Prevention of pancreatic cancer by the beta-blocker propranolol

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Pancreatic ductal adenocarcinoma (PDAC) is among the leading causes of cancer deaths and is unresponsive to existing therapy. Smoking and alcohol-induced pancreatitis are among the risk factors for PDAC. We have previously reported that beta-adrenergic receptors (β-ARs) stimulate the proliferation and migration of human PDAC cells *in vitro* by cAMP-dependent signaling and that the nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) activates this pathway directly *in vitro* while additionally stimulating the release of noradrenaline/adrenaline by binding to α7 nicotinic acetylcholine receptors (α7 nAChR) in hamsters. In this study, we have tested the hypothesis that the β-AR antagonist propranolol prevents the development of PDAC induced in hamsters with ethanol-induced pancreatitis by NNK. We found that propranolol had strong cancer preventive effects in this animal model. Western blots of pancreatic duct cells and PDAC cells harvested by laser capture microscopy showed significant upregulation of the α7 nAChR associated with significant inductions of p-CREB, p-ERK1/2, and increases in epidermal growth factor and vascular endothelial growth factor in PDAC cells of hamsters not treated with propranolol. These effects were reversed by treatment with propranolol. Our data suggest that propranolol may prevent the development of PDAC by blocking cAMP-dependent intracellular signaling, cAMP-dependent release of epidermal growth factor, and PKA-dependent release of vascular endothelial growth factor while additionally downregulating the α7 nAChR by inhibiting cAMP-mediated subunit assembly. We conclude that increased cAMP signaling is an important factor that drives the development and progression of PDAC and that the inhibition of cAMP formation is a promising new target for the prevention and adjuvant therapy of PDAC. *Anti-Cancer Drugs* 20:477–482 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Pancreatic cancer is the fourth leading cause of cancer mortality in both men and women in Western countries [1]. Smoking, diabetes mellitus, and pancreatitis from any etiology, including alcohol abuse, are risk factors for this malignancy [2]. Although pancreatic cancer can arise from endocrine or exocrine pancreatic cells, more than 95% of all pancreatic cancers are pancreatic ductal adenocarcinomas (PDACs). PDAC is one of the most aggressive human cancers, with extensive invasiveness and metastasis precluding surgical resection in the majority of patients at the time of diagnosis. In addition, PDAC is generally unresponsive to conventional radiotherapy and chemotherapy, resulting in a mortality rate of approximately 100% within 6 months of diagnosis.

The growth regulation of PDAC and its putative cells of origin, pancreatic duct epithelia, is poorly understood. The majority of PDACs (about 75%) harbor activating point mutations in K-ras while also overexpressing the epidermal growth factor receptor (EGFR), leading to the generally accepted view that EGFR signaling through ras and its downstream effector, the extracellular signal-regulated protein kinases (ERK1/2), play important roles in the regulation of this cancer. We have shown that human PDAC and pancreatic duct epithelial cells *in vitro* are stimulated in their growth by beta-adrenergic receptor (β-AR) agonists that activate signaling through adenylyl cyclase and its downstream effectors cAMP, PKA, and p-CREB as well as PKA-dependent transactivation of the EGFR pathway [3,4]. Interestingly, our studies also identified the powerful nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) as a β-AR agonist that stimulated the same signaling cascade in PDAC and pancreatic duct epithelial cells [3,4]. Beta-adrenergic activity of NNK resulting in mitogenic and/or antiapoptotic signaling has additionally been reported by us and others in cell lines derived from human small airway-derived adenocarcinomas, immortalized human small airway epithelia [5,6], and colon cancer cells [7]. In addition, it has been shown that the migration and invasiveness of adenocarcinomas of the colon, prostate, breast, and ovaries are under β-adrenergic control [8–11].

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In analogy to the increased risk of smokers and individuals with alcohol-induced panreatitis, studies in our laboratory have shown that pregnant hamsters given ethanol in the drinking water and treated with NNK give birth to offspring that developed a high incidence of pancreatitis-associated PDAC, whereas the offspring of females given ethanol alone developed pancreatitis [12,13]. Molecular analysis of the hamster PDACs showed upregulated expression levels of p-CREB and p-ERK1/2, suggestive of a hyperactive cAMP-dependent regulatory pathway [14]. By using this animal model, this study has tested the hypothesis that the general antagonist for β-ARs (synonym: β-blocker), propranolol, prevents the development of PDAC by blocking this signaling pathway.

Materials and methods

The animal experiment was approved by the Institutional Animal Care and Use Committee. Outbred male and female Syrian golden hamsters were purchased from Charles River (Wilmington, Massachusetts, USA) to establish a breeding colony at our laboratory animal facility. The animals were mated during evening hours under supervision of an experienced technician. Successful copulation was determined the next morning by the presence of a vaginal plug. Treatment of the pregnant hamsters with ethanol (10% in the drinking water from day 5 to end of pregnancy) alone or in combination with NNK (50 mg/kg by subcutaneous injection on day 15 of pregnancy) was done as in previous experiments that reproducibly yielded a 65–75% incidence of pancreatitis-associated PDAC in the offspring [12,13]. The offspring remained with their mothers until they were 4 weeks old and could feed themselves. One group ($n = 12$) treated prenatally with ethanol and NNK was subcutaneously injected five times a week with the β-blocker propranolol (0.3 mg/100 g bodyweight) until the end of the observation period (8 months), whereas a second group ($n = 12$) not treated with propranolol served as positive controls. A group of offspring exposed prenatally to ethanol alone ($n = 12$) served as negative control. All animals were killed 8 months after start of the cancer preventive treatments and all major organs harvested.

Pancreatic tissues were fixed in 70% ethanol and embedded in paraffin for histopathology evaluation of tissue sections stained with hematoxylin/eosin. Survival curves were established and assessed for significant differences among groups by using Prism Graphpad software (Graphpad Software Inc., San Diego, California, USA) and the χ^2 log-rank test. A contingency table was established with the columns representing the alternate outcome of pancreatic cancer or no pancreatic cancer and the rows representing the treatment groups. Association of the variable 'pancreatic cancer' with treatment group was ascertained by the χ^2 test.

Pancreatic duct epithelial cells and PDAC cells were harvested from tissue sections by laser capture microscopy as described earlier [14,15], using a PixCell II Laser Capture Microdissection system (Arcturus, Mountain View, California, USA). From each cell type in the treatment and control groups, 30 000 cells randomly allocated to three equal samples were analyzed. The proteins were extracted from the CapSure caps using protein extract buffer (Pierce, Rockford, Illinois, USA).

It has been shown in the nervous system and in colon cancer cells that the α7 nicotinic acetylcholine receptor (α7 nAChR) stimulates the synthesis and release of adrenaline and noradrenaline, which activate cAMP signaling downstream of β-ARs [16–18]. In accordance with these findings, we have shown that the α7 nAChR is upregulated in the hamster PDACs and that these animals have significantly higher levels of systemic noradrenaline and adrenaline than untreated controls or animals prenatally exposed to ethanol alone [14]. We therefore analyzed the protein expression of nAChRs expressing the α_7 subunits, as well as p-CREB and p-ERK1/2 in the harvested pancreatic cells. In addition, we assessed the protein levels of EGF and vascular endothelial growth factor (VEGF) that support the development and progression of pancreatic cancer. After lysis of the thawed tissues, protein was determined by using the BCA protein assay (Pierce). Equal amounts of protein were treated with loading buffer at 100°C for 5 min and applied to each lane for electrophoresis in 12% polyacrylamide gel. The electrophoresed proteins were transferred onto nitrocellulose membranes in transfer buffer at 100 mV for 1 h. After transfer, the membranes were treated with blocking buffer (5% nonfat dry milk in Tris-buffered saline with Tween 20) for 1 h, and incubated with primary antibody overnight at 4°C. Primary antibodies against the following signaling proteins were used: total CREB (Upstate Biotechnology, Lake Placid, New York, USA), p-CREB, p-ERK1/2, and ERK1/2 (Cell Signaling, Danvers, Massachusetts, USA), α7 nAChR (Millipore, Billerica, Massachusetts, USA), EGF (Santa Cruz Biotechology, Santa Cruz, California, USA), and VEGF (Abcam, Cambridge, Massachusetts, USA) and β-actin (Sigma). After being washed with Tris-buffered saline with Tween 20, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (goat anti-mouse, or goat anti-rabbit, Cell Signaling) for 1 h. Immunoreactive bands were detected using a chemiluminescent reaction (ECL, Amersham Biosciences, Piscataway, New Jersey, USA) by autoradiography on Kodak Bio-Max XAR film (Carestream Health, Paris, France). Three separate western blots were conducted for each antibody per sample and yielded similar data. Relative densities of the bands were determined by image analysis using NIH SCION image analysis software (National Institutes of health, Bethesda, Maryland, USA). Mean values and standard errors from five

densitometric readings per band were analyzed by one-way analysis of variance and the Tukey–Kramer multiple comparison test.

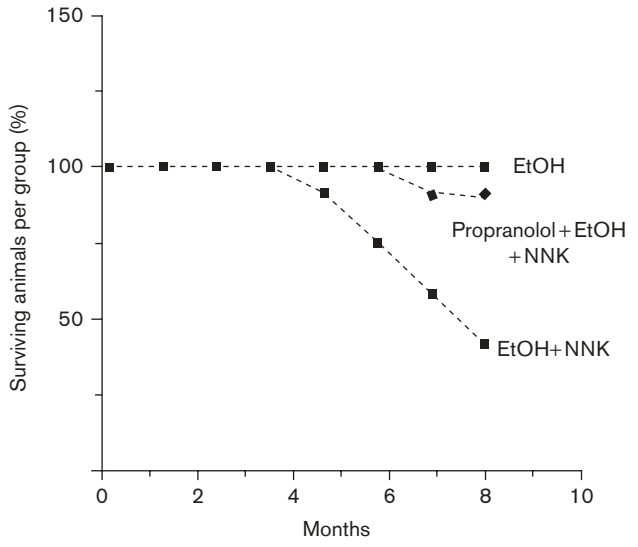
Results

As shown in Fig. 1, all offspring treated with the β-blocker propranolol survived until the end of the observation period. In contrast, numerous animals initiated for PDAC development by transplacental treatment with ethanol and NNK and maintained without further treatment showed clinical symptoms of pancreatic cancer (loss of appetite and bodyweight, diarrhea) and were therefore killed before the end of the experiment. The survival curve of this treatment group was significantly different from the propranolol-treated group ($P < 0.0001$ by log-rank test), with only 42% of the animals surviving until the end of the study (Fig. 1).

Histopathology evaluation showed pancreatitis in all of the animals treated transplacentally with ethanol and NNK and maintained postnatally without further treatment. In accordance with the classification of pancreatitis suggested by Klöppel [19], this fibroinflammatory disease was classified as alcoholic chronic pancreatitis, advanced stage (intensive perilobular and intralobular fibrosis affecting the entire pancreas and replacing most exocrine and endocrine pancreatic cells). In addition, 66.6% of the hamsters in this group ($n = 12$) had developed PDAC. In accordance with the validated 6th edition [20] of the American Joint Committee on Cancer, the induced PDACs were classified as T1 (tumor limited to pancreas, 2 cm or less in greatest diameter) or T2 (tumor limited to pancreas, greater than 2 cm in greatest diameter) and graded by histopathology as grade 2 (moderately differentiated duct-like structures and tubular glands) as suggested by Hruban and Fukushima [21]. In contrast, only one of the animals (8.3%, $n + 12$) treated with propranol developed PDAC, whereas all of these hamsters had pancreatitis. The hamsters exposed prenatally to ethanol alone all developed pancreatitis but no pancreatic cancer.

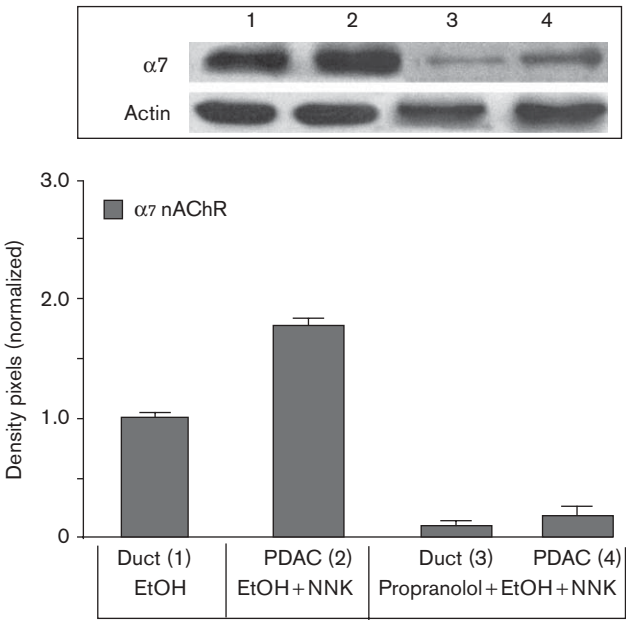
Protein analysis by western blotting of pancreatic cells harvested by laser capture microscopy showed a 1.9-fold increase ($P < 0.001$) in α7 nAChR protein in PDAC cells (Fig. 2). In contrast, the protein expression of this receptor in pancreatic duct epithelia and in cells from the single PDAC that developed in this group was reduced below the levels of the ethanol control group (Fig. 2). The upregulation of the α7 nAChR was accompanied by a 2.9-fold induction of p-CREB ($P < 0.001$) and a 2.2-fold increase in p-ERK1/2 ($P < 0.001$) in the ethanol (EtOH)/NNK-induced PDACs (Fig. 3). Both responses were reduced ($P < 0.001$) to levels not significantly different from the controls by treatment with propranolol

Fig. 1



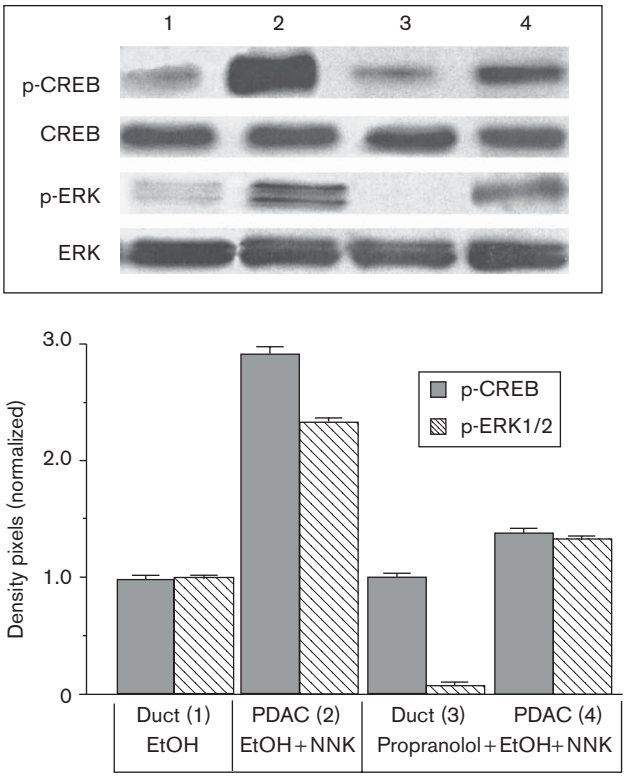
Survival curves of hamsters expressed as percentage of surviving animals per treatment group ($n = 12$ in each group) over time. Cancer preventive treatment with propranolol significantly ($P < 0.001$ by log-rank test) increased the survival time of hamsters induced for the development of pancreatic cancer by prenatal treatment with ethanol (EtOH) and NNK.

Fig. 2



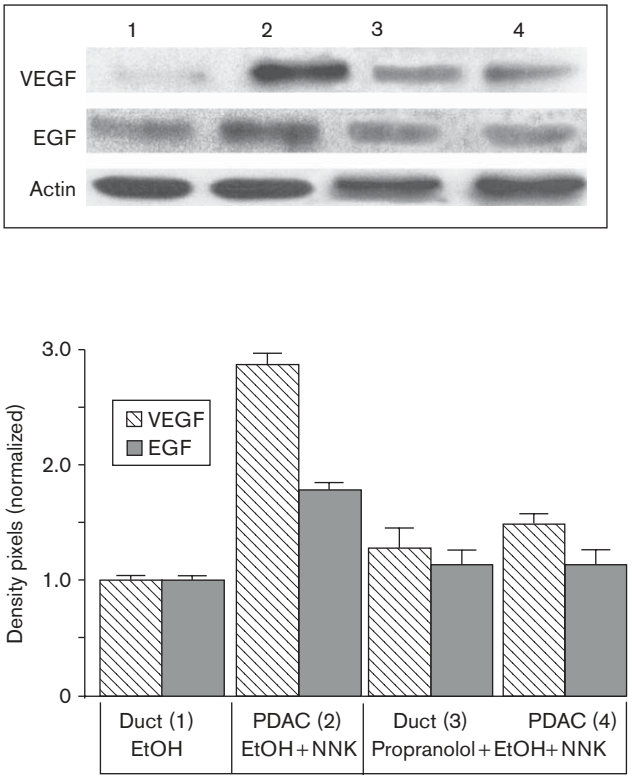
Western blot showing upregulation (1.9 fold; $P < 0.001$) of the α7 nAChR in cells harvested from ethanol (EtOH) + NNK-induced pancreatic ductal adenocarcinomas (PDACs) and dowregulation of this receptor below the levels of the EtOH controls by propranolol ($P < 0.001$). Columns in the graph represent mean values and standard errors of five densitometric readings per band expressed as the ratio of α7 nAChR over actin. This western blot was conducted three times with lysates from three separate samples per treatment group and yielded similar data.

Fig. 3



Western blots showing induction of p-CREB (2.9 fold, $P<0.001$) and p-ERK1/2 (2.2 fold, $P<0.001$) in cells harvested from ethanol (EtOH)/NNK-induced pancreatic ductal adenocarcinomas (PDACs) and inhibition of these responses ($P<0.001$) by treatment with propranolol. Columns in the graph represent mean values and standard errors of five densitometric readings per band expressed as the ratio of p-CREB over CREB or p-ERK1/2 over ERK1/2. Each western blot was conducted three times with lysates from three separate samples per treatment group and yielded similar data.

Fig. 4



Western blots exemplifying the induction of vascular endothelial growth factor (VEGF) (2.9 fold, $P<0.001$) and epidermal growth factor (EGF) (1.8 fold, $P<0.001$) in cells from ethanol (EtOH)/NNK-induced pancreatic ductal adenocarcinomas (PDACs) and inhibition of these responses ($P<0.001$) by propranolol. Columns in the graph represent mean values and standard errors of five densitometric readings per band expressed as ratio of VEGF or EGF over actin. Each western blot was conducted three times with lysates from three separate samples per treatment group and yielded similar data.

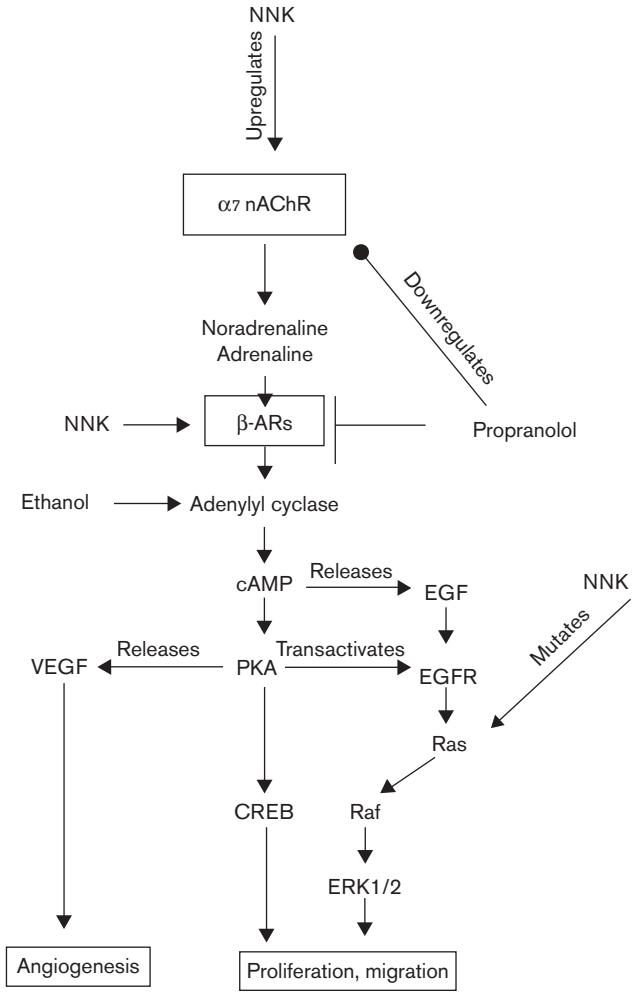
(Fig. 3), suggesting activation of these signaling protein as effectors of β-ARs. Interestingly, the expression of VEGF (2.9-fold, $P < 0.001$) and EGF (1.8-fold, $P < 0.001$) was also significantly ($P < 0.001$) increased in the EtOH/NNK-induced PDAC cells (Fig. 4). Both of these responses were significantly ($P < 0.001$) reduced by propranolol treatment ($P < 0.001$), suggesting that they were largely under β-AR control.

Discussion

Our data show that the β-blocker propranolol has strong cancer preventive effects on PDAC induced by prenatal exposure to EtOH/NNK in hamsters, an effect that involved the reversal of increases in EGF/VEGF and of CREB/ERK phosphorylation. In addition, a pronounced upregulation of the α7 nAChR in PDAC cells was not only reversed by propranolol but even downregulated below control levels. These findings are in accordance with in-vitro studies that have shown the activation of CREB and PKA-dependent transactivation of the EGFR downstream of β-ARs after ligand-binding of NNK to β-ARs in cell lines derived from human PDACs or lung adenocarcinomas and their respective cells of origin [3,4,6,22]. More recently, it has been shown that NNK additionally increases the systemic levels of the stress neurotransmitters noradrenaline and adrenaline in hamsters [14,23]. As noradrenaline and adrenaline are the physiological agonists for β-ARs, this effect of NNK further intensifies β-adrenergic signaling.

The observed upregulation of α7 nAChR protein in PDAC cells of the group treated with EtOH/NNK alone is in accordance with the documented function of NNK as an nAChR agonist [24–26] and mirror images the paradoxical upregulation of this receptor reported upon chronic exposure to nicotine in the nervous system [27]. However, in the current animal model, a single dose of NNK injected into pregnant hamsters 1 day before the delivery of the pups upregulated this receptor in the

Fig. 5



Working model of the proposed mechanisms and how propranolol prevented the development of pancreatic ductal adenocarcinomas (PDAC) induced in hamsters by ethanol+NNK. NNK-induced upregulation of the α7 nAChR increased the production and release of the stress neurotransmitter noradrenaline from which adrenaline is formed enzymatically. Both neurotransmitters bind as agonists to beta-adrenergic receptors (β-ARs), activating its effector adenylyl cyclase, the rate-limiting step for the formation of intracellular cAMP. In turn, cAMP causes the release of epidermal growth factor (EGF) [34] and activates PKA that phosphorylates the transcription factor CREB while additionally stimulating the release of vascular endothelial growth factor (VEGF) [32] and transactivating the epidermal growth factor receptor (EGFR). This signaling cascade is further intensified by direct agonist binding of NNK to β-ARs and activation of adenylyl cyclase by ethanol [36]. Propranolol blocks all signaling events downstream of β-ARs by binding as an antagonist to these receptors. In addition, propranolol downregulates the α7 nAChR by inhibiting cAMP-mediated assembly of its subunits [28], thus indirectly reducing the synthesis and release of noradrenaline and adrenaline. Collectively, these multiple actions of propranolol have strong inhibiting effects on the proliferation, migration and angiogenesis of PDAC.

offspring. This may be the reflection of the higher affinity of NNK to the α7 nAChR [24–26] or a greater sensitivity of the receptor to agonist-induced upregulation in fetal tissues. It has also been reported that cAMP positively regulates the subunit assembly of nAChRs, thereby increasing their protein expression [28]. The NNK-induced direct and indirect stimulation of cAMP signaling downstream of β-ARs may thus have contributed to the observed upregulation of the α7 nAChR. Conversely, inhibition of cAMP formation by propranolol may have triggered the observed reduction in expression levels of this receptor below the levels in controls. In light of the fact that the α7 nAChR stimulates the synthesis and release of noradrenaline and adrenaline in the nervous system [29,30] and in colon cancer cells [18], the observed induction of p-CREB and p-ERK1/2 in PDAC cells were in part caused by stress neurotransmitter-induced stimulation of β-adrenergic signaling. This interpretation is supported by our recent finding that NNK-treated hamsters have significantly increased systemic levels of noradrenaline and adrenaline [14,23]. The observed simultaneous increases in VEGF and EGF in PDAC cells and their reduction by propranolol are in accordance with the concept that both are stimulated by β-adrenergic signaling [31–34].

In summary, the β-blocker propranolol had strong cancer preventive effects on PDAC by inhibiting several important targets that drive the development and progression of this cancer (Fig. 5). These findings further emphasize the importance of cAMP signaling downstream of β-ARs in the regulation of PDAC. They suggest that interference with this signaling cascade is a promising target for PDAC prevention, a strategy that may also be applicable to other cancers that are stimulated by stress neurotransmitters and β-AR signaling, including adenocarcinoma of the colon [7,8], prostate [9], stomach [35], and ovarian cancer [11].

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