min and then lysed by sonication. After centrifugation, samples were analyzed for cAMP levels using a direct cAMP enzyme immunoassay kit according to the manufacturer's instructions (Assay Designs, Ann Arbor, MI). Color intensity was measured at 405 nm. Statistical analysis of data was by one-way ANOVA, Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test.

Assessment of phosphorylated ERK1/2 and CREB proteins by western blots

NCI-H322 or HPL1D cells were seeded into culture vessels (500 000 cell per 100 cm²) containing their respective growth media. When the cells had reached 60–65% confluence, they were rinsed one time with 1× PBS and starved of serum and supplements for 24 h. Following removal of the media and replacement with fresh basal media, isoproterenol (10 nM) was added in the presence or absence of pre-incubation with GABA (30 μM for 30 min) or baclophen (30 μM for 30 min) and cells were incubated for 10 min. Pre-incubation with the selective GABA_BR antagonist CGP-35348 (30 μM for 30 min; Sigma) was used to confirm the involvement of the GABA_BR in the observed inhibitory effects of GABA. The cultured cells were then washed once with cold PBS, lysed in 20 mM Tris-base, 200 mM NaCl, 1 M sodium fluoride, 0.5 M ethylenediaminetetraacetic acid, 100 mM Na₂VO₄, 100 mM phenylmethylsulfonyl fluoride, 1 ml pepstatin, 1 ml leupeptin, 1 ml aprotinin and 0.25 % NP-40. Then, protein samples were denatured by boiling at 95°C for 5 min, separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were blocked with 5% non-fat dry milk, probed with CREB and p-CREB or ERK1/2 and p-ERK1/2 antibodies, respectively, and developed by chemiluminescence with enhanced chemiluminescence reagents. Densitometry of the bands was performed using NIH Scion software. Briefly, the films were scanned onto a computer screen and five densitometric readings per band were taken. Data are expressed as mean values and standard errors of the ratios of p-CREB/CREB (Figure 4B) or p-ERK1/2 (Figure 6A and B). Each experiment was repeated twice and yielded similar results. Statistical analysis of data was by one-way ANOVA, followed by Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test.

Transient transfection with stealth select RNA_i for GABA_BR1

Cells (>90% viable) were plated at 3 × 10⁴ cells per well in 24-well plates in complete medium without antibiotics and allowed to reach 60% confluence. They were then transfected in triplicate for each treatment group with 100 μl of 40 nM of GABA_BR1 Stealth RNA_i (Invitrogen, San Diego, CA) complexed with 2 mg/ml Lipofectamine (Invitrogen). Following a 24 h incubation in a humidified incubator (5% CO₂, 37°C), transfection efficiency, transfection toxicity and percent of transfected cells were determined (Block-iT Alexa Fluor Red Fluorescent Control, dead cell stain ethidium homodimer-1, nuclear stain Hoechst 33342; Invitrogen). The growth medium was then replaced by basal medium without additives and responses to isoproterenol, NNK and GABA assessed in untransfected cells versus cells with GABA_BR knockdown, using the cyclic adenosine monophosphate response element (CRE)-luciferase reporter assay described below. Negative stealth RNA_i provided by the vendor served as negative control.

Assessment of CRE activation by CRE-luciferase reporter assay

Cells were cotransfected with 500 ng of pRSV-β-galactosidase and 1 μg of CRE-luciferase DNAs, alone, or along with 40 nM GABA_BR small interfering ribonucleic acid (siRNA) constructs. Forty-eight hours after transfection, cells were deprived of serum for 18 h before stimulation with the indicated agents. Untransfected and transfected cells with control siRNA provided by the vendor,

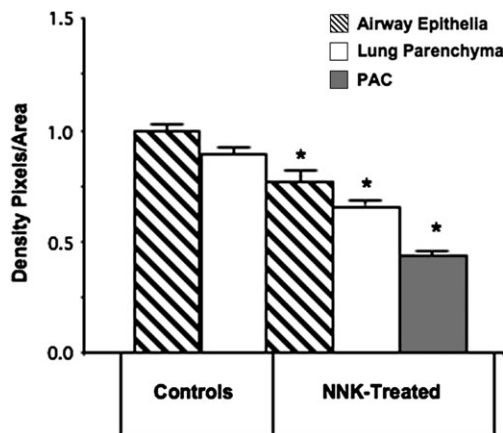


Fig. 2. Densitometry data of the immunostains with primary anti-GABA antibody showing mean values and standard errors of 100 densitometry readings from randomly chosen cytoplasmic areas (0.5 × 0.3 cm on photographs enlarged to ×400) of small airway epithelia, alveolar epithelia or tumor cells per hamster from 10 hamsters. Data significantly (*P* < 0.001) different from small airway epithelial controls are identified by asterisk.

either stimulated with the same agents or left alone, served as controls. Cells were harvested 24 h later, and luciferase and β-galactosidase activities were then measured using standard luciferase (Promega, Madison, WI) and β-galactosidase detection kits (Applied Biosystems, Bedford, MA).

Results

In accord with the role of GABA as a ubiquitous neurotransmitter, immunohistochemical analysis of lung tissues from control hamsters showed prominent positive immunoreactivity to GABA in all cells,

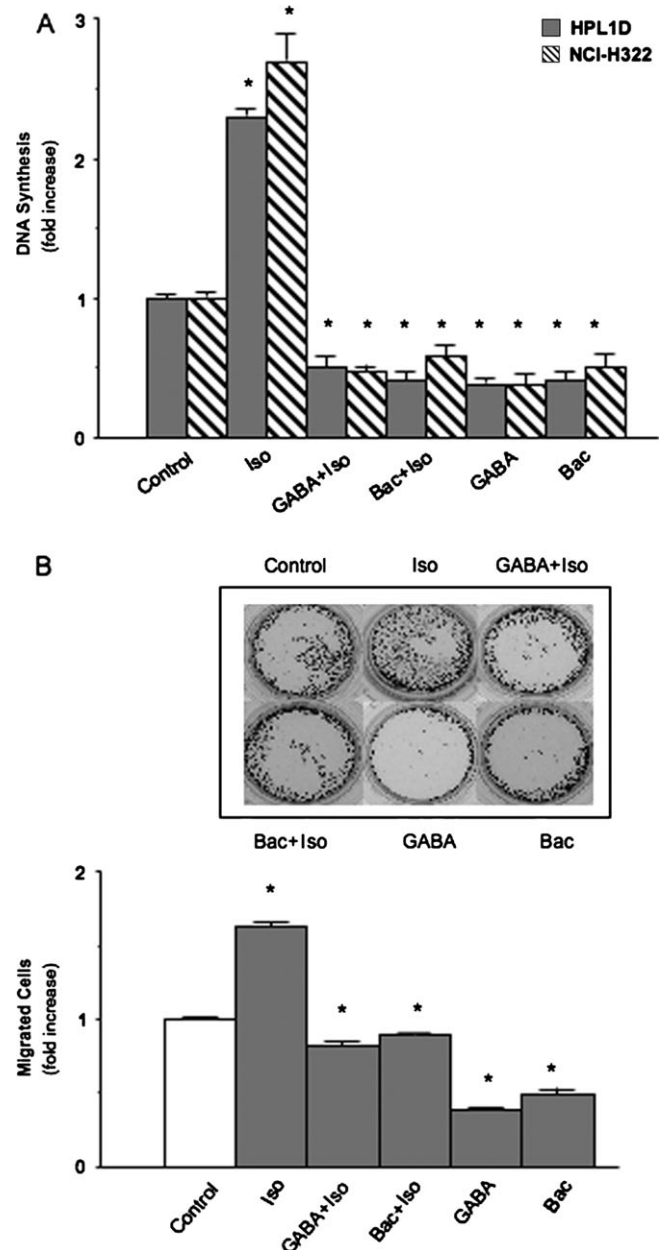


Fig. 3. Results of BrdU incorporation assays (A) and cell migration assays (B) in the PAC cell line NCI-H322 and immortalized human small airway epithelial cells HPL1D. Isoproterenol significantly (*P* < 0.001) stimulated each cell line in both assays and these responses were completely blocked by GABA or baclophen. Data in (A) are mean values and standard deviations of five replicates per data point. Data in (B) are mean values and standard deviation from three separate assays with identical treatment groups. The inset in (B) shows a photograph of the filter inserts with cells that have migrated to the bottom surface of the filters. Data significantly (*P* < 0.001) different from controls are identified by asterisk in both graphs.

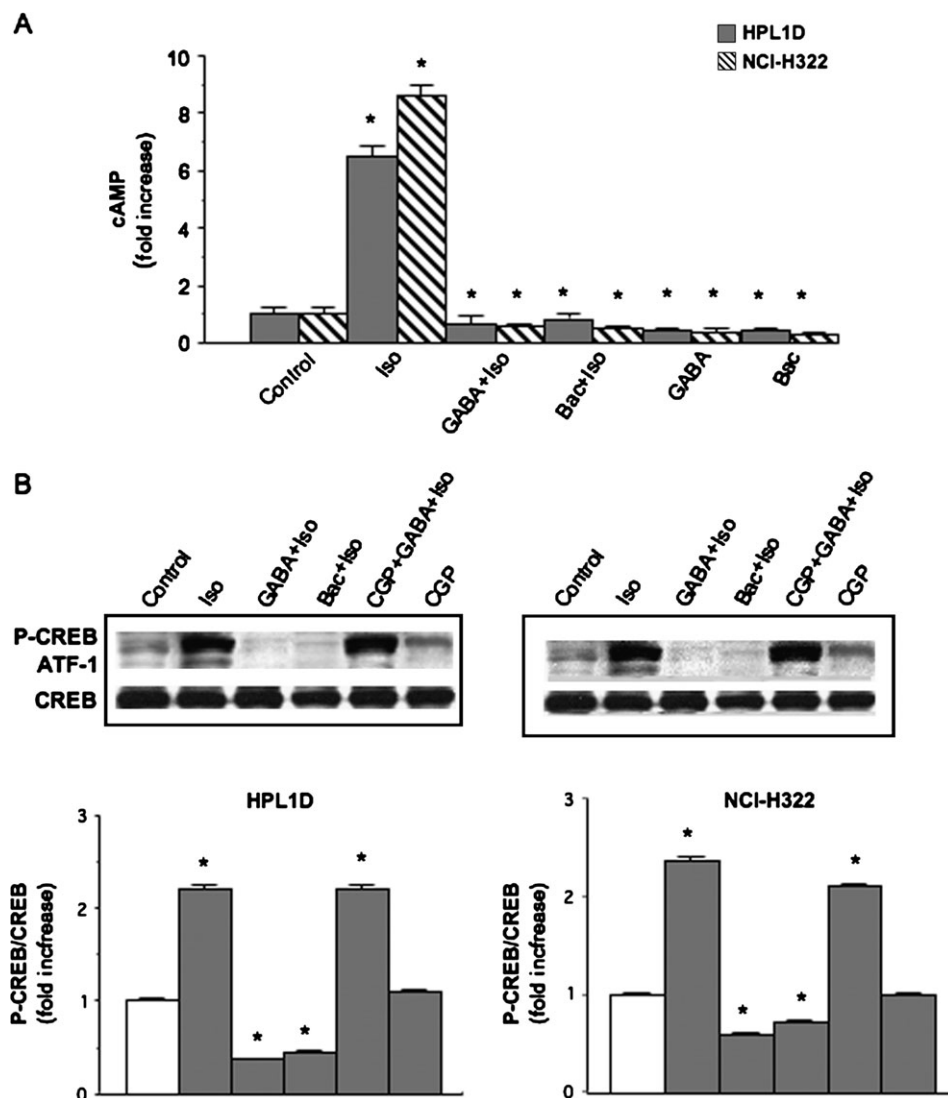


Fig. 4. Results of cAMP assays (A) showing strong stimulation by isoproterenol in HPL1D and NCI-H322 cells, responses completely blocked by GABA or baclophen. Data are mean values and standard deviations of triplicate samples. Western blots (B) showing strong induction of p-CREB by isoproterenol in HPL1D and NCI-H322 cells. This response was completely blocked by GABA or baclophen while the GABA_BR antagonist CGP-35348 disinhibited the effects of GABA. Data in the graphs are mean values and standard deviations of five densitometric readings per band. Data significantly ($P < 0.001$) different from controls are identified by asterisk in both graphs.

including the lining cells of large and small airways, as well as lung parenchyma, including alveolar epithelia and connective tissues (Figure 1B). Positive immunoreactivity to GABA was completely abolished when the primary anti-GABA antibody was preabsorbed with GABA (Figure 1A), thus verifying the specificity of the antibody. In addition, immunostains of lung sections exposed to diluent without primary antibody yielded no detectable immunoreactivity (data not shown). Positive immunoreactivity to GABA was reduced in all lung cells of animals treated with NNK, with PAC cells showing the strongest reduction (Figures 1C and 2). The differences in GABA reactivity between control and NNK-exposed airway epithelia ($P < 0.001$) or lung parenchyma ($P < 0.001$) were significant. In addition, the NNK-induced PACs (Figures 1C and 2) demonstrated $<40\%$ of the GABA reactivity than the control small airway epithelial cells ($P < 0.001$). These data suggest that NNK reduces GABA signaling in the lungs and prompted us to explore a potential tumor suppressor function of GABA by *in vitro* experiments.

As Figure 3A shows, the selective agonist for β -ARs, isoproterenol, significantly ($P < 0.001$) stimulated DNA synthesis in the human PAC cell line NCI-H322 and in immortalized human small airway

epithelial cells HPL1D. This response was suppressed significantly ($P < 0.001$) below base levels by pretreatment of the cells with GABA or the selective GABA_BR agonist baclophen (Figure 3B). Both of these agents also significantly ($P < 0.001$) reduced DNA synthesis below the levels of untreated cells when administered alone. In addition, isoproterenol significantly ($P < 0.001$) increased the number of migrated NCI-H322 cells in cell migration assays (Figure 3B), a response completely blocked by pretreatment of the cells with GABA or baclophen. Again, both agents significantly ($P < 0.001$) reduced base level migration below the levels of untreated cells when administered alone (Figure 3B).

The β_1 -AR is a member of the G-protein-coupled receptor family that increases intracellular cAMP upon agonist-induced activation of the stimulatory G-protein $G\alpha_s$. We have shown previously that the tobacco carcinogen NNK, which is a high affinity agonist for this receptor (8), induces cAMP-dependent activation of PKA and CREB, resulting in the stimulation of DNA synthesis in HPL1D and NCI-H322 cells (7). In accord with these findings, immunoassays for the detection of intracellular cAMP revealed a 6.4-fold (HPL1D) and 8.3-fold (NCI-H322) increase in cells exposed to isoproterenol

(Figure 4A). This response was completely blocked by pretreatment of the cells with GABA or baclophen (Figure 4A). Both of these agents also significantly ($P < 0.001$) reduced base levels of cAMP in cells not exposed to isoproterenol (Figure 4A).

The transcription factor CREB is a classic downstream effector of cAMP signaling that is activated by cAMP-dependent PKA. We therefore assessed the modulation of phosphorylated CREB protein in HPL1D and NCI-H322 cells by western blots. As Figure 4B shows, both cell lines responded to isoproterenol with a significant ($P < 0.001$) induction of p-CREB protein, an effect completely blocked by pre-exposure of the cells to GABA or baclophen ($P < 0.001$). The inhibitory effects of GABA on isoproterenol-induced p-CREB induction were disinhibited by pretreatment of the cells with the selective GABA_BR antagonist CGP-35348. These findings confirm an important role of the GABA_BR in the observed inhibitory effects of GABA that are additionally suggested by the almost identical responses of cells to GABA and the selective GABA_BR agonist baclophen.

Phosphorylation of CREB at the Ser133 site is an important step in the activation of genes with the CRE sites in their promoters. We therefore studied the modulation of CRE activity by CRE-luciferase reporter assays in HPL1D and NCI-H322 cells. As Figure 5A and B shows, isoproterenol as well as NNK significantly ($P < 0.001$) increased CRE activity and simultaneous exposure of HPL1D and NCI-H322 cells to both agents had significant ($P < 0.001$) additive effects. Pretreatment of the cells with GABA completely blocked the responses to isoproterenol or NNK while also significantly ($P < 0.001$) reducing the responses to the additive stimulation of isoproterenol and NNK (Figure 5A and B). GABA_BR knockdown disinhibited the inhibitory effects of GABA in cells simultaneously stimulated by isoproterenol and NNK (Figure 5A and B). These findings further emphasize the involvement of the GABA_BR in the observed effects of GABA.

We have shown previously that treatment of HPL1D or NCI-H322 cells with a classic β -AR agonist or NNK activates the ERK1/2 cascade via PKA-dependent transactivation of the EGFR (7). We therefore assessed the effects of GABA and baclophen on the expression of p-ERK1/2 in isoproterenol-stimulated and untreated HPL1D and NCI-H322 cells. As Figure 6A and B shows, isoproterenol significantly ($P < 0.001$) induced ERK1/2 phosphorylation in both cell lines. This response was completely blocked by pretreatment of the cells with GABA or baclophen while the GABA_BR antagonist CGP-35348 disinhibited the effects of GABA (Figure 6A and B).

Discussion

Our data provide evidence, for the first time, that the tobacco-specific nitrosamine NNK significantly reduces GABA in the lungs and that small airway-derived PACs induced by this potent lung carcinogen express particularly low levels of this inhibitory neurotransmitter. These findings are in accord with investigations by proton magnetic resonance studies that have revealed a reduction of GABA in the brain of smokers (22). While the mechanisms how NNK reduces tissue GABA levels remain obscure, our *in vitro* data indicate that GABA suppresses the proliferation and migration of human PAC cells and small airway epithelial cells via inhibition of base level and β -AR-stimulated cAMP signaling. The observed isoproterenol-induced increase in ERK1/2 phosphorylation and its inhibition by GABA and baclophen are consistent with our reports of PKA-dependent EGFR transactivation in these cells (7,9). Similarly, the observed additive effects of NNK and isoproterenol in the CRE-luciferase assays is in accord with the tumor-promoting effect of classic β -AR agonists on NNK-induced PAC in hamsters (12).

Pharmacological or genetic silencing of the GABA_BR disinhibited the effects of GABA. These findings indicate that the observed inhibitory effects of GABA were mediated by the GABA_BR and are consistent with the documented ability of this receptor to inhibit cAMP signaling via $G\alpha_i$ -induced inhibition of adenylyl cyclase (23–25). Collectively, these findings suggest that GABA may have

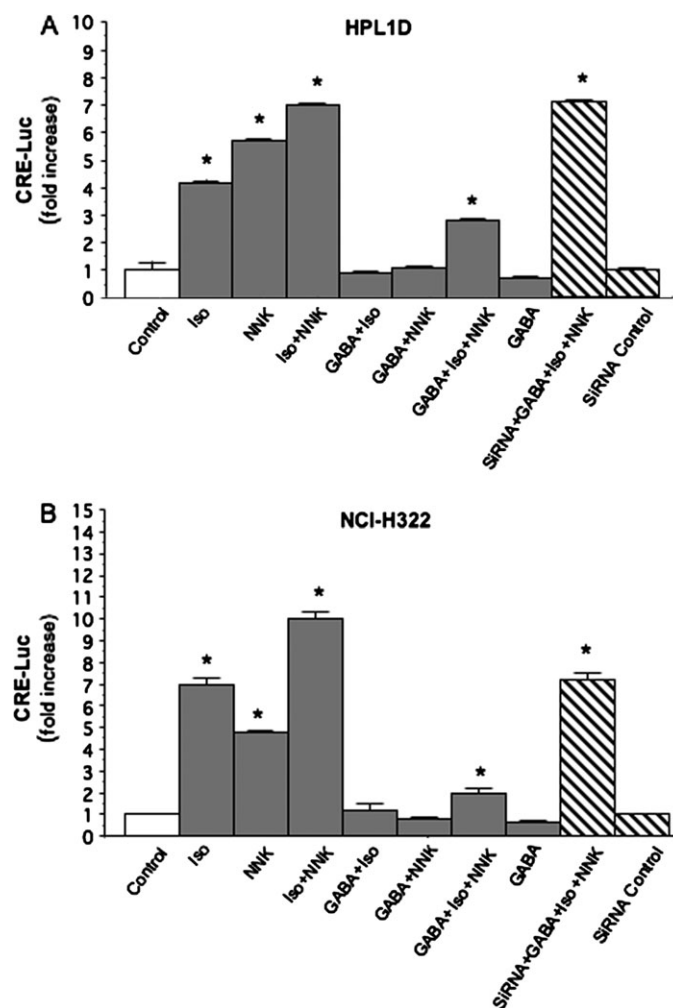


Fig. 5. Results of CRE-luciferase reporter assays in HPL1D (A) and NCI-H322 (B) cells. Isoproterenol and NNK significantly ($P < 0.001$) induced CRE-luciferase while exposure to both agents had additive effects. GABA completely inhibited the inductions of CRE-luciferase, an effect disinhibited by siRNA knockdown of the GABA_BR. Data are mean values and standard deviation of three replicate assays conducted under identical conditions. Data significantly ($P < 0.001$) different from controls are identified by asterisk.

tumor suppressor function in these cells and that its downregulation by NNK may contribute to the development and aggressive behavior of PAC in smokers.

As our current and published (7,9) *in vitro* data show, cAMP/PKA signaling has strong stimulatory effects on DNA synthesis and migration of human PAC cells with features of small airway epithelial cells. We have also shown that, this signaling pathway is activated by NNK and that it also stimulates the proliferation of the putative cells of origin for most PACs, small airway epithelial cells (7,9). The emerging novel concept of NNK-induced impaired GABA-ergic tumor suppressor function and concomitant hyperstimulation of cAMP signaling that drives cell proliferation and migration of these PACs goes far beyond a simple cause and effect model of lung cancer initiation by the mutational activities of this tobacco carcinogens and its reactive metabolites (26–28).

While we (7–9) and others (29,30) have unequivocally shown that NNK stimulates β -adrenergic signaling in PAC and their cells of origin, elevated systemic levels of the physiological agonists for β -ARs epinephrine and norepinephrine may additionally stimulate this pathway. This interpretation is in accord with recent reports that have emphasized an important role of these stress hormones in the aggressive behavior of adenocarcinoma of the colon (14), prostate

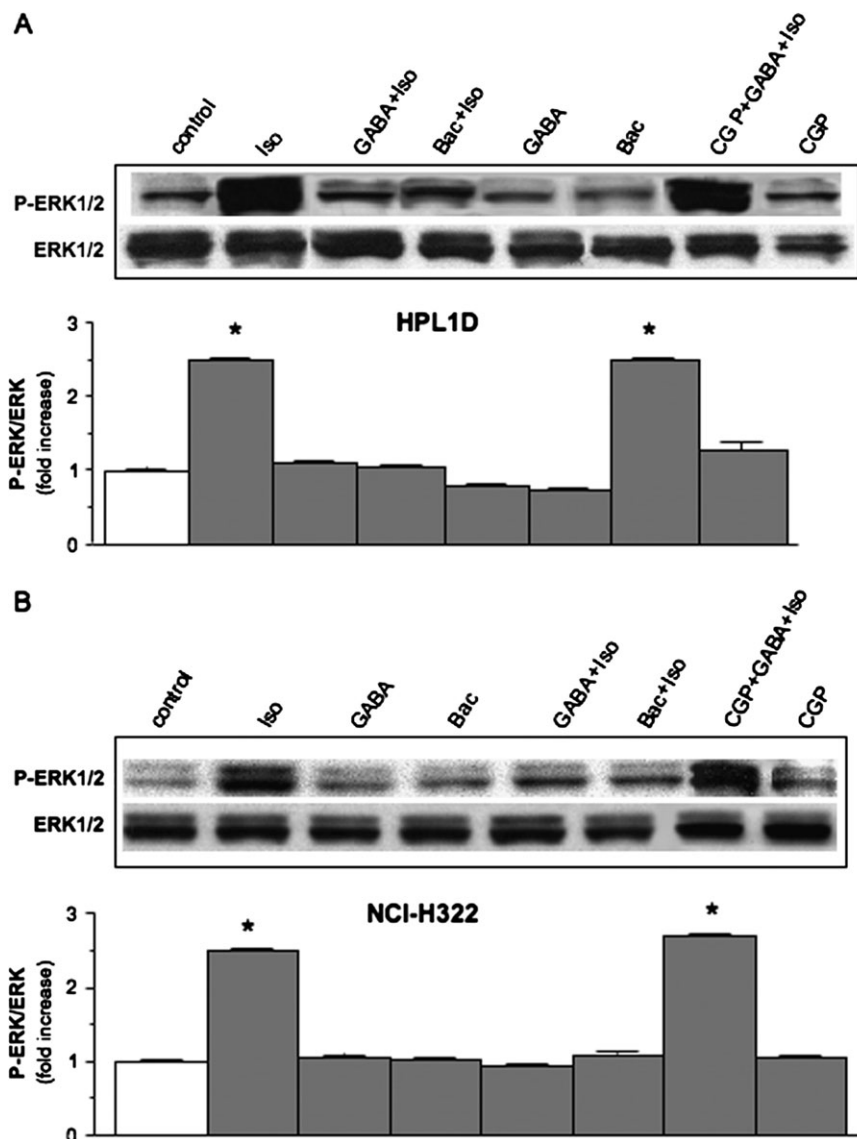


Fig. 6. Western blots showing the induction of p-ERK1/2 in HPL1D (A) and NCI-H322 (B) cells by isoproterenol and the inhibition of these responses by GABA or baclophen. The GABA_BR antagonist CGP-35348 blocked the inhibitory effects of GABA. Data in the graphs are mean values and standard deviations of five densitometric readings per band. Data significantly ($P < 0.001$) different from controls are identified by asterisk in both graphs.

(13), stomach (16), mammary gland (31) and ovary (17). Moreover, non-genomic estrogen signaling via the estrogen receptor beta also stimulates cAMP signaling and has been shown to cooperate with NNK-induced β_1 -AR signaling in HPL1D cells (9). In conjunction with these cited publications, our findings imply that environmental and lifestyle factors that reduce GABA signaling and enhance G-protein-coupled receptor-mediated cAMP signaling are risk factors for the development of PAC and contribute significantly to the aggressive behavior of this cancer. In turn, the hitherto neglected influence of stimulatory and inhibitory neurotransmitter signaling appears to be a promising target for the marker-guided prevention and therapy of PAC.

GABA_BR agonists are routinely prescribed for the treatment of muscle spasms due to spinal injuries, whereas GABA is widely used as a dietary supplement to reduce anxiety and sleeplessness. The concentrations of GABA and baclophen used in the current study are within the range of systemic concentrations at the maximum recommended dosages for these agents in people. Precautions taking into consideration the inhibitory effects of GABA-ergic agents on the central nervous system would thus be similar as with their current usage if they were used as anticancer drugs. However, the potential

exploitation of inhibitory GABA_BR signaling for the prevention or adjuvant therapy of small airway-derived PAC will require the careful monitoring of patients for pulmonary GABA levels and perhaps systemic cAMP levels. Environmental and lifestyle factors as well as diet, dietary supplements, drug treatments, stress and preexisting non-neoplastic diseases can profoundly modulate cell and tissue levels of GABA and cAMP, the number and sensitivity of GABA_BRs as well as G α_s -coupled receptors, including the β -ARs, that stimulate cAMP signaling. Large intraindividual differences in tissue GABA levels and responsiveness to agonists for GABA_BRs are therefore to be expected. The literature on the expression and function of GABA and its receptors in neoplastic diseases reflects this diversity. A recent publication from our laboratory thus reported underexpression of GABA in 29 of 30 investigated tissue arrays from human pancreatic ductal adenocarcinomas (PDACs) and GABA_BR-mediated inhibition of cAMP-dependent transactivation of the EGFR pathway, cell proliferation and cell migration in immortalized human pancreatic duct epithelial cells and in human PDAC cell lines Panc-1 and BXPC-3 (32). In contrast, a Japanese laboratory published at about the same time overexpression of GABA in 5 of 15 investigated tissue samples from the same histological type of pancreatic cancer that was

associated with overexpression of the pi subunit of the GABA_AR (33). Moreover, these investigators showed that two of seven investigated human PDAC cell lines overexpressed this GABA_AR subunit and were stimulated in their growth by GABA, whereas the remaining cell lines were not. Unfortunately, the responses to GABA of the five PDAC cell lines without overexpressed GABA_A-pi (including Panc-1 used in our study) were not shown although it was mentioned that they were not stimulated by GABA (33). Differences in GABA contents in a typical Western diet as opposed to a typical Asian diet due to the high GABA contents in rice (34) may have contributed to the high GABA levels observed in PDACs by the Japanese group. In addition, the described overexpression of the GABA_AR, which unlike the inhibitory GABA_BR mediates excitatory responses to GABA, may have reversed the effects of GABA from inhibitory to stimulatory. Similar discrepancies have been reported for colon cancer with GABA overexpression reported as an indication that GABA signaling may contribute to the carcinogenic process and GABA-ergic agonists may be of use for cancer intervention (35), whereas others have reported inhibition of colon cancer cell migration by GABA (36). In conjunction with our current data, these findings emphasize the need for marker-guided cancer intervention as the histopathology classification of cancer fails to provide reliable information on the presence or absence of hyperactive or hypoactive regulatory pathways that may be suitable drug targets in individual cases.

Funding

National Cancer Institute (RO1CA096128).

Acknowledgements

Conflict of Interest Statement: None declared.

References

- Albertine, K.H. *et al.* (1998) Analysis of cell type and radiographic presentation as predictors of the clinical course of patients with bronchioalveolar cell carcinoma. *Chest*, **113**, 997–1006.
- Garber, M.E. *et al.* (2001) Diversity of gene expression in adenocarcinoma of the lung. *Proc. Natl Acad. Sci. USA*, **98**, 13784–13789.
- Devesa, S.S. *et al.* (2005) International lung cancer trends by histologic type: male:female differences diminishing and adenocarcinoma rates rising. *Int. J. Cancer*, **117**, 294–299.
- Paez, J.G. *et al.* (2004) EGFR mutations in lung cancer: correlation with clinical response to Gefitinib therapy. *Science*, **304**, 1497–1500.
- Roskoski, R., Jr (2004) The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem. Biophys. Res. Commun.*, **319**, 1–11.
- Yu, Z. *et al.* (2007) Resistance to an irreversible epidermal growth factor receptor (EGFR) inhibitor in EGFR-mutant lung cancer reveals novel treatment strategies. *Cancer Res.*, **67**, 10417–10427.
- Laag, E. *et al.* (2006) NNK activates ERK1/2 and CREB/ATF-1 via beta-1-AR and EGFR signaling in human lung adenocarcinoma and small airway epithelial cells. *Int. J. Cancer*, **119**, 1547–1552.
- Schuller, H.M. *et al.* (1999) The tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is a beta-adrenergic agonist and stimulates DNA synthesis in lung adenocarcinoma via beta-adrenergic receptor-mediated release of arachidonic acid. *Cancer Res.*, **59**, 4510–4515.
- Majidi, M. *et al.* (2007) Nongenomic beta estrogen receptors enhance beta1 adrenergic signaling induced by the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in human small airway epithelial cells. *Cancer Res.*, **67**, 6863–6871.
- Schuller, H.M. *et al.* (2005) NNK-induced hamster lung adenocarcinomas over-express beta2-adrenergic and EGFR signaling pathways. *Lung Cancer*, **49**, 35–45.
- Cekanova, M. *et al.* (2007) Overexpressed Raf-1 and phosphorylated cyclic adenosine 3'-5'-monophosphate response element-binding protein are early markers for lung adenocarcinoma. *Cancer*, **109**, 1164–1173.
- Schuller, H.M. *et al.* (2000) Beta-adrenergic modulation of NNK-induced lung carcinogenesis in hamsters. *J. Cancer Res. Clin. Oncol.*, **126**, 624–630.
- Palm, D. *et al.* (2006) The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers. *Int. J. Cancer*, **118**, 2744–2749.
- Masur, K. *et al.* (2001) Norepinephrine-induced migration of SW 480 colon carcinoma cells is inhibited by beta-blockers. *Cancer Res.*, **61**, 2866–2869.
- Drell, T.L. IV *et al.* (2003) Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells. *Breast Cancer Res. Treat.*, **80**, 63–70.
- Shin, V.Y. *et al.* (2007) Functional role of beta-adrenergic receptors in the mitogenic action of nicotine on gastric cancer cells. *Toxicol. Sci.*, **96**, 21–29.
- Sood, A.K. *et al.* (2006) Stress hormone-mediated invasion of ovarian cancer cells. *Clin. Cancer Res.*, **12**, 369–375.
- Franeek, M. (2004) History and the present of metabotropic GABA_B receptor. *Cesk Fysiol*, **53**, 117–124.
- Chapman, R.W. *et al.* (1993) GABA_B receptors in the lung. *Trends Pharmacol. Sci.*, **14**, 26–29.
- Nikitin, A.Y. *et al.* (2004) Classification of proliferative pulmonary lesions of the mouse: recommendations of the mouse models of human cancers consortium. *Cancer Res.*, **64**, 2307–2316.
- Masuda, A. *et al.* (1997) Establishment of human peripheral lung epithelial cell lines (HPL1) retaining differentiated characteristics and responsiveness to epidermal growth factor, hepatocyte growth factor, and transforming growth factor beta1. *Cancer Res.*, **57**, 4898–4904.
- Epperson, C.N. *et al.* (2005) Sex, GABA, and nicotine: the impact of smoking on cortical GABA levels across the menstrual cycle as measured with proton magnetic resonance spectroscopy. *Biol. Psychiatry*, **57**, 44–48.
- Gladkevich, A. *et al.* (2006) The peripheral GABAergic system as a target in endocrine disorders. *Auton. Neurosci.*, **124**, 1–8.
- Oka, M. *et al.* (2006) Functional expression of metabotropic GABA_B receptors in primary cultures of astrocytes from rat cerebral cortex. *Biochem. Biophys. Res. Commun.*, **341**, 874–881.
- Osawa, Y. *et al.* (2006) Functional expression of the GABA receptor in human airway smooth muscle. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **291**, 923–931.
- Wogan, G.N. *et al.* (2004) Environmental and chemical carcinogenesis. *Semin. Cancer Biol.*, **14**, 473–486.
- Hecht, S.S. (2006) Cigarette smoking: cancer risks, carcinogens, and mechanisms. *Langenbecks Arch. Surg.*, **391**, 603–613.
- Hecht, S.S. (2006) Smoking and lung cancer—a new role for an old toxicant? *Proc. Natl Acad. Sci. USA*, **103**, 15725–15726.
- Jin, Z. *et al.* (2005) Survival function of protein kinase C[ι] as a novel nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-activated bad kinase. *J. Biol. Chem.*, **280**, 16045–16052.
- Wu, W.K. *et al.* (2005) 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone from cigarette smoke stimulates colon cancer growth via beta-adrenoceptors. *Cancer Res.*, **65**, 5272–5277.
- Lang, K. *et al.* (2004) Induction of a metastatogenic tumor cell type by neurotransmitters and its pharmacological inhibition by established drugs. *Int. J. Cancer*, **112**, 231–238.
- Schuller, H.M. *et al.* (2007) GABA(B) receptor is a novel drug target for pancreatic cancer. *Cancer*, **112**, 767–778.
- Takehara, A. *et al.* (2007) Gamma-aminobutyric acid (GABA) stimulates pancreatic cancer growth through overexpressing GABA_A receptor pi subunit. *Cancer Res.*, **67**, 9704–9712.
- Oh, C.H. *et al.* (2004) Effects of germinated brown rice extracts with enhanced levels of GABA on cancer cell proliferation and apoptosis. *J. Med. Food*, **7**, 18–23.
- Kleinrok, Z. *et al.* (1998) GABA content and GAD activity in colon tumors taken from patients with colon cancer or from xenografted human colon cancer cells growing as s.c. tumors in athymic nu/nu mice. *J. Physiol. Pharmacol.*, **49**, 303–310.
- Joseph, J. *et al.* (2002) The neurotransmitter gamma-aminobutyric acid is an inhibitory regulator for the migration of SW 480 colon carcinoma cells. *Cancer Res.*, **62**, 6467–6469.

Received January 8, 2008; revised January 29, 2008; accepted January 29, 2008