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Acetylcholine signaling system in progression of lung cancers

Jamie R. Friedman [a,1], Stephen D. Richbart [a,1], Justin C. Merritt [a], Kathleen C. Brown [a], Nicholas A. Nolan [a], Austin T. Akers [a], Jamie K. Lau [b], Zachary R. Robateau [a], Sarah L. Miles [a], Piyali Dasgupta [a,*]

[a] Department of Biomedical Sciences, Joan C. Edwards School of Medicine, 1700 Third Avenue, Huntington, WV 25755
[b] Biology Department, Center for the Sciences, Box 6931, Radford University, Radford, Virginia 24142

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ABSTRACT

The neurotransmitter acetylcholine (ACh) acts as an autocrine growth factor for human lung cancer. Several lines of evidence show that lung cancer cells express all of the proteins required for the uptake of choline (choline transporter 1, choline transporter-like proteins) synthesis of ACh (choline acetyltransferase, carnitine acetyltransferase), transport of ACh (vesicular acetylcholine transport, OCTs, OCTNs) and degradation of ACh (acetylcholinesterase, butyrylcholinesterase). The released ACh binds back to nicotinic (nAChRs) and muscarinic receptors on lung cancer cells to accelerate their proliferation, migration and invasion. Out of all components of the cholinergic pathway, the nAChR-signaling has been studied the most intensely. The reason for this trend is due to genome-wide data studies showing that nicotinic receptor subtypes are involved in lung cancer risk, the relationship between cigarette smoke and lung cancer risk as well as the rising popularity of electronic cigarettes considered by many as a "safe" alternative to smoking. There are a small number of articles which review the contribution of the other cholinergic proteins in the pathophysiology of lung cancer. The primary objective of this review article is to discuss the function of the acetylcholine-signaling proteins in the progression of lung cancer. The investigation of the role of cholinergic network in lung cancer will pave the way to novel molecular targets and drugs in this lethal malignancy.

Contents

* Corresponding author at: Department of Biomedical Sciences, Joan C. Edwards School of Medicine, Marshall University, 1700 3rd Avenue, Huntington, WV 25755
E-mail address: dasgupta@marshall.edu (P. Dasgupta).
[1] These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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List of abbreviations used 3 or more times

α-NETA	2-(alpha-naphthoyl)ethyltrimethylammonium
ACh	Acetylcholine
AChE	Acetylcholinesterase or acetylcholineacetylhydrolase
AFDX-116	{11-[[2-[(Diethylamino)methyl]-1-piperidinyl] acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4] benzodiazepin-6-one}
BAC	Bronchioalveolar carcinoma
BChE	Butyrylcholinesterase or acylcholine acylhydrolase
BCX	β-Cryptoxanthin
BECs	Bronchial epithelial cells
BrdU	Bromodeoxyuridine
CarAT	Carnitine acetyltransferase
ChAT	Choline acetyltransferase
ChE	Cholinesterase
ChT	Choline transporter
ChT1/SLC5A7	High affinity choline transporter 1
COPD	Chronic obstructive pulmonary disease
CTL1-5	Choline transporter-like proteins 1-5
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular related kinase
H69	NCI-H69
H82	NCI-H82
HC-3	Hemicholinium-3
HMEC-L	Human microvascular endothelial cells of the lung
hsa-miR-132	microRNA-132
HUVECs	Human umbilical cord microvascular endothelial cells
IGF-2	Insulin growth factor-2
LAC	Lung adenocarcinoma
LCC	Large cell carcinoma
Ly-6	Lymphocyte antigen 6
M1R to M5R	Muscarinic Receptor 1-5
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinases
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
nAChR	Nicotinic acetylcholine receptors
NEP	Norepinephrine
NHBE	Normal bronchial epithelial cells
NNK	Nicotine-derived nitrosamine ketone, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NSCLC	Non-small cell lung cancer
OAT	Organic anion transporter
OCT	Organic cation transporter
OCT1-3/SLC22A1-2	Polyspecific organic transporters
OCTN1 and OCTN2	Carnitine/cation transporters
PI-3 kinase	Phosphoinositol-3 kinase
poly-APS	3-alkyl pyridium polymers
SAEC	Primary small airway epithelial cells
SCC-L	Squamous cell carcinoma of the lung
SCLC	Small cell lung cancer
shRNA	Short-hairpin RNA
siRNA	Small interfering RNA
SLURP	Secreted ly6/urokinase-type plasminogen activator receptor related peptide
SNPs	Single nucleotide polymorphisms
STAT3	Signal transducer and activator of transcription 3
TGF-β1	Transforming growth factor β1
VAChT	Vesicular acetylcholine transporter
VEGF	Vascular endothelial growth factor

1. Introduction

Lung cancer is comprised of a spectrum of malignancies. Small cell lung cancer (SCLC; formerly known as oat cell carcinoma) is a neuroendocrine carcinoma and accounts for about 15-20% of all lung cancer cases (Gazdar, Bunn, & Minna, 2017). All other forms of lung cancers are included in a heterogeneous group called non-small cell lung cancer (NSCLC). NSCLC includes lung adenocarcinoma (LAC), squamous cell carcinoma (SCC-L), large cell carcinoma (LCC) and neuroendocrine lung carcinoid tumors (**Fig. 1**; Doroshow & Herbst, 2018; Herbst, Morgensztern, & Boshoff, 2018). LAC originates from the mucus secreting glands in the lungs (Meza, Meernik, Jeon, & Cote, 2015). A substantial number of early published reports involved a type of lung cancer called bronchioalveolar carcinoma (BAC). According to the new WHO classification, BAC is now included in the category of LAC. SCC-Ls usually develop in the tissues comprising the air passages of the lung. Due to cigarette smoking, SCC-L is often preceded by a columnar-to-squamous metaplasia, which lasts for years before developing into an *in situ* carcinoma (Soldera & Leighl, 2017). Traditionally SCC-L has also been called as epidermoid carcinoma, arising in central large bronchi which join the trachea to the lung.

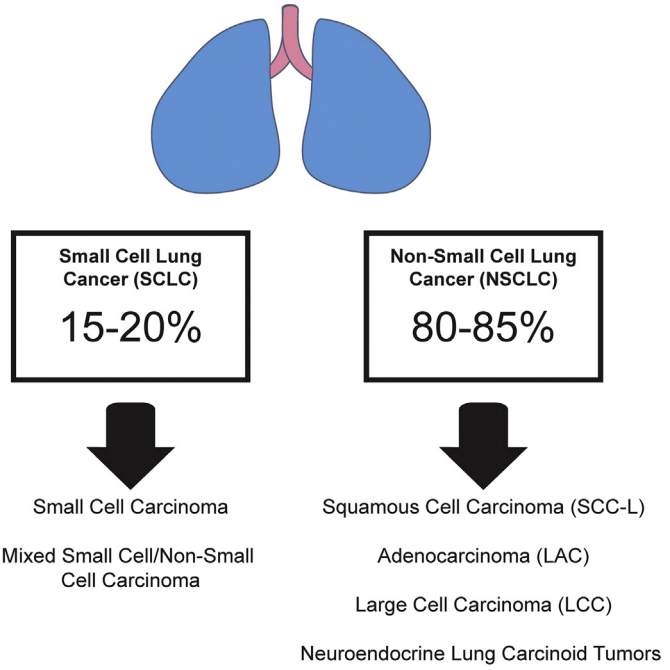

Fig. 1. The spectrum of malignancies which comprise lung cancers. Small cell lung cancer (SCLC; also called oat cell carcinoma) comprises of morphologically tiny cells. All other lung malignancies are put into a heterogenous group termed non-small cell lung cancer (NSCLC). Out of NSCLCs lung adenocarcinoma (LAC) accounts for majority of cases followed by squamous cell carcinoma of the lung (SCC-L). Large cell carcinoma (LCC) and neuroendocrine carcinoid tumors of the lung are relatively less common than LAC and SCC-Ls.

Epidemiological data indicates that cigarette smoking bears a strong etiological association with the development of all histological types of lung cancer (Furrukh, 2013). The association between smoking and lung cancer is stronger with SCLC and SCC-L than with other forms of lung cancer (Khuder, 2001; Khuder & Mutgi, 2001). Nicotine is the addictive component of cigarette smoke. Several lines of evidence show that nicotine accelerates the growth, angiogenesis and metastasis of lung cancers (Dasgupta & Chellappan, 2006; Dasgupta et al., 2011; Dasgupta et al., 2009; Davis et al., 2009; C Heeschen et al., 2001; C. Heeschen, Weis, Aicher, Dimmler, & Cooke, 2002; Singh, Pillai, & Chellappan, 2011; Spindel, 2016; Zoli, Pucci, Vilella, & Gotti, 2018). Furthermore, nicotine protects lung cancers from cell death induced by chemotherapeutic drugs, oxidative stress and ionizing radiation (Dasgupta, Kinkade, et al., 2006; Egleton, Brown, & Dasgupta, 2008; Jin, Gao, Flagg, & Deng, 2004; Mai, May, Gao, Jin, & Deng, 2003; Maneckjee & Minna, 1994; West, Linnoila, Belinsky, Harris, & Dennis, 2004; Zeidler, Albermann, & Lang, 2007). The growth-stimulatory effects of nicotine are mediated via nicotinic acetylcholine receptors (nAChRs) on lung tumors and the surrounding stroma (S. Wang & Hu, 2018; Zhao, 2016; Zoli et al., 2018). The endogenous ligand for nAChRs is the neurotransmitter acetylcholine (ACh; Kirkpatrick et al., 2001; Kummer & Krasteva-Christ, 2014; Mucchietto, Crespi, Fasoli, Clementi, & Gotti, 2016; Niu & Lu, 2014; Saracino, Zorzetto, Inghilleri, Pozzi, & Stella, 2013). Genome-wide association studies (GWAS) identified a genetic component of the association between tobacco components and the development of lung cancer. Data collected from European populations have discovered a locus in the long arm of chromosome 15 (15q24/15q25.1) as the 'top hit' for genomic association with lung

cancer. The region includes three genes that encode nicotinic acetylcholine receptor subunits α5, α3, and β4-nAChR (CHRNA5, CHRNA3 and CHRNB4; Amos et al., 2008; Hung et al., 2008; Improgo, Scofield, Tapper, & Gardner, 2010; P. Liu et al., 2008; Thorgeirsson et al., 2008a). Such observations underscore a role for the cholinergic pathway in the development and progression of lung cancer (Gao, Zhang, Breitling, & Brenner, 2016; Tournier & Birembaut, 2011; Wen, Jiang, Yuan, Cui, & Li, 2016; I. A. Yang, Holloway, & Fong, 2013).

Traditionally, ACh is a neurotransmitter and mediates synaptic transmission (Arias et al., 2009; Barman, Barrett, Boitano, & Brooks, 2016; Kopelman, 1986; Lindstrom, 1996; Phillips et al., 2010; Picciotto, Higley, & Mineur, 2012). ACh and cholinergic proteins have been detected in non-neuronal tissues like lung, colon, pancreas, skin, gall bladder, and small/large intestine tissues (Beckmann & Lips, 2013; S. A. Grando, 2008; S.A. Grando, Kist, Qi, & Dahl, 1993; Lindstrom, 1997; Wessler, Kirkpatrick, & Racke, 1998). The bronchial epithelium has been shown to synthesize, transport and degrade ACh (Kistemaker & Gosens, 2015; Kummer & Krasteva-Christ, 2014; Proskocil et al., 2004; Saracino et al., 2013; Wessler et al., 1998). These observations suggest that ACh plays a vital role in the lung homeostasis (Pieper, 2012). Published data demonstrate that ACh functions as an autocrine and paracrine growth factor for lung epithelial cells (Proskocil et al., 2004). It is also a regulator of airway remodeling, airway muscle contraction, mucus secretion and immune functions of the lungs (Fujii et al., 2017a, 2017b; Koarai & Ichinose, 2018; Kummer & Krasteva-Christ, 2014; Pieper, Chaudhary, & Park, 2007; Proskocil et al., 2004; Wessler et al., 1998). ACh is synthesized in the cytoplasm by the enzyme choline acetyltransferase (ChAT) from choline and acetyl-coenzyme A (acetyl-coA; Kummer & Krasteva-Christ, 2014). An alternative route for ACh synthesis is provided by carnitine acetyltransferase (CarAT), which has been detected in the respiratory tract (**Fig. 2**; Kummer & Krasteva-Christ, 2014; Kummer, Lips, & Pfeil, 2008; Lips, Wunsch, et al., 2007b). Subsequently, the ACh is packaged in vesicles by the vesicular acetylcholine transporter (VAChT) and transported to the plasma membrane, where it is released into the extracellular space by exocytosis (Barman et al., 2016; de Castro et al., 2009). In addition, a V-ATPase containing proteolipid complex called "mediatophore" also releases ACh from the cytoplasm to the extracellular space (Birman et al., 1990; Brochier, Israel, & Lesbats, 1993; Brochier & Morel, 1993; Fujii, Takada-Takatori, Horiguchi, & Kawashima, 2012). The released ACh binds to its cognate receptors, namely the nAChRs and muscarinic receptors, on the target cells. The excess ACh is rapidly degraded by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) to generate choline (Patocka, Kuca, & Jun, 2004; Silman & Sussman, 2005; Xi, Wu, Liu, Zhang, & Li, 2015). The choline is then transported back to the cytoplasm by choline transporter1 (ChT1) for another round of ACh synthesis (Barman et al.,

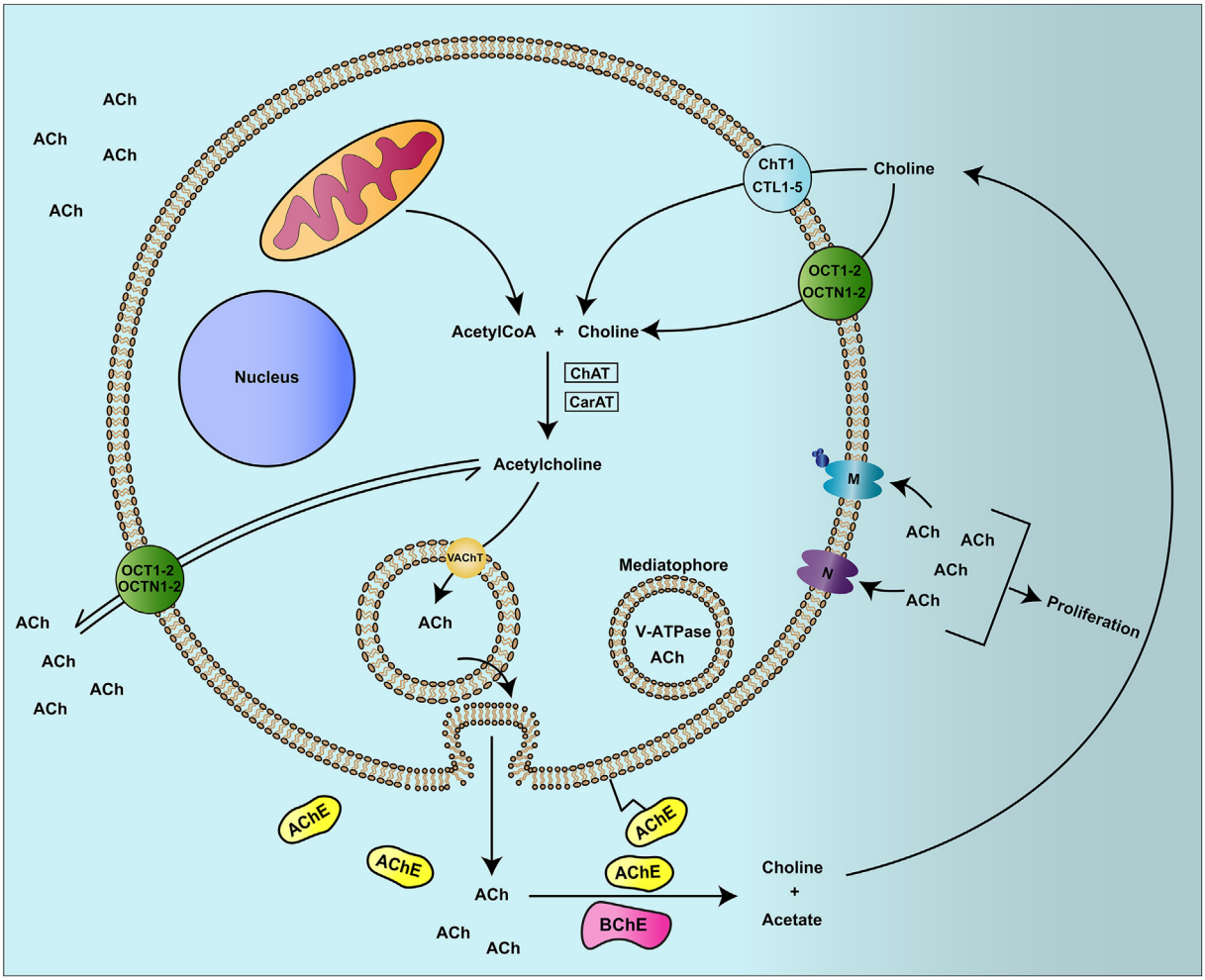

Fig. 2. A simplified diagram of the acetylcholine (ACh)-signaling pathway in human lung cells. ACh is synthesized in the cytoplasm by the enzyme choline acetyltransferase (ChAT). In the absence of ChAT, an enzyme carnitine acetyltransferase (CarAT) synthesizes ACh from choline by adding an acetyl group to it (from acetyl-CoA). The ACh is packaged into vesicles by the vesicular acetylcholine transporter (VAChT) and exocytosed into the extracellular milieu. The ACh released binds back to its cognate receptors namely the nicotinic acetylcholine receptor (nAChR) and the muscarinic acetylcholine receptor in an autocrine (or paracrine) manner to recruit downstream cellular signaling pathways. The excess ACh is quickly hydrolyzed by the enzymes acetylcholinesterase (AChE) and butrylcholinesterase (BChE) to generate choline. This choline is transported back in the cytoplasm by choline transporter 1 (ChT1). In the absence of ChT1 choline transporter like proteins (CTL) 1-5 facilitate the uptake of choline back to the cytoplasm for another round of ACh synthesis. Polyspecific organic cations OCTs and OCTNs have the ability to transport ACh and choline bidirectionally in and out of the cell.

2016). Apart from ChT1, choline transporter-like proteins 1-5 (CTL1-5) have been shown to have a role in choline uptake and its transport to the cytoplasm in non-neuronal cells (Inazu, 2014; Song, Sekhon, Duan, Mark, & Spindel, 2004; Traiffort, O'Regan, & Ruat, 2013). Similarly, polyspecific organic cationic transporters (OCT1-2 and OCTN1-2) facilitate the bidirectional transport of choline and ACh in lung cells (Lips et al., 2005; Pochini, Scalise, Galluccio, & Indiveri, 2012a; Pochini, Scalise, Galluccio, & Indiveri, 2013; Tamai, 2013; Volk, 2014).

A survey of literature shows that several components of the cholinergic pathway are altered in human lung cancers (Dang, Meng, & Song, 2016; S. A. Grando, 2008; Sergei A. Grando, 2014; Improgo, Soll, Tapper, & Gardner, 2013; Spindel, 2016; S. Wang & Hu, 2018). Pioneering studies by Song et al., (2003, 2007, 2008) showed that ACh acts as a growth factor for human SCLC and NSCLC (Song et al., 2003a; Song et al., 2008; Song, Sekhon, Duan, et al., 2004). ACh also promotes migration and invasion of human lung cancers (Niu & Lu, 2014; Spindel, 2016; Wessler et al., 1998). Data from several studies suggest that the ACh signaling pathway in lung cancers is modified to elevate the production of the growth factor ACh. This may include upregulation of ACh, ChAT, VAChT, CTLs and OCTs or a decrease in the function/expression of AChE (Lau et al., 2013; Niu & Lu, 2014; Spindel, 2016; Wessler et al., 1998; Zoli et al., 2018). The present review describes the functional role of the ACh signaling pathway in human lung cancers. We will discuss the feasibility of the cholinergic network as a molecular target for detection and treatment of lung cancer. Out of all components of the cholinergic signaling axis, nAChRs have been most extensively studied in the context of lung cancer (Sergei A. Grando, 2014; Improgo et al., 2013; Schaal & Chellappan, 2016; Schuller, 2012; Spindel, 2016). The primary reason for this trend may be due to the fact that cigarette smoking is closely correlated with lung cancer (Pesch et al., 2012; Proctor, 2012). Tobacco components like nicotine, the nicotine-derived nitrosamine ketone 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-Nitrosonornicotine (NNN) and N-Nitrosodiethylamine (DEN) are high-affinity ligands for nAChRs (Schuller, 1992, 2007; Schuller, Jull, Sheppard, & Plummer, 2000; Schuller & Orloff, 1998; Schuller, Plummer III, & Jull, 2003). The rising popularity of electronic cigarettes has led to further research in the field of nAChR signaling in the lungs. Many people view electronic cigarettes as a "cessation device" or a "safe alternative" to cigarettes (Dinakar & O'Connor, 2016; M. Hua & Talbot, 2016; Springer, 2014). Furthermore, single nucleotide polymorphisms (SNPs) involving the nAChRs locus chromosome 15q25 region (CHRNA5, CHRNA3, CHRNB4) have been associated with an increased risk of lung cancer in European populations comprised of heavy smokers (Amos et al., 2008; Hung et al., 2008; Improgo et al., 2010; P. Liu et al., 2008; Thorgeirsson et al., 2008a). All these factors have led to intense research involving the role of nAChRs in progression of lung cancer. Several state-of-the-art reviews are already published on this subject (Sergei A. Grando, 2014; Improgo et al., 2013; Schaal & Chellappan, 2016; Schuller, 2012; Spindel, 2016; S. Wang & Hu, 2018; Zoli et al., 2018). On the other hand, there is a paucity of reviews which contain in-depth knowledge involving the role of other cholinergic proteins in lung cancer. The primary emphasis of this review is to discuss the role of the acetylcholine-signaling pathway in lung cancer. In the light of this rationale, we will only discuss the most recent (past three years) findings involving nicotine-NNK-nAChRs signaling pathway. The potential applications of cholinergic modulators in the detection and treatment of human lung cancer will be summarized. This review will include recently identified nAChR modulators which have potential applications in lung cancer therapy. Finally, we will discuss the signaling pathways underlying the anti-neoplastic activity of cholinergic modulators in lung cancer and normal lung cells.

2. Acetylcholine (ACh)

The presence of ACh in non-neuronal tissues has raised intriguing questions about its role in non-neuronal systems (for review articles see Spindel, 2016; Niu & Lu, 2014; Fujii et al., 2017a, 2017b; Koarai & Ichinose, 2018; Kummer & Krasteva-Christ, 2014; Pieper et al., 2007; Proskocil et al., 2004; Wessler et al., 1998). ACh has important immunomodulatory functions and triggers both initiation and termination of cytokine synthesis (Fujii et al., 2017a, 2017b). The synthesis of ACh in immune cells is sensitive to phytohemagglutinin (PHA), lipopolysaccharide and toll-like receptors (TLR), which emphasize its role in immune functions (for review articles see Fujii et al., 2017a, 2017b; Fujii, Takada-Takatori, Horiguchi, & Kawashima, 2012; Kawashima, Fujii, Moriwaki, & Misawa, 2012; Koarai & Ichinose, 2018; Yoo & Mazmanian, 2017).

ACh acts as an autocrine and paracrine growth factor for bronchial epithelial cells (BECs). ACh has been detected in human bronchi, mouth, trachea and pulmonary pleura (Kummer et al., 2008; Kummer & Krasteva-Christ, 2014; Wessler et al., 1998). High-performance liquid chromatography (HPLC) analysis revealed that ACh was secreted in cultured BECs isolated from one-year-old monkeys and from humans (Proskocil et al., 2004). When SV-40 immortalized human BECs were stimulated with cigarette smoke extract elevated production of ACh was observed both in lysates and supernatant (Albano et al., 2018; Montalbano et al., 2014; Profita et al., 2009). This phenomenon is believed to play a role in the context of pro-inflammatory lung diseases like chronic obstructive pulmonary disease (COPD; Profita et al., 2009). ACh is also generated by pulmonary arteries, human umbilical cord endothelial cells (HUVEC) and human angiosarcoma endothelial cells (HAEND; Haberberger, Bodenbenner, & Kummer, 2000; Kirkpatrick, Bittinger, Nozadze, & Wessler, 2003). ACh released by the endothelium plays a vital role in endothelial calcium signaling, vasodilation/relaxation of arteries and maintenance of vascular homeostasis (Chataigneau et al., 1999; Wilson, Lee, & McCarron, 2016; Zhang et al., 2015).

Schuller, McGavin, Orloff, Riechert, and Porter (1995) induced lung carcinogenesis in hamsters via subcutaneous injection of nicotine and simultaneous exposure to 60% hyperoxia for 12 weeks (Schuller et al., 1995). Subsequently, they isolated neuroendocrine lung tumor epithelial cells from these tumors. They observed that ACh stimulated the proliferation of these neuroendocrine lung cancer cell lines via nAChR receptors (Schuller et al., 1995). Song, Sekhon, Jia, et al. (2003a) demonstrated (for the first time) that ACh is produced by a panel of human SCLC cell lines, namely H345, NCI-H69 (H69), NCI-H82 (H82), H1694 and H592 (Song, Sekhon, Duan, et al., 2004; Song, Sekhon, Jia, et al., 2003a; Song & Spindel, 2008). Furthermore, they went on to show that ACh acts as an autocrine growth factor for H82 human SCLC cells (Song, Sekhon, Jia, et al., 2003a). The magnitude of ACh secreted by H82 human SCLC cells was upregulated by neostigmine (**Fig. 3A**, an antagonist of acetylcholinesterase; see section 7; Song, Sekhon, Jia, et al., 2003a) and choline (Song et al., 2013). In contrast, ACh production in H82 cells was inhibited by vesamicol (**Fig. 3B**, an antagonist of vesicular acetylcholine transporter; section 3.1; Song, Sekhon, Jia, et al., 2003a) and hemicholinium-3 (HC-3; **Fig. 3C**, an antagonist of choline transporters, see section 3.2; Song, Sekhon, Jia, et al., 2003). The treatment of quiescent SBC3 human SCLC cells with 100 μM-1 mM ACh increased the viability of these cells at 48 and 72 hours (S. Zhang et al., 2010). ACh activated mitogenic pathways, namely the mitogen-activated protein kinase (MAPK) pathway, intracellular calcium pathway and Akt pathway in H82 human SCLC cells (Song, Sekhon, Duan, et al., 2004). Subsequent studies from their research group showed that homogenates of human SCC-L (isolated from patients) produced an increased amount of ACh relative to adjacent normal lung tissue (Song et al., 2008). The role of ACh as a growth factor for human lung cancer is further reenforced by the co-expression of ChAT (the enzyme synthesizing ACh) and muscarinic receptor type 3 (M3R) in human lung cancers (Song, Sekhon, Duan, et al., 2004; Spindel, 2012). Tobacco components like nicotine elevate the levels of ACh in human lung cancer cells (Lau et al., 2013; Song et al., 2008). Data from our laboratory show that the treatment of A549, H358 and H650 human LAC cells with 10 nM-10 μM nicotine caused a concentration-dependent increase in the levels of ACh over 24 hours (Lau et al., 2013; Song et al., 2008). Subsequently, we

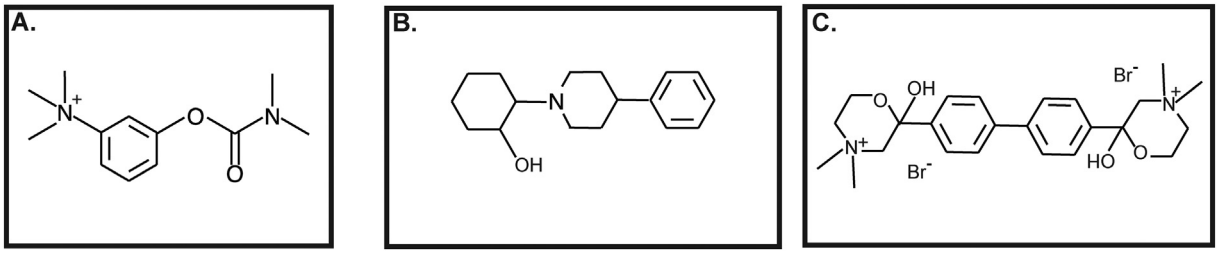

Fig. 3. ACh production is sensitive to cholinergic pathway ligands. A. Neostigmine, an antagonist to AChE B. Vesamicol, an inhibitor of VAChT C. Hemicholinum-3, an antagonist of choline transporters.

analyzed the mitogenic effects of ACh (at levels produced in nicotine-treated cells) in A549 and H358 human LAC cells ACh, using the bromodeoxyuridine (BrdU) assay. BrdU is a thymidine analog which gets incorporated into the DNA of cells entering S-phase (Lau et al., 2013). We found that ACh (at levels present in the supernatant of nicotine treated LAC cells) induced a 4-4.5 fold increase in the proliferation of A549 and H838 human LAC cells. A relevant aspect of the above-mentioned studies was that they were performed using nicotine concentrations found in the plasma of moderate-heavy smokers (Lau et al., 2013). Xi et al. (2015) studied the mitogenic effects of ACh in A549 and H1299 human NSCLC cells using the cell counting kit-8 (CCK-8) assay (R. Xu et al., 2015). They observed that ACh stimulated the proliferation of the above-mentioned cell lines in a concentration-dependent manner from 50-300 μM in 24 hours, with the maximal cell proliferation being observed at 200 μM (N. Hua et al., 2012; R. Xu et al., 2015). In contrast, Hua et al. (2012) reported that exogeneous ACh caused no change in cell viability in H1299 cells within the concentration range 0-100 μM at 72 hours. These differences may be attributed to the time points used in these studies. It is probable that ACh produces a rapid proliferative response in human lung cancer cells at 24 hours, which is ablated by 72 hours. The basal concentration of ACh secreted by the human lung cancer cells ranges from 5-50 nM. When the AChE inhibitor neostigmine is added the levels of ACh range between 125-175 nM (Lau et al., 2013; Song et al., 2013; Song, Sekhon, Duan, et al., 2004; Song et al., 2008; Song, Sekhon, Jia, et al., 2003a; Song, Sekhon, Duan, et al., 2004; Song, Sekhon, Proskocil, et al., 2003; Spindel, 2012, 2016). Such elevation in ACh levels are observed due to neostigmine-induced blockage of ACh degradation by AChE. Data from Song et al., (2007) estimates the basal ACh content of SCLC tumors (xenografted on athymic mice) as approximately 400 nM (Song, Sekhon, Duan, et al., 2004). Therefore, it is unclear why several of the above-mentioned studies have used unusually high concentrations of ACh for their experiments (N. Hua et al., 2012; R. Xu et al., 2015). The reason may have been that they did not use neostigmine for their experiments. Furthermore, ACh is rapidly degraded and a high initial concentration may be required for physiological steady-state levels of ACh in the extracellular milieu.

Apart from being an autocrine growth factor, ACh potently stimulates the adhesion, migration and invasion of human lung cancer cells (**Fig. 4**). The treatment of SBC3 human SCLC cells with 100 μM ACh caused 2-3-fold increase in adhesion to fibronectin and migration through fibronectin-coated filters (S. Zhang et al., 2010). The pro-adhesive and pro-migratory effect of ACh involved functional regulation of αvβ1 and α5β1 integrins (S. Zhang et al., 2010). Xu et al. (2015) found that the ACh displayed robust pro-invasive and pro-migratory activity in human NSCLC cell lines within the concentration range of 100-300 μM (R. Xu et al., 2015). The highest magnitude of invasion and migration was observed at 200 μM ACh in A549 and H1299 human NSCLC cells (R. Xu et al., 2015). This data agrees with the observations of Lin, Sun, Wang, Guo, and Xie (2014) that 100 μM ACh stimulated the invasion (and migration) of two NSCLC cell lines, A549 and L78 (Lin et al., 2014). Real-time PCR analysis showed that 200 μM ACh induced the expression of cytokines IL-1, IL-6, IL-8 from A549 human NSCLC cells. Out of these genes, ACh-induced upregulation of IL-8 was confirmed by ELISA (R. Xu et al., 2015). The cytokines IL-1, IL-6 and IL-8 induce growth, angiogenesis and metastasis of human NSCLCs (Neufeld & Kessler, 2006; Nishida, Yano, Nishida, Kamura, & Kojiro, 2006; Z. Wang et al., 2015). Lin et al., (2014) observed that 100 μM ACh increased the expression (and functional activity) of MMP-9, as well as downregulated E-cadherin expression in A549 and L78 human NSCLC cells (Lin et al., 2014). Both of these signaling events required the phosphoinositol-3 kinase (PI-3 kinase)/Akt signaling pathway in A549 and L78 cells (Lin et al., 2014; R. Xu et al., 2015). MMPs play a vital role in the invasion and metastasis of human lung cancers (Gong et al., 2016; Merchant et al., 2017). The downregulation of E-cadherin is a marker for epithelial-to-mesenchymal transition (EMT), which confers a migratory phenotype on tumor cells, allowing them to invade into the surrounding stroma, blood vessels and lymph (Nieto, Huang, Jackson, & Thiery, 2016; Tsoukalas et al., 2017; Xiao & He, 2010). The fact that ACh is upregulating the levels of these proliferative, angiogenic and pro-invasive pathways suggests that it plays an essential role in the progression and metastasis of human NSCLC. A drawback of these experiments is that the authors have used very high concentrations of ACh in their studies (100 μM and 200 μM), which makes it difficult to extrapolate their results to the pathophysiology of NSCLC (Lin et al., 2014; Song, Sekhon, Duan, et al., 2004; R. Xu et al., 2015). Once again, a plausible explanation may be the lack of neostigmine (AChE antagonist) in their experiments. ACh-induced proliferation, migration and invasion of A549 and H1299 human NSCLC cells were found to require the M3R (see section 4) which transactivated the epidermal growth factor receptor (EGFR) followed by downstream activation of PI-3 kinase/Akt pathway.

The cholinergic signaling axis has been found to play a role in TGF-β1-induced EMT in A549 human NSCLC and immortalized human BECs (K. Yang et al., 2014). The treatment of A549 human NSCLC cells with 5 ng/ml TGF-β1 caused a 1.5-fold increase in ACh secretion from

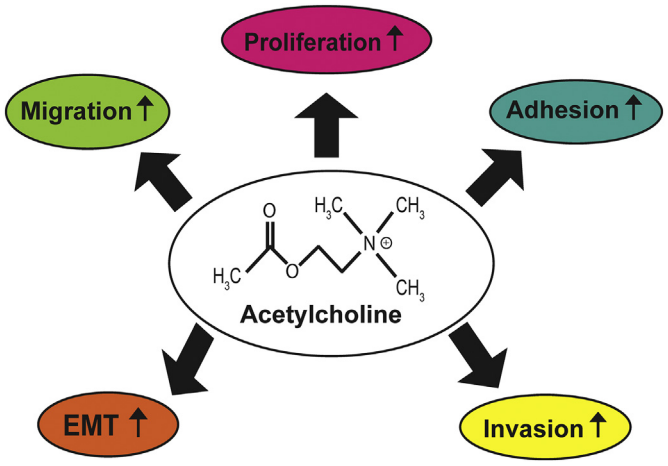

Fig. 4. A simplified schematic of the multiple functions of acetylcholine (ACh) in human lung cancer.

A549 human NSCLC cells (K. Yang et al., 2014). TGF-β1-induced EMT was primarily mediated by the muscarinic receptor subtype 1 (M1R) and M3R in human NSCLCs.

2.1. Choline Acetyltransferase (ChAT)

The enzyme ChAT catalyzes the synthesis of ACh from choline. The gene encoding ChAT is comprised of multiple exons, which undergo alternate splicing to generate six transcripts of the gene (Barman et al., 2016; Oda, 1999). A unique feature of the ChAT gene locus is that the open reading frame of the VAChT gene is located within the first intron of the ChAT gene (Oda, 1999). Out of all the ChAT transcripts, four (denoted as H, R, N1 and N2) translate to a 69 kDa protein which is the predominant form of ChAT. The remaining two transcripts (called M and S) yield two isoforms of ChAT of molecular weights 74 kDa and 82 kDa, respectively (Oda, 1999). In addition, Tooyama and Kimura (2000) have identified a new form of ChAT called pChAT (molecular weight of 50 kDa) generated by alternate splicing and exon skipping of the regions between exon 6 and 9 (Bellier & Kimura, 2011; Nakajima, Tooyama, Yasuhara, Aimi, & Kimura, 2000; Tooyama & Kimura, 2000). A smaller ChAT protein (molecular weight of 27 kDa) has also been characterized. This protein lacks catalytic activity but is believed have a regulatory role on the activity of full-length ChAT (Grosman, Lorenzi, Trinidad, & Strauss, 1995).

Recombinant ChAT (69 kDa) and its isoform (82 kDa) are modified by phosphorylation via protein kinase-C (PK-C), protein kinase CK2, and α-Ca2+/calmodulin-dependent protein kinase II (CaM-kinase; Dobransky, Davis, & Rylett, 2001; Dobransky, Davis, Xiao, & Rylett, 2000; Dobransky et al., 2004; Dobransky & Rylett, 2003, 2005; Pahud, Bontron, & Eder-Colli, 2001; Schmidt and Rylett, 1993). SNPs in the ChAT gene have been correlated with nicotine dependence and prospective smoking cessation (R. Ray et al., 2010).

Immunoreactive ChAT and ChAT activity has been detected in multiple tissues of the human lung epithelium (Krasteva et al., 2011; Kummer et al., 2008; Kummer & Krasteva-Christ, 2014; Proskocil et al., 2004; Song & Spindel, 2008). This includes immortalized normal lung epithelial cells, primary normal human alveolar epithelial cells, and normal BECs (**Table 1**). Electron microscopy experiments show that ChAT in airway epithelial cells is localized to the cytosol, nucleus and extracellular fluids like plasma (Kummer et al., 2008; Kummer & Krasteva-Christ, 2014; Matsuo et al., 2011). The expression of ChAT on normal airway epithelium is regulated by inflammatory stimuli, cigarette smoke and nicotine (Albano et al., 2018; Lau et al., 2013; Montalbano et al., 2014; Profita et al., 2009). ChAT is vigorously expressed in immortalized human BECs, SCLC and NSCLC (Akers et al., 2018; Dasgupta et al., 2018; Dasgupta et al., 2016; N. Hua et al., 2012; Song, Sekhon, Jia, et al., 2003a; Song et al., 2003b; Song & Spindel, 2008). Song, Sekhon, Jia, et al. (2003a) demonstrated for the first time, the existence of a functional cholinergic loop in human SCLC. They performed Southern blotting to demonstrate the presence of N, R and S ChAT transcript in a panel of six human SCLC cell lines (Song, Sekhon, Jia, et al., 2003; Song, Sekhon, Jia, et al., 2003a; Song & Spindel, 2008). Apart from SCLCs, ChAT has been detected in many human LAC and SCC-Ls cell lines (**Table 1**). The expression of ChAT in human lung cancer cells is sensitive to mitogenic factors like TGF-β1 and nicotine (K. Yang et al., 2014; Lau et al., 2013). The treatment of human LAC cell lines with 100 nM nicotine (which is within the range of nicotine concentrations found in the plasma of average smoker) increased ChAT levels, ACh production and cell proliferation (Lau et al., 2013). Similarly, the multifunctional cytokine TGF-β1 increased ChAT expression and ACh secretion in A549 human NSCLC cells, which in turn correlated with the induction of EMT in these cells (K. Yang et al., 2014). The aforesaid findings confirm the mitogenic, pro-migratory and pro-invasive activity of ACh in human lung cancer cells. Hence, we surmised that human LAC cells should express higher amounts of ChAT (which in turn would produce increased amounts of ACh) relative to normal BECs. We performed ELISA and immunoblotting experiments to analyze the expression of ChAT in a panel of human LAC cell lines and in primary normal bronchial epithelial cells (NHBEs). We observed elevated amounts of ChAT in human LAC cells, relative to NHBEs (Dasgupta et al., 2016; Dasgupta et al., 2018). We repeated the experiments using two other types of normal lung epithelial cells namely small airway epithelial cells (SAEC) and human pulmonary alveolar epithelial cells (HPAEpiCs) and obtained similar results.

Numerous research studies have demonstrated the presence of ChAT in human SCLC and NSCLC tumors, isolated from patients (**Table 2**; Dasgupta et al., 2018; Dasgupta et al., 2016; Song et al., 2008; Song, Sekhon, Jia, et al., 2003a; Song & Spindel, 2008; Spindel, 2012). A noteworthy observation is that the muscarinic receptor M3R is co-expressed with ChAT in a large fraction of SCLC, SCC-Ls and LAC tumors isolated from patients (Song et al., 2007; Spindel, 2012). Such co-

Table 1
ChAT Expression in normal human bronchial epithelial cells and lung cancer cell lines

Cell Line	Nature of lung cells	Expression of ChAT	References
NHBE	Normal human bronchial epithelium	+	Akers et al., 2018 Akers et al., 2017 Dasgupta et al., 2018 Dasgupta et al., 2016
SAEC	Small airway epithelial cells	+	Akers et al., 2018 Akers et al., 2017 Dasgupta et al., 2018 Dasgupta et al., 2016
HLF-1	Human lung fibroblasts	+	Profita et al., 2009
16-HBE	Immortalized human bronchial epithelial cells	+	Albano et al., 2018 Montalbano et al., 2014 Profita et al., 2009
BEP2D	Immortalized human bronchial epithelial cells	+	Hua et al., 2012
HPAEpics	Human pulmonary alveolar epithelial cells	+	Lau et al., 2013
H82, H69, H345, H417, H378, H592	Small cell lung cancer	+	Martinez-Lopez de Castro et al., 2005 Song et al., 2003a Song et al., 2003b
H520, H226, SK-MES, H157	Squamous cell carcinoma of the lung	+	Dasgupta et al., 2018 Dasgupta et al., 2016 Hua et al., 2012 Martinez-Lopez de Castro et al., 2005
H460, H1299, A549, H358, H650, H2228, H1975, H1563, H1650, H23, H1944, H838, H1355, PC-9	Lung adenocarcinoma	+	Akers et al., 2018 Akers et al., 2017 Dasgupta et al., 2018 Dasgupta et al., 2016 Hua et al., 2012 Lau et al., 2013 Martinez-Lopez de Castro et al., 2005 Yang et al., 2014 Zhao et al., 2015a Zhao et al., 2015b

Table 2
Expression of ChAT in lung cancer tissue adjacent normal tissue (isolated from patients)

Type of lung tissue	Expression of ChAT	References
Normal lung tissue	+	Lau et al., 2013 Song et al., 2008 Song et al., 2003b
Small cell lung cancer	+	Song et al., 2003b Spindel, 2012
Squamous cell lung cancer tissue	+	Song et al., 2008 Song et al., 2003b Song et al., 2007
Lung adenocarcinoma tissue	+	Lau et al., 2013 Song et al., 2003b Song et al., 2007 Spindel, 2012

expression may have important ramifications for the progression of lung cancers. Song et al. (2008) compared the levels of ChAT between human SCC-L tumors and adjacent normal tissue isolated from patients using real-time PCR techniques. They found that ChAT mRNA was virtually undetectable in normal tissue whereas it was highly expressed in the SCC-L tissue (Song et al., 2008; Spindel, 2012, 2016). They also measured the ChAT levels in a panel of well differentiated to poorly differentiated human SCC-L tumors from patients. They did not find any statistically significant differences in ChAT expression between human well differentiated SCC-Ls and poorly differentiated SCC-L tumors (Song et al., 2008). Studies in our laboratory examined relative ChAT expression patterns in human LACs tumor tissues (isolated from patients) and adjacent matched normal lung tissue using ELISA, immunoblotting and immunohistochemistry techniques (Lau et al., 2013). The expression of ChAT in all human LAC tumor tissue was higher than adjacent normal lung tissue. Song et al. (2008) measured the abundance of ChAT in human SCC-L tumors isolated from patients (Dasgupta et al., 2016; Dasgupta et al., 2018). They found that about 60% of all the SCC-L tumors expressed ChAT, which underscores the vital function of this protein in the progression of human lung cancers (Song et al., 2008).

The initial quest for pharmacological ligands of ChAT was aimed at using these compounds for the diagnosis and treatment of neurological diseases like Alzheimer's disease, related dementias, Down's syndrome and Lewy body disorders (Barman et al., 2016; Oda, 1999). An early research study describing small molecule ChAT inhibitors was that of Mehta, Musso, & White (1985) who synthesized water soluble styryloxazine compounds that displayed potent ChAT-inhibitory activity in isolated brain tissue (Mehta, Musso, & White (1985)). Out of these compounds BW813U (**Fig. 5A**) is an irreversible non-competitive inhibitor of ChAT, which has been studied extensively in neuronal systems (Mehta et al., 1985). BW813U does not show any effect on AChE activity. The possible side-effects of ChAT disrupters on the brain and nervous system may be of concern for clinical applications of ChAT-ligands. While early studies aiming to disrupt ChAT activity by inducing morphological lesions in a murine model resulted in a worsened performance in the radial maze test, recent studies using small molecule inhibitors of ChAT activity demonstrated no such disruption in spatial brain functions (Russell, 1988). The administration of BW813U (50 mg/kg bodyweight intraperitoneally) in rats did not impair their performance in the radial maze test (Meck, 2006; Wenk, Sweeney, Hughey, Carson, & Olton,

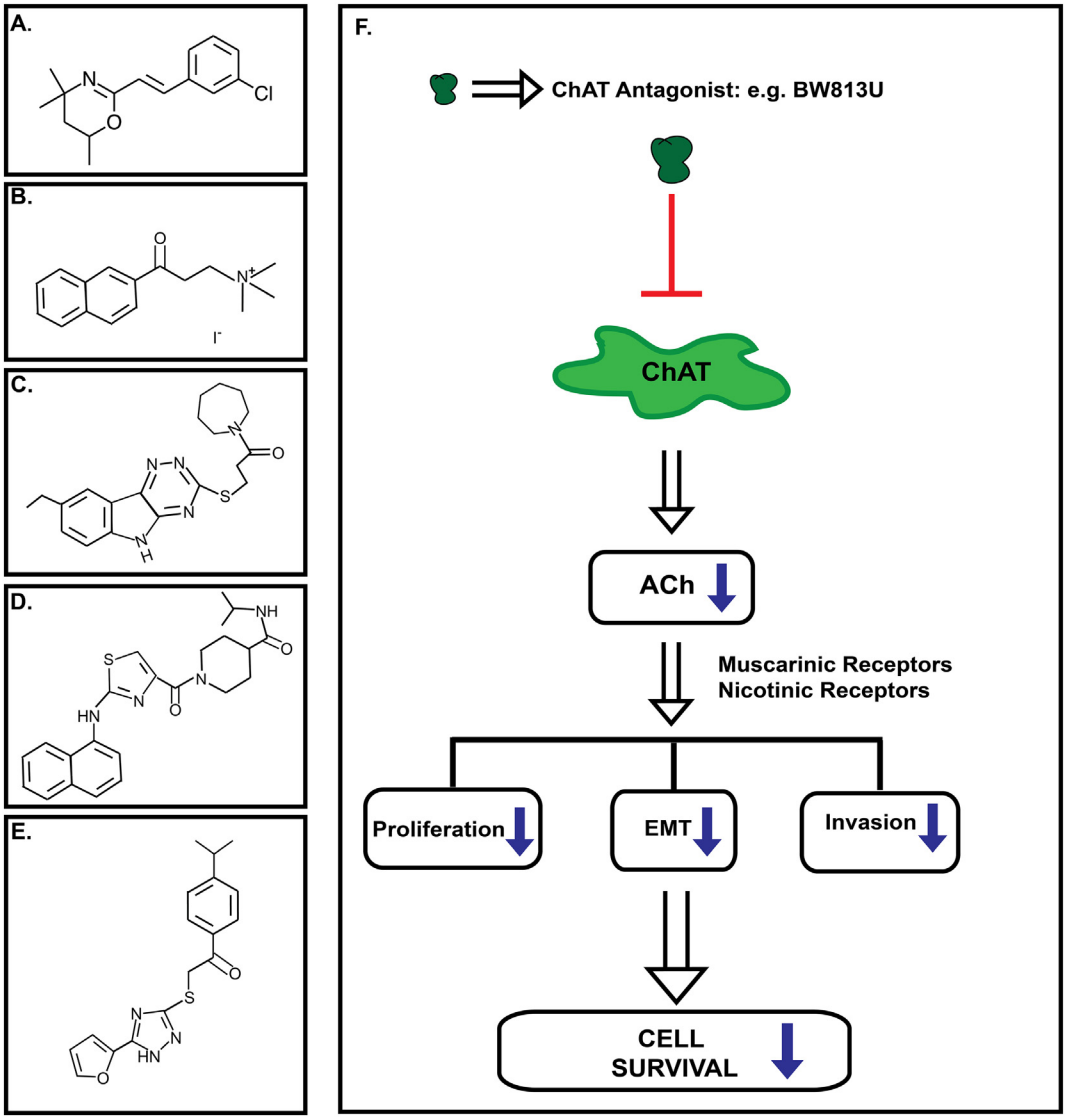

Fig. 5. Structure of synthetic ChAT inhibitors. A. BW813U B. alpha-NETA C. ASN07441713 D. BAS11101702 E. BAS03014741 F. Pharmacological disruptors of ChAT may be useful for suppressing the growth of human lung cancers. ChAT antagonists like BW813U bind and inhibit ChAT enzymatic activity, which diminishes the downstream production of acetylcholine (ACh). The decreased ACh levels translates to reduced growth and progression of human lung cancer cells.

1986). The authors showed that BW813U did indeed reduce ChAT activity by 66-80%. Such experiments confirm that BW813U does not cause detrimental side-effects on spatial memory and cognition. This may be explained by the fact that the functional activity of ChAT is not the rate-limiting step for ACh synthesis. The inhibition of ChAT activity (close to 90%) had only minimal effect on ACh production. Another plausible explanation may be the compensatory effects by non-cholinergic neural systems.

The elucidation of the crystal structure of ChAT has enabled the rational design of potent ChAT inhibitors. Sastry, Jaiswal, Janson, Day, and Naukam (1988a) examined the ability of two compounds namely 2-(α-naphthoyl) ethyltrimethylammonium (α-NETA; **Fig. 5B**) and its beta isomer to suppress the enzymatic activity of ChAT in isolated organ systems. They observed that both α-NETA and β-NETA functioned as specific ChAT inhibitors (Sastry, Jaiswal, Janson, et al., 1988a; Sastry, Jaiswal, Owens, Janson, & Moore, 1988b). However, the binding affinity of α-NETA for ChAT (IC_{50}=9 μM) is higher relative to the beta isomer (IC_{50}=76 μM). Both of these compounds displayed no cross-reactivity at carnitine acetyltransferase, cholinesterases, muscarinic and nicotinic receptors. Recent experiments have used high-throughput virtual screening of commercial compound libraries (comprising of about 300,000 compounds) to identify potential ChAT modulators. The hits obtained after the virtual screen were tested for their ability to suppress ChAT activity *in vitro* (Kumar, Kumar, Langstrom, & Darreh-Shori, 2017) and subjected to molecular docking studies. The authors found that the three compounds namely ASN07441713 (**Fig. 5C**), BAS11101702 (**Fig. 5D**) and BAS03014741 (**Fig. 5E**) to be the most effective inhibitors of ChAT activity. The authors intend to use these compounds as a starting platform for developing a second generation of ChAT ligands which would be used as imaging probes for early diagnosis of neurodegenerative diseases (Kumar et al., 2017).

The decrease in ChAT expression or disruption in its enzyme activity has been investigated as a possible drug target in the treatment of human lung cancers. The depletion of ChAT by small interfering RNA (siRNA) methodology decreased the viability of H1299 human LAC cells at 48 and 72 hours (N. Hua et al., 2012). Studies in our laboratory have attempted to assess the anti-cancer activity of the small molecule water-soluble ChAT antagonist BW813U. We observed that BW813U decreased the viability of human SCC-L and LAC cell lines *in vitro* in a concentration dependent manner (Akers et al., 2017; Dasgupta et al., 2018). Subsequently we analyzed the anti-tumor activity of BW813U in athymic mouse models of human LAC. The administration of BW813U (at a dose of 2.5 mg/kg bodyweight, thrice a week by intraperitoneal injection) robustly decreased the growth rate of H838 human LAC tumors xenografted into athymic mice. Most importantly, the treatment of tumor-bearing athymic mice with BW813U did not cause in any gross toxicity or behavioral discomfort to mice; weights and food/water consumption of the BW813U-treated athymic mice were similar to vehicle-treated athymic mice (Akers et al., 2018). The small molecule ChAT enzyme inhibitors namely ASN07441713, BAS11101702 and BAS03014741 decreased the viability of HEK293 human embryonic kidney fibroblasts at 10 and 50 μM (Kumar et al., 2017). The growth-inhibitory activity of α-NETA has not been tested in human cell lines. Our ongoing studies are aimed at dissecting out the molecular mechanisms underlying the anti-neoplastic activity of ChAT antagonists like BW813U. We believe that ChAT antagonists (like BW813U) block ChAT enzyme activity, which in turn induces a decline in the secretion of ACh by human lung cancer cells. Traditionally, ACh acts via nAChR and muscarinic receptors to stimulate the proliferation, induction of EMT, migration and invasion of human lung cancer cells. The fall in ACh levels will suppress the abovementioned signaling pathways and abrogate the growth and survival of human lung cancers (**Fig. 5F**).

Therefore, ChAT disruptors may represent a new generation of drugs relevant for lung cancer therapy. However, a majority of these compounds have not been tested for their growth-inhibitory activity in human cancer cells.

3. Choline Transporters (ChTs)

3.1. Vesicular Acetylcholine Transporter (VAChT)

The primary function of VAChT is to package ACh (synthesized in the cytoplasm) into vesicles, which store ACh at much higher concentrations than that available in the cytoplasm (Prado, Roy, Kolisnyk, Gros, & Prado, 2013; Usdin, Eiden, Bonner, & Erickson, 1995). These vesicles transport ACh to the cellular membrane where it is released into the extracellular space by exocytosis (Barman et al., 2016). Several lines of evidence show that VAChT is localized in the vesicle membrane (Y. Liu & Edwards, 1997; Weihe, Tao-Cheng, Schafer, Erickson, & Eiden, 1996). Each molecule of ACh transported by VAChT is exchanged for two vesicular protons, which leads to loading of synaptic vesicles with ACh (Barman et al., 2016). Molecular cloning and hydrophobic analysis studies have revealed that the structure of VAChT is comprised of twelve transmembrane domains. The carboxy terminus of VAChT contains structural motifs such as di-leucine motif, which are vital for its cellular trafficking and localization (Eiden, Schafer, Weihe, & Schutz, 2004; Erickson et al., 1996; Prado et al., 2013; Usdin et al., 1995).

The detection of VAChT in multiple types of cells in normal lung tissue and lung cancer tissue has led to intense about its possible role in lung maintenance and homeostasis (Song, Sekhon, Proskocil, et al., 2003b; Song & Spindel, 2008; Spindel, 2016; Wessler et al., 1998). Studies in VAChT-mutant mice have indicated role for VAChT in pulmonary inflammation (Lips et al., 2007a; Pinheiro et al., 2015). VAChT has been robustly expressed in a diverse array of human lung cancer cell lines (**Table 3**). VAChT also has been detected in human LAC and SCC-L tissues (isolated from patients) and in matched normal tissue (**Table 4**). Immunohistochemistry experiments reveal that HUVECs express VAChT (Kirkpatrick et al., 2001; Kirkpatrick et al., 2003). Electron microscopy experiments demonstrate that endothelial VAChT is localized to endocytotic vesicles (Kirkpatrick et al., 2001). This observation supports the possibility that VAChT is responsible for packaging ACh and transporting it to extracellular space, in a manner analogous to neuronal cells. Shao et al. (2016) explored the effect of autonomic nervous infiltration on the risk and prognosis of patients diagnosed with LAC (Shao et al., 2016). VAChT was used as a biomarker for cholinergic nerve infiltration (Prado et al., 2013). They observed that the upregulation of VAChT was correlated with increased risk and increased recurrence in surviving LAC patients (Shao et al., 2016).

Studies in our laboratory have analyzed the effect of nicotine on VAChT levels in human LAC cell lines. The treatment of A549 and H358 human LAC cell lines with 100 nM nicotine (which is within the range of nicotine concentrations found in the plasma of an average smoker) elevated the magnitude of VAChT. We observed that VAChT was robustly expressed in human LAC tumors (isolated from patients) and adjacent normal lung tissue (Lau et al., 2013). Furthermore, Song

Table 3
VAChT Expression in normal human bronchial epithelial cells and lung cancer cell lines

Cell Line	Nature of lung cells	Expression of VAChT	References
NHBE	Normal human bronchial epithelium	+	Dasgupta et al., 2018 Dasgupta et al., 2016
SAEC	Small airway epithelial cells	+	Dasgupta et al., 2018 Dasgupta et al., 2016
HPAEpics	Human pulmonary alveolar epithelial cells	+	Lau et al., 2013
H82, H69, H345, H417, H592, H378	Small cell lung cancer	+	Song et al., 2003b Song et al., 2007
H520, H226, SK-MES	Squamous cell carcinoma of the lung	+	Dasgupta et al., 2018 Dasgupta et al., 2016
A549, H358, H650, H2228, H1975, H1563, H1650, H23, H1944, H1355	Lung adenocarcinoma	+	Dasgupta et al., 2018 Dasgupta et al., 2016 Lau et al., 2013

Table 4
VAChT Expression in lung cancer tissue and adjacent normal tissue (isolated from patients)

Type of lung tissue	Expression of VAChT	References
Normal lung tissue	+	Lau et al., 2013 Song et al., 2008 Song et al., 2003b
Squamous cell lung cancer tissue	+	Dasgupta et al., 2018 Dasgupta et al., 2016 Song et al., 2008
Lung adenocarcinoma tissue	+	Lau et al., 2013

and Spindel (2008) detected the presence of VAChT mRNA in human SCC-L tumor tissue (Song et al., 2008). They performed VAChT immunohistochemistry on 31 SCC-L tumors and observed that VAChT is robustly expressed by about 65% of the tumors (Song et al., 2008).

The vesicular transporter activity of VAChT is blocked by the non-competitive antagonist, vesamicol. The growth-inhibitory activity of vesamicol has been studied in both SCLCs and NSCLCs (**Table 5**). Song, Sekhon, Jia, et al. (2003a) demonstrated that vesamicol suppressed the viability of asynchronous H82 human SCLC cells (in a concentration-dependent manner) at nine days and twelve days post treatment (Song, Sekhon, Jia, et al., 2003a). Our laboratory examined the growth-suppressive activity vesamicol in a panel of human LAC cells (**Table 5**). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays revealed that vesamicol potently decreased the viability of nicotine-treated human LAC cell lines (Dasgupta et al., 2016; Dasgupta et al., 2018; Lau et al., 2013). Caspase-3 activity and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assays revealed that vesamicol induced 2.5-3.0-fold apoptosis in nicotine-treated A549 and H358 human LAC cells. The administration of vesamicol (at a dose of 50 mg vesamicol/kg food) decreased the growth rate of nicotine-treated A549 human LAC tumors xenografted in athymic mice (Lau et al., 2013). Although, Song, Sekhon, Jia, et al. (2003a) showed the growth-inhibitory effects of vesamicol in H82 human SCLC cells in cell culture, they did not examine the anti-neoplastic activity of vesamicol *in vivo* (Song, Sekhon, Jia, et al., 2003a). SCLCs have a robust cholinergic signaling axis and occur exclusively in smokers. Therefore, it may be probable that vesamicol will display anti-neoplastic activity in athymic mouse models of SCLC.

3.2. ChT1, CTLs, OCTs and OCTNs in lung cancer

Choline plays a vital role in cellular homeostasis and survival. Mammalian cells utilize choline for the synthesis of membrane phospholipids namely phosphatidylcholine, sphingomyelin and betaine (Farine, Niemann, Schneider, & Butikofer, 2015; Lagace & Ridgway, 2013; Ridgway, 2013). Additionally, choline is the precursor for the synthesis of ACh, which acts as an autocrine and paracrine growth factor for bronchial epithelium, SCLCs, SCC-Ls, and LACs. (S. A. Grando, 2008; Kummer & Krasteva-Christ, 2014; Kummer et al., 2008; Proskocil et al., 2004; Song & Spindel, 2008; Wessler et al., 1998; Lau et al., 2013; Song et al., 2008; Song, Sekhon, Proskocil, et al., 2003b; Song & Spindel, 2008; Spindel, 2012, 2016). The choline transport system in the lung is mediated by three major families of proteins: i) High affinity choline transporter 1 (ChT1/SLC5A7; Okuda & Haga, 2003), ii) Choline transporter like proteins (CTL1-5; Inazu, 2014; Traiffort et al., 2013) with moderate affinity towards choline, and iii) Polyspecific organic transporters (OCT1-3/SLC22A1-2) and carnitine/cation transporters (OCTN1 and OCTN2; Pochini et al., 2013; Tamai, 2013; Volk, 2014). OCTN3 has been only detected in mouse tissues (Tamai et al., 2000). OCT1 and OCT2 also transport ACh in BECs (Kummer et al., 2006; Lips et al., 2005). Pochini et al., (2012) investigated the ability of human OCTN1 to transport ACh using proteoliposomal model systems. They developed human OCTN1 reconstituted proteoliposomes and analyzed their ability to mediate the uptake, transport and efflux of ACh. The results from their experiments revealed that OCTN1 (in proteoliposomal preparation) efficiently catalyzed the bidirectional transport of ACh, and this process was asymmetrically regulated by sodium ions (Pochini, Scalise, Galluccio, & Indiveri, 2012a; Pochini, Scalise, Galluccio, Pani, et al., 2012b). However, these studies have not been extended to normal lung or lung cancer cells.

Five types of CTL like proteins have been characterized in humans (Inazu, 2014; Traiffort et al., 2013). Studies in rat models indicate that the major form of CTL1 originates from a 3.5 kb transcript and is present in the diverse regions of the brain and spinal cord. A minor form of CTL1 (arising from a 5 kb transcript) is detected in the colon, lung and spinal cord (O'Regan et al., 2000; Traiffort et al., 2013). The CTL1 protein has lower affinity for choline than ChT1. However, both CTL1 and ChT1 are inhibited by HC-3 (Inazu, Takeda, & Matsumiya, 2005; Kouji et al., 2009; Uchida et al., 2009). The lung expresses two isoforms of CTL2, namely CTL2-P1 and CTL2-P2. Human CTL-P1 does not participate in choline transport. However, CTL2-P2 is a functional choline transporter (Kommareddi et al., 2010). CTL2 and CTL4 have been shown to transport choline in human lung cells (Nakamura et al., 2010; Song et al., 2013). No studies have addressed the choline transport properties of CTL3 and 5. The affinity of OCT1 and OCT2 for choline is lower than CTL1 and CTL2 (Inazu, 2014). OCT3 does not have the ability to transport choline (Inazu, 2014). Both CTL1 and CTL2 play a vital role in physiological functions of the lung like surfactant production, cell growth and cell repair (Traiffort et al., 2013). Choline transporters play a vital role in multiple lung diseases, like infant respiratory distress syndrome, drug-induced interstitial lung diseases, transfusion-related lung injury and lung cancer (Curtis et al., 2010; Greinacher et al., 2010; Inazu, 2014; Nakamura et al., 2010; Traiffort et al., 2013).

Data from several research laboratories have shown that cancer cells have enhanced choline uptake and transport in comparison to normal cells (Inazu, 2014; Ingoglia et al., 2015; Salomon et al., 2014; Tamai, 2013; Volk, 2014). The reason for this may be due to the vital role of choline transport in cell growth, membrane integrity and cell repair process (Glunde, Bhujwalla, & Ronen, 2011; Glunde, Penet, Jiang, Jacobs, & Bhujwalla, 2015; Mori, Wildes, Takagi, Glunde, & Bhujwalla, 2016). The enhanced uptake of choline has formed the basis of imaging of tumors by magnetic resonance imaging (MRI) and positron emission tomography (PET; Glunde et al., 2015; Hara, Bansal, & DeGrado, 2006). Radiolabeled choline transporter ligands like tritiated HC-3, ^{18}F-Choline, ^{18}F-FA-4 and ^{11}C-pipzA-4 (**Fig. 6**) have been investigated as imaging agents for a variety of human tumors including lung cancer (Challapalli & Aboagye, 2016; Gilissen et al., 2003; M. Li et al., 2013; Ramirez de Molina et al., 2007). CTLs, OCTs and OCTNs are robustly expressed by multiple human lung cancer cell lines (**Table 6**). CTL1 and CTL2 mediate choline transport in human SCLCs and LACs (Inazu, Yamada, Kubota, & Yamanaka, 2013; Nakamura et al., 2010). The choline-transport activity of CTL4 has been only investigated in SCLC cells (Song et al., 2013). Song et al., (2013) studied expression of CTLs in two SCLC tumors isolated from patients. Both the tumors expressed CTL1-5 (Song et al., 2013). OCT1-2 and OCTN1-2 were detected in lung tumors (isolated

Table 5
Growth-suppressive activity of VAChT inhibitor vesamicol in human lung cancer cell lines and athymic mouse models

Cell Line	Nature of lung cells	Growth-inhibitory Activity of Vesamicol	Model used	References
H82	Small cell lung cancer	+	Cell culture	Song et al., 2003b Spindel, 2016
A549	Lung adenocarcinoma	+	Cell culture, athymic mice	Lau et al., 2013
H1975, H838 H358	Lung adenocarcinoma	+	Cell culture	Dasgupta et al., 2018 Dasgupta et al., 2016 Lau et al., 2013

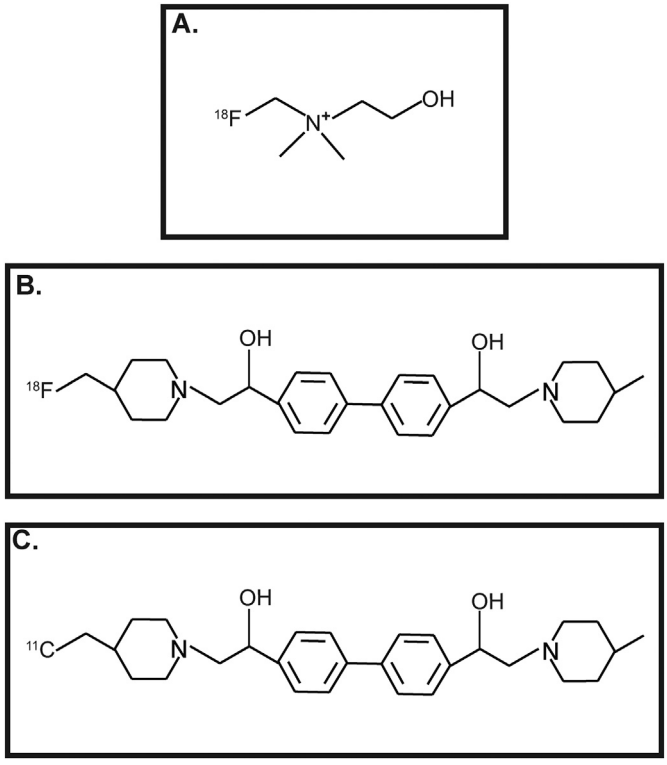

Fig. 6. Choline-transporter-based imaging agents used in PET scans. A. ^{18}F-choline B. ^{18}FA-4 C. ^{11}C-pipzA-4.

from patients) and matched normal lung tissue samples (**Table 7**; More et al., 2010; T. Wang et al., 2007). However, no clear-cut trends were obtained in the expression pattern of OCTs or OCTNs between normal and lung tumor tissue.

The observation that neoplastic cells have higher choline metabolism and uptake (relative to normal cells) has been exploited to develop innovative therapeutic approaches in lung cancer. The depletion of CTL4 by siRNA methodology suppressed the proliferation of SCLC cells (Song et al., 2013). Inazu et al. (2013) found that the transfection of CTL1-siRNA decreased the viability and enhanced apoptosis of H69 human SCLC cells (Inazu et al., 2013). Similarly, CTL1-siRNA displayed small yet significant anti-proliferative activity in H82 human SCLC cells (Song, Mark, & Spindel, 2010a). H82 is a variant human SCLC cell line, whereas H69 is a classical human SCLC cell line. The variant SCLC cells are associated with accelerated doubling time, greater invasive phenotype and lower sensitivity to growth-inhibitory agents than classical SCLC cell lines like H69 (Broers et al., 1988; Gazdar, Carney, Nau, & Minna, 1985). This may explain the lower growth-suppressive activity of CTL1-siRNA in H82 relative to H69 human SCLC cells.

The CLT1 inhibitor HC-3, shows growth-inhibitory activity in human SCLC and LAC cell lines (**Table 8**). Data from our laboratory indicates that 50 μM HC-3 induces robust apoptosis in nicotine-treated H1975 and H838 human LAC cells over 24 hours (Dasgupta et al., 2016; Dasgupta et al., 2018). We did not observe any apoptotic activity of HC-3 in untreated human LAC cells. Our data is divergent relative to the results of Inazu et al. (2013) who treated H69 human SCLC cells with 1 mM HC-3 for 2 days (Inazu et al., 2013). Subsequently, they measured cell viability by a luminescence ATP detection assay. They found that 1 mM HC-3 decreased the viability of H69 cells at 48 hours (Inazu et al., 2013). Such differences in results may be due to the fact the two studies involved two different histological types of lung cancer (LAC versus SCLC). Inazu et al. (2013) explored the apoptotic activity of HC-3 using caspase-3/7 activity assay and immunofluorescence. They observed that HC-3 induced 2-2.5-fold increase in apoptosis in H69 cells. DAPI staining of HC-3 treated H69 cells showed morphology typical of apoptotic cells including condensed nuclei and apoptotic bodies (Inazu et al., 2013). Such data indicate that multiple dosing with high concentrations of the drug may be needed for the pro-apoptotic activity of HC-3 in asynchronous human SCLC cells. This is confirmed by the data of Wang et al. (2007) who observed that HC-3 induced cell cycle arrest in A549 and SPC-A-1 human LAC cells. They added 200 μM HC-3 daily for eight days, changing the drug daily and measured S-phase entry by BrdU assay (T. Wang et al., 2007). They found that HC-3 decreased S-phase entry of A549 and SPC-A-1 cells by approximately 64% relative to the vehicle-treated cells. Choline uptake blockers like phenoxybenzamine (PbA), tetraethylammonium (TEA) and norepinephrine (NEP) block choline uptake and proliferation of A549 and SPC-A-1 LAC cells (T. Wang et al., 2007). Likewise, organic cationic drugs like quinine, quinidine, desipramine, imipramine, clomipramine, fluvoxamine, diphenhydramine,

Table 6
The expression of choline transporters in normal human bronchial epithelial cells and lung cancer cell lines

Cell Line	Nature of lung cells	ChT1	CTL1-5	OCT1-2	OCTN1	References
NHBE	Normal human bronchial epithelium	+				Dasgupta et al., 2018 Dasgupta et al., 2016
SAEC	Small airway epithelial cells	+				Dasgupta et al., 2018 Dasgupta et al., 2016
HPAEpics	Human pulmonary alveolar epithelial cells	+				Lau et al., 2013
H146, H417, H82	Small cell lung cancer		+			Song et al., 2013
H69, H345	Small cell lung cancer	+	+			Inazu et al., 2013 Song et al., 2013
H592	Small cell lung cancer		CTL1-3, CTL5			Song et al., 2013
H1694	Small cell lung cancer	+				Song et al., 2013
H520, H226	Squamous cell carcinoma of the lung	+				Dasgupta et al., 2018 Dasgupta et al., 2016 Lau et al., 2013
H1703	Squamous cell carcinoma of the lung			OCT1, OCT2		More et al., 2010
H1975, H1563, H1734, H1650, H23, H650, H358	Lung adenocarcinoma	+				Dasgupta et al., 2018 Dasgupta et al., 2016 Lau et al., 2013
H838	Lung adenocarcinoma	+		OCT1, OCT2		Dasgupta et al., 2018 Dasgupta et al., 2016
A549	Lung adenocarcinoma	+	CTL1	OCT1	OCTN1, OCTN2	Lau et al., 2013 Wang et al., 2007
H460	Lung adenocarcinoma			OCT1, OCT2		More et al., 2010
SPC-A-1, H1299	Lung adenocarcinoma		CTL1		OCTN1, OCTN2	Wang et al., 2007
H441	Lung adenocarcinoma			OCT1, OCT2	OCTN1, OCTN2	Salomon et al., 2014

Blanks indicate Not Determined (ND).

paroxetine, reboxetine, citalopram and fluoxetine inhibit choline uptake and the viability of H69 human SCLC cells (T. Wang et al., 2007). Such results seem to emphasize the importance of choline metabolism regulating the viability of lung cancer cells. This is confirmed by the data of More et al., (2010) showing a role for OCT1 and OCT2 in enhancing the cytotoxicity of picoplatin in a panel of human LAC cell lines (More et al., 2010). The overexpression of OCT1 and OCT2 (but not OCT3) augmented the cytotoxicity of picoplatin in both cell culture and mice models of human LAC. Cimetidine, a small molecule inhibitor of OCT1 and OCT2, reduced the growth-inhibitory activity of picoplatin in human LAC cell lines (More et al., 2010). This appears to be contradictory to the results obtained with ChT1 and CTLs antagonists. However, the effect of OCT1 and OCT2 on the anti-cancer activity of picoplatin is mediated by their effects on the uptake of the drug and increasing the formation of intracellular DNA-picoplatin adducts (More et al., 2010). Therefore, cationic transporters like OCT1 and OCT2 also regulate drug trafficking and biodistribution in lung cancer cells, apart from their role in choline uptake and transport.

4. Muscarinic Receptors

The biological activity of ACh is mediated by its binding to the nicotinic acetylcholine receptors and the muscarinic receptors on target cells (Barman et al., 2016). Muscarinic receptors belong to the superfamily of G-protein-coupled receptors. These receptors are comprised of seven hydrophobic domains and an intracytoplasmic loop between hydrophobic domains 5 and 6 (Wess, 1996). This loop is considered to be responsible for the G-protein coupling functions of the muscarinic activity. Five subtypes of the muscarinic receptor (M1R to M5R) have been identified in mammalian cells (Barman et al., 2016; Wess, 1996). These receptors may be broadly divided in two categories, based on their G-protein coupling activity (D. A. Brown, 2018; Kruse et al., 2014; Zenko & Hislop, 2018). The M1R, M3R and M5R are coupled to G_q-type proteins and activate phospholipase C to recruit phosphoinositol triphosphate-signaling cascade. The M2R and M4R receptors are coupled to pertussis toxin-sensitive Gi/0 proteins which inhibit adenylyl cyclase activity (D. A. Brown, 2018; Kruse et al., 2014; Zenko & Hislop, 2018). The muscarinic receptor system regulates several lung functions including airway remodeling, airway smooth muscle contraction, inflammation and wound healing (Kistemaker & Gosens, 2015; Roth, 2015).

Immunoblotting and RT-PCR reveal that muscarinic receptor subtypes are expressed in human lung cancer cell lines (**Table 9**). However, research studies aimed at analyzing the role of muscarinic receptors in lung cancer have yielded divergent results, which may be attributed to the nature of the muscarinic ligand used to activate the muscarinic receptors on human lung cancer cells (Figueroa, Griffin, & Ehlert, 2009). Early studies revealed that the activation of muscarinic receptors (by carbachol) inhibited cell cycle progression and voltage-dependent calcium influx in SCC-9 human SCLC cells (Williams, 2003; Williams & Lennon, 1990, 1991). In contrast, Song et al., (2003) observed that carbachol at concentrations of 1 μM and 10 μM increased the viability of H82 human SCLC cells at nine and twelve days post-treatment (Song, Sekhon, Jia, et al., 2003a). Such variations in the results could be due to the fact that carbachol activates nicotinic and muscarinic receptors. Therefore, the biological effects of carbachol is dependent on the cell membrane specific expression (and abundance) of nAChRs and muscarinic receptors on SCLC cells (Spindel, 2012, 2016). The expression pattern of nAChRs and muscarinic receptors varies across diverse human SCLC cell lines and such differences may be playing a central role in mediating the observed differences in response to carbachol treatment.

Carbachol upregulated the invasive phenotype of A549 human NSCLC cells by inducing EMT. The treatment of A549 cells with 1 μM carbachol produced a dose-dependent and time-dependent decrease of the epithelial junction protein E-cadherin with concomitant increase in the levels of mesenchymal proteins namely vimentin and α-smooth muscle actin. These results were confirmed in immortalized BECs (K. Yang et al., 2014). Apart from inducing EMT, carbachol upregulated the expression of MMP-9 in BECs (K. Yang et al., 2014). MMPs degrade the basement membrane to enable the invasion of tumor cells in the blood and lymph thereby facilitating distant metastasis of tumors (Gong et al., 2016; Merchant et al., 2017).

Yang et al., (2014) examined the role of the muscarinic receptor pathway in carbachol-induced EMT in A549 human NSCLC and immortalized BECs. Carbachol stimulated the production of TGF-β1 and MMP-9 from A549 human NSCLC cells (K. Yang et al., 2014). Carbachol-induced EMT and MMP-9 expression was reversed by the M1R antagonist pirenzepine (**Fig. 7A**) and M3R antagonist 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP; **Fig. 7B**) indicating that these biological activities of carbachol were mediated by M1R and M3R. The authors further showed that carbachol induced EMT required downstream activation of the Smad and ERK pathways (K. Yang et al., 2014). Conflicting reports exist about the biological role of M2R in lung cancer. The activation of M2R inhibits proliferation of H1694 human SCLC cells (Song, Sekhon, Duan, et al., 2004). These findings are in alignment with the fact that M2R staining in poorly differentiated human SCC-L tissue (isolated from patients) is decreased relative to adjacent normal tissue (Song et al., 2008). Data by Zhao, He, et al. (2015a and 2015b) reveals that the activation of muscarinic receptors by pilocarpine induces proliferation, EMT, migration and invasion of human NSCLCs via the MAP kinase and the Akt pathway (**Fig. 8A**; Zhao, Gu, et al., 2015b; Zhao et al., 2015c). These biological effects of pilocarpine are antagonized by M2R-short-hairpin RNA (shRNA; QZhao, Yue, et al., 2015c). An innovative aspect of this study was that the authors demonstrated the growth-inhibitory effects of M2R-shRNA in athymic mouse model (Q. Zhao, X. Gu, et al., 2015). A549 cells transfected with control (non-targeting)-shRNA or transfected with M2R-shRNA were subcutaneously injected in the flank of athymic mice. After four weeks, the authors observed that the growth rate of the A549-M2R-shRNA tumors were significantly lower ($P \leq 0.05$) than A549-control-shRNA tumors (Q. Zhao, X. Gu, et al., 2015). The results obtained from M2R-shRNA were verified by using the synthetic M2R antagonist namely methoctramine (**Fig. 9A**). Methoctramine suppressed the viability of A549 and PC9 cells in a concentration dependent manner over 72 hours. Furthermore, methoctramine displayed anti-neoplastic activity in athymic mouse models of human NSCLCs (Q. Zhao, X. Gu, et al., 2015; Q. Zhao, J. Yue, et al., 2015).

A majority of research papers have explored the role of M3R in the proliferative and pro-invasive effects of ACh in lung cancer (**Fig. 8B**). M3R is robustly expressed on several SCLC and NSCLC cell lines (**Table 9**). The depletion of M3R by siRNA abolished ACh-induced cell growth and elevation of intracellular calcium in H82 and H1694 human SCLC cells (Song, Sekhon, Lu & Jiang, 2017). ACh-induced calcium influx in H82 cells was unaffected by the M1R antagonist pirenzepine, M2R/M4R antagonist {11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one; (AFDX-116, **Fig. 9B**), M1R-siRNA and M5R-siRNA. Immunoblotting experiments show that ACh induces the activation of MAP kinase via M3R in H82 human SCLC cells (Song, Sekhon, Lu, et al., 2007). Furthermore, M3R is involved in the adhesion and migration of lung cancers. Boyden chamber assays confirmed that ACh accelerated the migration of SBC3 human SCLC cells towards fibronectin (as a chemoattractant). Similarly, ACh increased the adhesion of SBC3 cells on fibronectin-coated dishes (S. Zhang et al., 2010). The proliferative effects of ACh on SBC3 cells required both nicotinic and muscarinic receptors. However, ACh-induced adhesion and migration of SBC3 were exclusively mediated by the M3R (S. Zhang et al., 2010). In contrast, Quigley, Shafer, and Williams (1998) found that carbachol increased the adhesion of SCLC cells on laminin and collagen, but not on fibronectin (Quigley et al., 1998). It must be recognized that ACh and carbachol activate both nAChRs and muscarinic receptors. The muscarinic receptor antagonist atropine blocked carbachol-induced adhesion of SCC-9

cells on collagen IV, suggesting that the adhesion-stimulatory activity of carbachol was primarily mediated by muscarinic receptors on SCC-9 cells. Both the studies by Zhang et al. (2010) and Quigley et al. (1998) found that muscarinic receptors alter the functional activity of α1-containing integrins to elevate the adhesion of SCLC to extracellular matrix proteins (Quigley et al., 1998; S. Zhang et al., 2010).

The treatment of SCC-9 human SCLC cells with 100 μM carbachol increased cell-cell adhesion and compaction by about 2-3 fold relative to untreated SCC-9 cells. Carbachol-induced cell-cell adhesion was reversed by the muscarinic receptor antagonist atropine, indicating that the cell-compaction activity of carbachol was mediated by the muscarinic receptor family. Furthermore, carbachol-induced cell-cell adhesion of SCC-9 (human SCLC cells) was ablated by the M3R receptor antagonist 4-DAMP suggesting that the cell-cell adhesion activity of carbachol required the activation of M3Rs on SCC-9 human SCLC cells (Williams, 2003). Subsequently, the authors overexpressed GFP-tagged constitutively active Rac (Rac1Val-12) or GFP-tagged dominant negative Rac1 (Rac1Asn-17) in SCC-9 cells and activated muscarinic receptors using carbachol. Phase contrast microscopy revealed that the adhesion protein GFP-Rac1val-12 was localized to cell-cell junctions in carbachol-treated cells, probably indicating a role for the Rac pathway in this process (Williams, 2003). Taken together, these studies emphasize the vital role of M3R in the growth and progression of human SCLCs.

Lin et al. (2014) compared the expression of M3R in a panel of human NSCLC cell lines with normal human lung fibroblasts. Out of these NSCLC cell lines, four were human LACs and one was SCC-L (Lin et al., 2014). All NSCLC cell lines showed elevated levels of M3R, relative to normal human lung fibroblasts. However, no difference in M3R expression was observed between LAC and SCC-L cell lines. Subsequently, the authors explored the role of M3R in the progression of NSCLC using siRNA methodology. The transfection of M3R-siRNA caused a small but significant reduction of cell viability in A549 human LAC and L78 human SCC-L cells (Lin et al., 2014). However, the depletion of M3R (by siRNA techniques) had dramatic effects on the migration and invasion in both A549 and L78 human NSCLC cells (Lin et al., 2014). The knockdown of M3R in these two human NSCLC cell lines attenuated cell invasion and migration by 50-70% relative to control scrambled-siRNA-transfected cells. M3R-siRNA-transfected A549 human LAC cells and L78 human SCC-L cells showed decreased expression of MMP-9 expression and activity relative to control-siRNA-transfected cells. M3R-siRNA also decreased the activation of Akt and increased levels of E-cadherin in both A549 and L78 cells. An intriguing observation was that the changes in expression/activity of the above-mentioned genes was more pronounced in L78 human SCC-L cells than A549 human LAC cells (Lin et al., 2014). Both E-cadherin and MMP-9 play a vital role in the invasion and distant metastasis of human NSCLC cells (Gong et al., 2016; Merchant et al., 2017; Nieto et al., 2016; Tsoukalas et al., 2017; Xiao & He, 2010). A noteworthy observation is that the authors performed these experiments on only one LAC cell line (A549) and one SCC-L cell line (L78). It would be interesting to determine if this pattern is maintained in a panel of human LAC and SCC-L cell lines.

A role for cross-talk between M3R and EGFR has been indicated in the proliferative, pro-migratory and pro-invasive activity of ACh (**Fig. 8B**). The treatment of A549 cells with 200 μM ACh led to phosphorylation of EGFR, PI-3 kinase and Akt (R. Xu et al., 2015). The knockdown of M3R using siRNA methodology abrogated ACh-induced proliferation, migration and invasion. M3R-siRNA also suppressed ACh-induced activation of EGFR, PI-3 kinase and Akt (R. Xu et al., 2015). Such interaction between cell surface receptors and EGFR has been reported for other muscarinic receptor subtypes and nicotinic receptors in human cancer cells (Di Bari et al., 2018; H. Li et al., 2015).

Immunohistochemistry experiments demonstrate that M3R is expressed by 70% of SCLC, 85% of LAC and 70% of SCC-L tissues (Song et al., 2008; Song, Sekhon, Lu, et al., 2007; Spindel, 2012). Apart from M3R, SCC-Ls also express M2R and M4R (**Table 10**). The M3R co-localizes with ChAT in about 70% of SCLC and LACs, which further confirms the growth-stimulatory role of ACh in human lung cancers (Song, Sekhon, Duan, et al., 2004; Spindel, 2012). Lin et al. (2014) observed that M3R levels in NSCLC tumors was considerably higher than adjacent matched normal tissue. They further confirmed their results using immunohistochemistry in 148 cases of archived paraffin-embedded sections of NSCLC tumors (Lin et al., 2014). M3R was robustly expressed in approximately 57% samples. In agreement with their cell culture data, no trends were observed within the histological types of NSCLC (LAC vs. SCC-L vs. LCC; Lin et al., 2014). Statistical analysis revealed that the levels of M3R were inversely correlated with five-year survival rate of patients. There are no significant trends of M3R expression for age and sex of patients. A similar study was performed by Wu et al. (2013) which analyzed the levels of M3R in 192 NSCLC tumors isolated from patients. They observed that M3R levels were elevated in metaplasia/dysplastic tissue relative to matched adjacent normal tissue (J. Wu et al., 2013). The expression of M3R showed a strong association with stage, Ki67 (biomarker for proliferation) expression, tumor size, lymphatic vessel size and lymph node metastasis. M3R staining was higher in LAC relative to other types of NSCLC. M3R expression was elevated in Stage II and III NSCLC relative to Stage I of the disease (J. Wu et al., 2013). Furthermore, NSCLC patients whose tumors expressed high levels of M3R displayed lower disease free survival and overall survival relative to NSCLC patients with low M3R levels.

COPD is an independent risk factor for NSCLC (Durham & Adcock, 2015; Takiguchi, Sekine, Iwasawa, Kurimoto, & Tatsumi, 2014). Muscarinic receptors play a crucial role in the pathophysiology and airway remodeling associated with COPD (Gosens, Zaagsma, Meurs, & Halayko, 2006; Mastrodicasa et al., 2017). This led to extensive research on the status of muscarinic receptors in NSCLC (Song & Spindel, 2008; Spindel, 2012, 2016). Lin et al. (2014) examined the status of the M3R in NSCLC patients suffering from COPD (hereby referred to as NSCLC-COPD patients). They observed that high levels of M3R expression in NSCLC-COPD patients correlated with poorer survival rates than NSCLC-COPD patients displaying low M3R expression (Lin et al., 2014). Elevated levels of M3R in NSCLC-COPD patients correlated with high smoking history and poor lung function. Furthermore, tumors

Table 7
Expression of choline transporters in lung cancer tissue and adjacent normal tissue (isolated from patients)

Type of lung tissue	ChT1	CTL1-5	OCT1-2	OCTN1-3	References
Normal lung tissue	+	CLT-1	OCT1	OCTN1	More et al., 2010 Song et al., 2008 Song et al., 2003a Wang et al., 2007
Small cell lung carcinoma tissue	+	+	+		Song et al., 2003b Song et al., 2013
Squamous cell lung cancer tissue	+		OCT1, OCT2		Song et al., 2008 Song et al., 2003b
Lung adenocarcinoma tissue	+	CTL1		OCTN1, OCTN2	Song et al., 2003b Wang et al., 2007

Blanks indicate Not Determined (ND).

Table 8
The choline transporter antagonist hemicholinium-3 blocks the growth of lung cancer cell lines *in vitro*

Cell Line	Nature of lung cells	Growth-inhibitory Activity of Hemicholinium-3	Model used	References
H69, H82	Small cell lung cancer	+	Cell culture	Inazu et al., 2013 Song et al., 2003b Spindel, 2016
A549, SPC-A-1	Lung adenocarcinoma	+	Cell culture	Wang et al., 2007
H1975, H838	Lung adenocarcinoma	+	Cell culture	Dasgupta et al., 2018 Dasgupta et al., 2016

Blanks indicate Not Determined (ND).

isolated from NSCLC-COPD patients showed substantially higher M3R expression than NSCLC patients without COPD (Lin et al., 2014). Both univariate and multivariate Cox's regression analyses showed that M3R expression was an independent predictor of prognosis in NSCLC patients.

The muscarinic acetylcholine signaling system has been extensively used for cancer drug discovery (**Table 11**). Cell culture experiments reveal that generalized muscarinic antagonists like atropine decrease the viability of human SCLC cell lines (Song, Sekhon, Proskocil, et al., 2003b; Spindel, 2016). In addition, atropine abrogates TGF-β1-induced EMT in A549 human NSCLC cells at concentrations ranging from 0.1-10 μM over 72 hours (K. Yang et al., 2014).

The dual nicotinic/muscarinic receptor agonist carbachol induced EMT in A549 human NSCLC and immortalized BECs in a concentration-dependent manner at 72 hours post treatment. ELISA experiments revealed that carbachol-induced EMT in A549 human NSCLC cells correlated to increased secretion of TGF-β1 from A549 cells (K. Yang et al., 2014). The pro-EMT effects of carbachol were abolished by the M1R receptor antagonist pirenzepine and unaffected by the M2R antagonist methoctramine (**Fig. 9A**). However, Zhao, He, et al. (2015a) observed that the M2R antagonist methoctramine inhibited EMT, invasion and migration of A549 and PC-9 human NSCLC cells by multiple pathways (Zhao, Gu, et al., 2015b; Zhao, Yue, et al., 2015c). The anti-invasive, anti-migratory and EMT-inhibitory activity of methoctramine was triggered by inhibition of the PI-3kinase/Akt, MAPK, ERK and NF-κB pathway (Zhao, Yue, et al., 2015c). Methoctramine showed robust anti-tumor activity in A549 human NSCLC tumors xenografted in athymic mice. Two doses of methoctramine (2 mg/kg bodyweight/day and 5 mg/kg bodyweight/day) were administered intraperitoneally to these tumor-bearing athymic mice for three weeks (Zhao, Gu, et al., 2015b). Methoctramine (5 mg/kg bodyweight/day) decreased the growth of A549 tumors (in athymic mice) by about five-fold relative to vehicle-treated mice. Saline was used as the vehicle in the study. The methoctramine dose 2 mg/kg bodyweight/day did not show significant anti-tumor activity ($P \leq 0.05$) relative to saline-treated mice. The studies by Yang et al. (2014) used carbachol to activate the muscarinic receptor whereas Zhao, He, et al. (2015a) used pilocarpine as the muscarinic agonist in A549 human NSCLC cells. Such differences in data may be due to the fact that carbachol activates to both nicotinic receptors and muscarinic receptors, whereas pilocarpine is thought to be a generalized muscarinic receptor agonist with minimal nAChR-activating ability (Figueroa et al., 2009).

The M3R antagonist 4-DAMP, abrogated ACh-induced cell proliferation and calcium influx in human SCLCs (Song, Sekhon, Lu, et al., 2007). The treatment of H82 human SCLC cells with 100 pM-100 nM 4-DAMP robustly decreased ACh-induced activation of Akt and MAP kinase. Furthermore, 4-DAMP also decreased the viability of asynchronous H82, H1694 and SBC3 human SCLC cells in a concentration-dependent manner. The growth-inhibitory activity of 4-DAMP on asynchronous H82 human SCLC cells correlated with downregulation of phospho-Akt and phospho-MAPK (Song, Sekhon, Duan, et al., 2004; Spindel, 2012; S. Zhang et al., 2010). Data from Yang et al. (2014) demonstrated that 4-DAMP decreases carbachol-induced EMT in A549 human NSCLC cells (K. Yang et al., 2014). The growth-inhibitory activity of 4-DAMP was investigated in athymic mice models xenografted with SBC3 human SCLC cells (Caihong & Shuxiang, 2017). Real time PCR and immunoblotting experiments were used to monitor M3R expression, vascular endothelial growth factor (VEGF) levels and microvessel density (MVD) in these tumor-bearing athymic mice. Three doses of 4-DAMP (0.5, 1, and 2 mg/kg body weight) were administered intraperitoneally to these mice. They observed that all three doses of 4-DAMP decreased the growth rate and weights of SBC3 tumors implanted in athymic mouse (Caihong & Shuxiang, 2017). Furthermore, 4-DAMP decreased M3R expression, VEGF levels and MVD in these SBC3 tumors. This is the first study which demonstrates the anti-angiogenic activity of M3R antagonists (Caihong & Shuxiang, 2017).

Apart from 4-DAMP, multiple M3R antagonists like darifenacin (**Fig. 9C**), tiotropium (**Fig. 9D**) and para-fluoro-hexahydrosila-difenidol (P-F-HHSiD) suppressed the growth of human SCLC and SCC-L cell lines *in vitro* (Song et al., 2008; Song, Olivas, & Spindel, 2009; Song, Sekhon, Duan, et al., 2004; Spindel, 2012, 2016). The M2R/M4R antagonist AFDX-116 had no impact on the viability of asynchronous H82 and H1694 human SCLC cells (Song, Sekhon, Lu, et al., 2007; Spindel, 2012).

Song & Spindel (2008) observed that the M3R-antagonist darifenacin suppressed nicotine-induced proliferation of H520 human SCC-L cells (Song et al., 2008). Subsequently, they investigated the anti-tumor activity of darifenacin in athymic mouse model of human SCC-Ls using osmotic pumps. They injected H520 human SCC-L cells subcutaneously in the right flank of male athymic mice (Song et al., 2008). The tumors were administered darifenacin at a dose of 6 mg/kg bodyweight/day using osmotic pumps. The mice in the control group were administered the vehicle (50% DMSO in PBS) via osmotic pumps. After four weeks of treatment, the authors observed that darifenacin decreased the tumor weight and volume of SCC-L tumors xenotransplanted in athymic mice (Song et al., 2008). A later study by the same research group demonstrated the anti-tumor activity of tiotropium in athymic mice bearing SCC-L tumors. An interesting aspect of this experiment was that tiotropium was administered via inhalation, which would minimize any possible pleotropic effects of this compound on other tissues (Song, Olivas, & Spindel, 2010b).

Song and Spindel (2008) continued their studies to investigate the anti-tumor activity of darifenacin in H82 human SCLC tumors xenografted on athymic mice (Song et al., 2008; Spindel, 2012). The methodology of the experiment was similar to the previously discussed study involving the anti-cancer activity of H520 SCC-L tumor-bearing athymic mice. Three doses of darifenacin (0.3, 1, 3 mg/kg/day) were administered (using osmotic pumps) to the athymic mice xenografted with H82 human SCLC cells. Two of the doses of darifenacin, namely 1 mg/kg/day and 3 mg/kg/day, significantly decreased the growth rate of H82 tumors, whereas the dose of 3 mg/kg/day darifenacin decreased both tumor volume and tumor weight of H82 human SCLC tumor-bearing mice (Song, Sekhon, Lu, et al., 2007). The authors detected darifenacin in the plasma in all the three doses of drug administered to mice. The anti-tumor activity of darifenacin correlated with decreased levels of phospho-Akt and phospho-MAPK (Song, Sekhon, Lu, et al., 2007). A noteworthy aspect of these studies was that the authors did not report any gross toxicity of darifenacin on mice in either of the studies.

Hua et al. (2012) examined the anti-neoplastic activity of a novel synthetic muscarinic receptor antagonist R2HBJJ (**Fig. 9E**) in human NSCLC. The design of R2HBJJ was based on structure-activity relationship (SAR) studies on the potent anti-cholinergic compound R2-PHC (**Fig. 9F**), developed in their laboratory (N. Hua et al., 2012). Receptor binding assays showed that the R2HBJJ showed maximal affinity for M3R followed by M1R, M4R, M5R and M2R. The authors investigated the growth-inhibitory effect of R2BHJJ on a panel of four NSCLC cell lines (A549, H1299, H157, and H460) and an immortalized normal lung epithelial cell line BEP2D. The growth-suppressive activity of R2BHJJ in H460, H157 and H1299 cells was greater than in BEP2D normal lung cells (N. Hua et al., 2012). R2BHJJ minimally affected the viability of A549 human NSCLC cells. This may be due to the fact that A549 expressed the lowest amount of M3R out of all the cell lines tested. The authors also compared the growth-inhibitory activity of R2BJJ with atropine, pirenzepine, AFDX-116, darifenacin and their parent compound R2PHC in H1299 cells (N. Hua et al., 2012). The cells were treated with varying concentrations of the drugs (1-100 μM) for 72 hours. Out of all of the compounds tested, atropine and pirenzepine had the least effect on the viability of H1299 cells. AFDX-116 displayed growth inhibitory activity at high concentrations, namely 30 μM and 100 μM. The growth-inhibitory activity of R2BHJJ was similar to the parent compound R2-BHC. However, R2BHJJ displayed a better side effect profile than R2-PHC. The growth-suppressive effect of darifenacin was intermediate between AFDX-116 and R2BHJJ. The transfection of M3R-

Table 9
Expression of muscarinic receptors in normal human bronchial epithelial cells and lung cancer cell lines

Cell line	Nature of cells	Nature of muscarinic receptor					Reference
		M1R	M2R	M3R	M4R	M5R	
BEP2D	Immortalized normal human bronchial epithelium					+	Hua et al., 2012
16-HBE	Immortalized normal human bronchial epithelium			+			Albano et al., 2018 Montalbano et al., 2014
HLF	Human lung fibroblasts			+			Lin et al., 2014 Profita et al., 2009
SCC-15, SCC-9	Small cell lung cancer			+			Quigley et al., 1998 Williams, 2003 Williams and Lennon, 1990, 1991
SBC3	Small cell lung cancer			+			Caihong and Shuxiang, 2017 Zhang et al., 2010
NCI-H146	Small cell lung cancer			+			Williams, 2003
NCI-H209	Small cell lung cancer			+			Williams, 2003
H69, H82, H345, H592, H417, H1694	Small cell lung cancer	+	+	+	+	+	Song et al., 2003a Song et al., 2007
H398	Small cell lung cancer			+		+	Song et al., 2003a
H157	Squamous cell carcinoma	+	+	+	+	+	Hua et al., 2012
L78, SPCA-1	Squamous cell carcinoma			+			Lin et al., 2014
A549, PC9	Lung adenocarcinoma	+	+	+	+	+	Hua et al., 2012 Lin et al., 2014 Zhao et al., 2015a Zhao et al., 2015b
GLC82	Lung adenocarcinoma			+			Lin et al., 2014
H1299, H460	Lung adenocarcinoma	+	+	+	+	+	Hua et al., 2012

(Blanks indicate Not Determined (ND).

siRNA ablated the growth-inhibitory activity of R2BHJJ in human NSCLC cells, suggesting that R2BHJJ predominantly targeted the M3R for its growth-suppressive activity. The binding of R2BHJJ to M3R induced cell cycle arrest (G0/G1 phase) in H1299 cells (N. Hua et al., 2012). R2BHJJ was shown to have the ability to inhibit Rb phosphorylation and downregulate the expression of cell cycle-regulatory proteins.

The muscarinic receptor antagonist J-115311 (**Fig. 9G**) has a high binding affinity for M3R and is about 50- to 400-fold more selective for M3R than other muscarinic receptor subtypes. J-115311 had a greater affinity and selectivity for M3R than darifenacin (Sagara et al., 2002; Song et al., 2008; Song, Sekhon, Lu, et al., 2007). Ami et al. (2011) explored the anti-cancer activity of J-115311 in human SCLCs. The authors compared the ability of J-115311 and darifenacin to decrease the viability of H82 human SCLC cells. WST-8 assays reveal that the growth-inhibitory activity of J-115311 was about three-times greater than darifenacin in H82 cells (Ami et al., 2011). J-115311 suppressed the growth of H82 cells by inducing apoptosis. This report by Ami et al., (2011) is the first to examine the anti-tumor activity of an M3R antagonist using the orthotopic mouse model of SCLC (Ami et al., 2011). Several congruent studies show that the orthotopic mouse model reproduces the morphology, tumor microenvironment and metastatic pattern of human lung cancers (Justilien & Fields, 2013; Kellar, Egan, & Morris, 2015). H82 cells were injected into the upper left lung of athymic mice. The tumors were allowed to grow for a week after which the mice were daily administered two doses of J-115311 of 25 mg/kg bodyweight and 50 mg/kg bodyweight subcutaneously. After 10 days, the tumors were visualized by microCT scans (Ami et al., 2011). The administration of 50 mg/kg bodyweight J-115311 caused a reduction in tumor weight and tumor volumes by about 40%. An interesting observation was that the anti-tumor activity of darifenacin was observed at lower doses (6 mg/kg bodyweight/day) than J-115311 (Ami et al., 2011; Song et al., 2008). This may be due to differences in mouse models and duration of experiments used in both studies. Data from Sangara et al. (2002) also indicates that darifenacin has a higher cross-reactivity towards other muscarinic receptor subtypes relative to J-115311 (Sagara et al., 2002). The M2R has been shown to play a role in promoting invasion and EMT in human lung cancers (Zhang et al., 2015a and 2015b). It is possible that the greater anti-tumor activity of darifenacin may be due to its ability to target multiple muscarinic receptor subtypes. The design and discovery of synthetic compounds with improved specificity for M2R, M3R or both may have potential applications in the management and treatment of lung cancer.

5. Nicotinic Receptors (nAChRs)

The role of nAChRs has been widely studied in the growth, angiogenesis and metastasis of lung cancer (S. A. Grando, 2008; Sergei A. Grando,

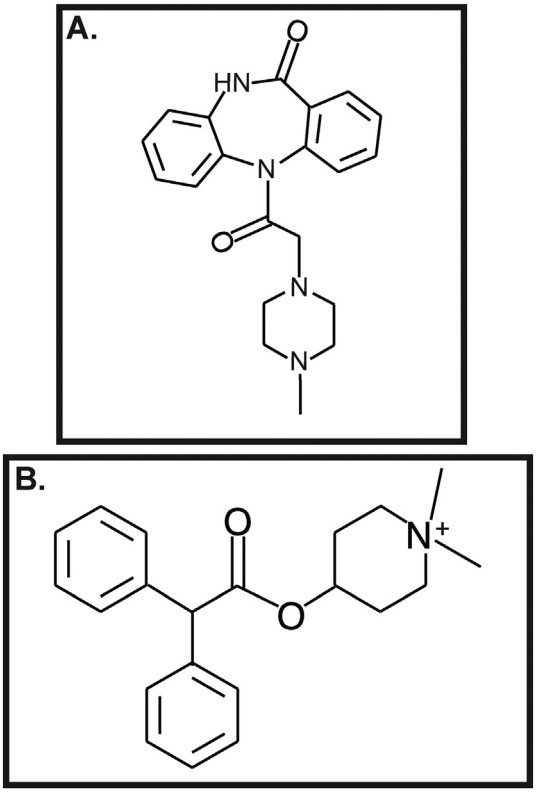

Fig. 7. Muscarinic receptor antagonists display growth suppressive activity in human lung cancers. A. Pirenzepine B. 4-DAMP.

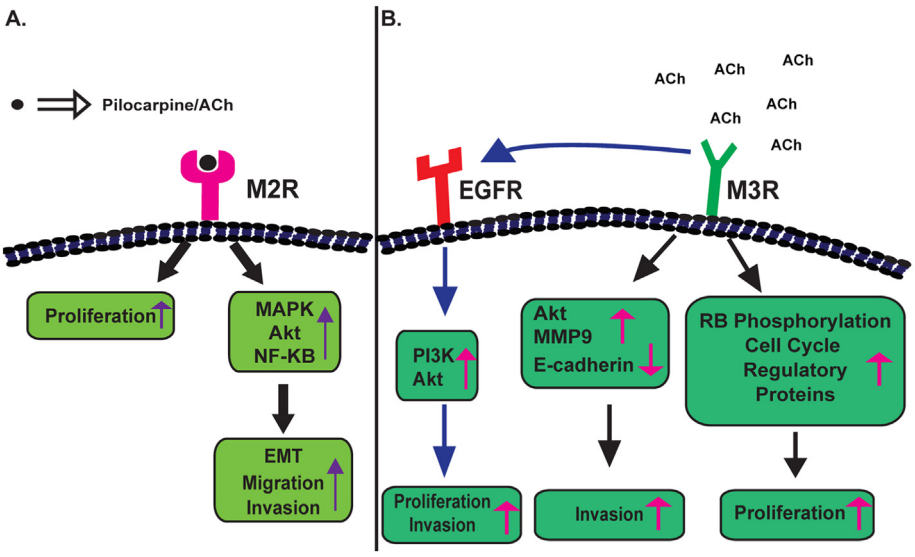

Fig. 8. A flow chart summarizing muscarinic receptor-induced signaling pathways. A. Signal-transduction pathways downstream of M2R in human lung cancers. B. Cellular signaling pathways underlying the proliferative activity of M3R. EMT: Epithelial to mesenchymal transition.

2014; Pillai & Chellappan, 2012; Schuller, 2012; Spindel, 2016; S. Wang & Hu, 2018; Zoli et al., 2018). The proliferative effects of ACh in SBC3 human SCLC cells are partially mediated by nAChRs (S. Zhang et al., 2010). In addition, nAChRs protect lung cancer cells against cell death induced by chemotherapeutic drugs, ionizing radiation, EGFR-tyrosine kinase inhibitors (EGFR-TKI) and other extracellular stress signals (Campoy et al., 2016; Jin et al., 2004; Mai et al., 2003; Maneckjee & Minna, 1994; Mucchietto et al., 2016; Togashi et al., 2015; West et al., 2003; West et al., 2004; Wright, Zhong, Zheng, & Larrick, 1993; J. Xu et al., 2007; Zeidler et al., 2007; T. Zhang et al., 2006). The reviews (mentioned above) comprehensively discuss the role of nAChRs in lung cancer progression and therapy. We will chronicle the most recent

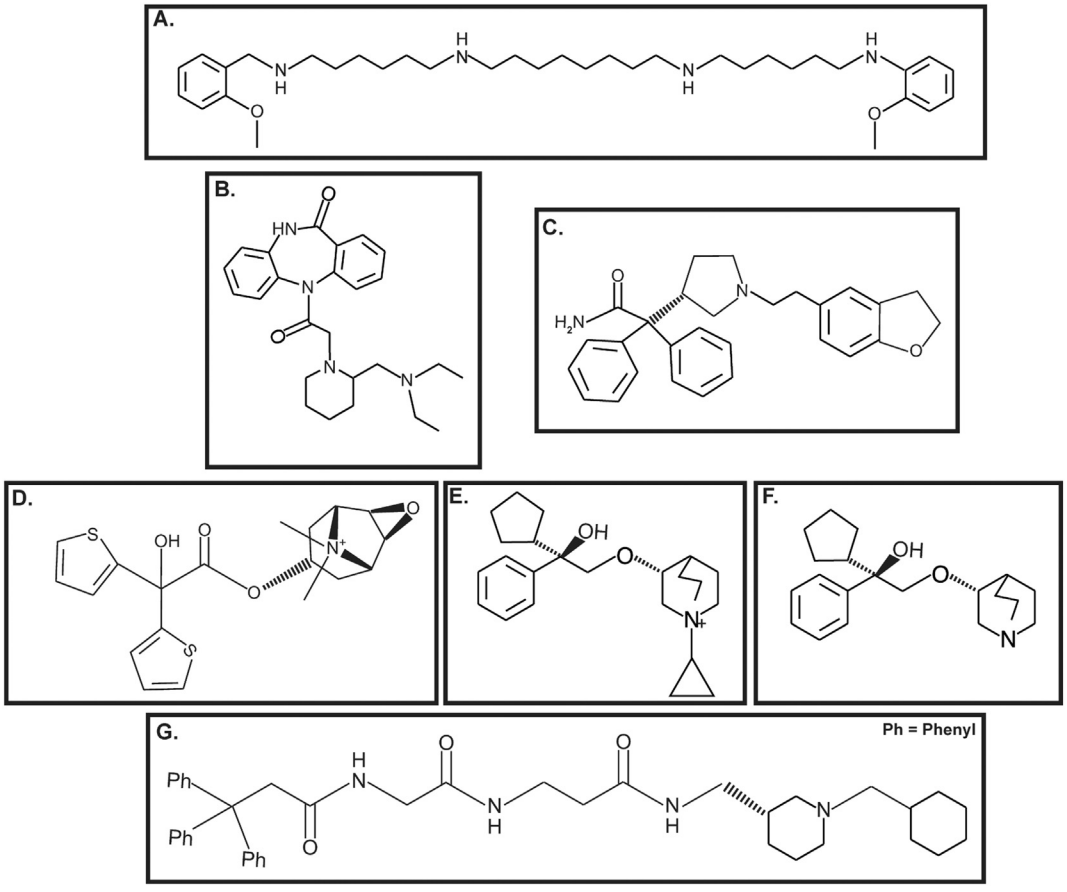

Fig. 9. Structure of muscarinic receptor antagonists analyzed for their growth-inhibitory activity in human lung cancer A. Methoctramine B. AFDX-116 C. Darifenacin D. Tiotropium E. R2HBJJ F. R2-PHC G. J-115311.

developments in the field of nAChR-biology (over the past three years) in this review.

The nAChRs are ligand-gated ion channel receptors, with five subunit proteins tightly wrapped around a central ion pore (Barman et al., 2016; Gotti & Clementi, 2004). These pentameric proteins are comprised of α and β subunits (Egleton et al., 2008; Egleton, Brown, & Dasgupta, 2009; Gotti & Clementi, 2004). The α-subunit contains the binding site for ACh (Lindstrom, 1996, 1997). Non-neuronal nAChRs are broadly classified into two categories, heteromeric and homomeric nAChRs. Heteromeric nAChRs contain a combination of α and β subunits (Dang et al., 2016; Improgo et al., 2013; Improgo, Tapper, & Gardner, 2011; Mucchietto et al., 2016; Zhao, 2016; Zoli et al., 2018). Homomeric nAChRs are composed of five α subunits (Gotti & Clementi, 2004; Lindstrom, 1996, 1997). There are many isoforms of α (α1-10) and β (β1-4) subunits. Examples of homomeric nAChRs are α7-nAChR, α6-nAChR and α9-nAChR (Gotti & Clementi, 2004). Apart from ACh, physiological modulators of nAChRs include lynx1, lynx2, SLURP-1 and SLURP-2 (section 6; Chernyavsky, Arredondo, Galitovskiy, Qian, & Grando, 2010; Durek et al., 2017; Fu, Rekow, & Spindel, 2012; Ibanez-Tallon et al., 2002; Lyukmanova et al., 2018; Lyukmanova et al., 2016a; Miwa et al., 1999; O'Neill et al., 2012; Sekhon, Song, Jia, Lindstrom, & Spindel, 2005; Tekinay et al., 2009). The nAChRs are ubiquitously expressed in all tissues of the lung, even in fetal lung tissue (Sekhon et al., 2005). Such observations underscore the importance of nAChRs in lung development and homeostasis (Mucchietto et al., 2016; Schuller, 2012; Song & Spindel, 2008; Spindel, 2016; Zoli et al., 2018).

GWAS studies have indicated that SNPs in the α5-α3-β4 nAChR cluster (CHRNA5-CHRNA3-CHRNB4) located on chromosome 15q25 region confers risk for lung cancer in European populations who are heavy smokers (Amos et al., 2008; Hung et al., 2008; Spitz, Amos, Dong, Lin, & Wu, 2008; Thorgeirsson et al., 2008a). Genetic variations in the 15q25 chromosome (in smokers) are associated with increased risk of death from lung cancer, COPD and tobacco-related cancers (Hallden et al., 2016; Nedeljkovic et al., 2018; Qu et al., 2016; Saccone et al., 2010). Polymorphisms in CHRNA3 and CHRNA5 are also associated with familial lung cancer (Byun et al., 2018). Other genomic variances near CHRNA2 gene were correlated to increased overall risk for developing lung cancer (McKay et al., 2017). It must be remembered that the occurrence of these SNPs is dependent of race and ethnicity of the population selected for the study (Luo et al., 2008). Studies in Chinese and African-American populations have implicated different SNPs of CHRNA3 and duplicated CHRNB4 as risk factors for lung cancers (Amos et al., 2010; Hansen et al., 2010; Zhang et al., 2016b; Zhou et al., 2015). Studies in European populations of heavy smokers have shown that the rs1051370 variant of CHRNA3 correlates with larger tumor size in SCC-L patients (X. Chen et al., 2011; Ware, van den Bree, & Munafo, 2012). The rs16969968 genotype of CHRNA5 may comprise of a G to A (G-A), G to G (G-G) or A to A (A-A) missense variant in the CHRNA5 gene. The G-A variant translates to a substitution of aspartic acid 398 to asparagine (Lassi et al., 2016; Pandey et al., 2017; Russo et al., 2011; Wen et al., 2016). This is also known as the D398N form of CHRNA5, which displays a reduced response to the nAChR agonist epibatidine (Bierut et al., 2008; Hung et al., 2008; Kuryatov, Berrettini, & Lindstrom, 2011). The rs16969968 genotype of CHRNA5 containing the A-A variant is considered to be a high-risk variant for the development of lung cancer. In contrast, the CHRNA5 rs16969968 genotype containing G-G variant is a low-risk variant for lung cancer (Bierut et al., 2008; Kuryatov et al., 2011). Epidemiological studies in European populations of active heavy smokers show that the high risk variant (A-A; rs1696996 CHRNA5) displayed a strong association with lung cancer risk, developing lung cancer four years earlier than individuals having the low risk variant (G-G; rs16969968 CHRNA5; L. S. Chen et al., 2016; L. S. Chen et al., 2015; Hall, 2016).

Recent studies have focused on the role of α5-nAChR in the development and progression of lung cancer. Conventionally, the α7-nAChR is thought to mediate the proliferative, pro-angiogenic and pro-metastatic activity of nicotine in lung cancer (C Heeschen et al., 2001; C. Heeschen et al., 2002; Pillai & Chellappan, 2012; Schuller, 2012; S. Wang & Hu, 2018; Zhang, et al., 2016a). The role of α7-nAChR is further reinforced by the anti-tumor activity of α7-nAChR antagonists in multiple experimental models of lung cancer (K. C. Brown et al., 2012; Mucchietto et al., 2018; S. Wang & Hu, 2018). Zhang et al., (2017) have shown that tobacco compounds like nicotine increase the levels of α5-nAChR in A549 human NSCLC cells (Zhang et al., 2017b). Nicotine-induced elevation of α5-nAChR coincided with increased levels of phopho-STAT3 and recruitment of the JAK-STAT pathway (Zhang et al., 2017b). Chromatin immunoprecipitation experiments confirmed the presence of STAT binding sites and STAT-regulatory elements on the α5-nAChR promoter (Zhang et al., 2017b). The α5-nAChR receptor potently influences addiction, cigarette consumption and nicotine dependence (Berrettini & Doyle, 2012; Bierut et al., 2008; Kuryatov et al., 2011; Lassi et al., 2016; Spitz et al., 2008; Thorgeirsson et al., 2008b). These factors have to be taken into consideration when translating basic research findings to patient-oriented studies involving the role of α5-nAChR in lung cancer.

A recent study mapped the transcriptome of A549 cells that were transfected with or without α5-nAChR-siRNA (H. J. Sun, Jia, & Ma, 2017). The gene expression profile revealed that α5-nAChR modulates cell cycle genes, DNA replication genes, oncogenes and the p53 signaling pathway. Out of all of the signaling pathways, genes involved in cell cycle progression (cyclin D1, E2 and D3) were most significantly down-regulated by α5-nAChR-siRNA (H. J. Sun et al., 2017). The treatment of α5-nAChR-siRNA transfected A549 human NSCLC cells with nicotine caused a robust decrease in S-phase entry. Notably, the depletion of α5-nAChR also suppressed basal cell cycle progression in A549 cells. Flow cytometry experiments revealed that α5-nAChR-siRNA triggered apoptosis in both untreated and nicotine-treated A549 cells. Such observations argue for a role for α5-nAChR in cell cycle progression and apoptosis (H. J. Sun et al., 2017). Sun et al., (2015) showed that nicotine-induced migration and invasion of A549 human NSCLC cells was mediated by α5-nAChR (H. Sun & Ma, 2015). The ablation of α5-nAChR by siRNA techniques abrogated nicotine-induced invasion and migration of A549 cells. The transfection of α5-nAChR-siRNA led to an increase in the cell adhesion protein E-cadherin (H. Sun & Ma, 2015). The decrease of E-cadherin is a vital event in the EMT of lung cancer cells, which endows the cells with a migratory phenotype, allowing them to invade surrounding blood vessels and eventually metastasize to distant sites (Gheldof & Berx, 2013; Wong, Fang, Chuah, Leong, & Ngai, 2018). This is in alignment with studies of athymic mouse models demonstrating a role for α5-nAChR in the progression of NSCLCs. A549 human NSCLC cells transfected with α5-nAChR-siRNA were subcutaneously implanted in the flank of athymic mice. The presence of α5-nAChR-siRNA decreased the tumor growth rates of both nicotine-treated and untreated A549 tumors (H. J. Sun et al., 2017).

An analysis of α5-nAChR expression in NSCLC tumors isolated from patients reveal that α5-nAChR expression in tumor tissue is greater than adjacent matched normal tissue. Zhang et al., (2017) analyzed the levels of α5-nAChR in 130 LAC specimens. They observed that 60% of the tumors showed elevated levels of α5-nAChR and about 67% contained phosphorylated STAT3 (Y. Zhang, et al., 2017). Statistical analysis showed an association between α5-nAChR and phopho-STAT3 expression. The expression of α5-nAChR in LAC tumors isolated from patients showed a positive correlation with smoking status, STAT3 expression and decreased survival times in LAC patients (Y. Zhang, et al., 2017). Emerging data show that α5-nAChR accelerates the development of lung cancer by indirect mechanisms. The indirect mechanisms by which α5-nAChR facilitates the development of lung cancers are thought to be behavioral in nature. The α5-nAChR mediates tobacco addiction, deeper inhalation of cigarettes and increased intake of nicotine (Berrettini & Doyle, 2012; Brunzell, Stafford, & Dixon, 2015; L. S. Chen et al., 2015; Kuryatov et al., 2011; Lassi et al., 2016).

Table 10
Expression of muscarinic receptor subtypes in lung cancer tissue and adjacent normal tissue (isolated from patients)

Nature of cells	Nature of muscarinic receptor					Reference
	M1R	M2R	M3R	M4R	M5R	
Normal lung tissue		+	+	+		Lin et al., 2014 Song et al., 2008 Wu et al., 2013
Lung tissue (metaplasia/dysplasia)			+			Wu et al., 2013
Small cell lung carcinoma tissue			+			Song et al., 2003b Spindel, 2012
Squamous cell lung cancer tissue		+	+	+		Lin et al., 2014 Song et al., 2008 Spindel, 2012
Lung adenocarcinoma tissue			+			Lin et al., 2014 Song et al., 2007 Spindel, 2012 Wu et al., 2013

Blanks indicate Not Determined (ND).

All of these factors translate to the development of aggressive lung tumors, reduced response to chemotherapy and poorer survival rates in lung cancer patients who are smokers.

There are no known synthetic antagonists to α5-nAChR. Polymorphisms in the α5-α3-β4 nAChR cluster are associated with both nicotine dependence and lung cancer risk (Amos et al., 2010; Hung et al., 2008; Thorgeirsson et al., 2008b). However, it is not known how these three subunits regulate each other in the brain or in the lung. The discovery of synthetic molecules specific for α5-nAChR may shed light on how this receptor regulates other nAChR subunits. An interaction between the α5-nAChR and α7-nAChR-signaling pathways has already been reported in normal and malignant lung cells. With this background, Ray et al. (2017) used a directed compound library of 275 nAChR antagonists to identify compounds which would display selectivity for α5-nAChR over α3-nAChR and β2-nAChRs. In addition, they screened for compounds which would differentially bind to α5- and α5-D398N-nAChR (C. Ray et al., 2017). These landmark studies are the first to report of small molecule antagonists which selectively bind to α5-nAChR. They identified three compounds AK-968/12117231, AN-038/15563010 and AE-641/30177001 (as named by the authors; **Fig. 10A-C**) display differential binding to α5- and α5-D398N-nAChRs. Furthermore, the compounds AE-641/30177001 and AK-968/40218701 (**Fig. 10C-D**) distinguish between α3β4- and α3β4α5-D398N-nAChRs (C. Ray et al., 2017). It would be interesting to determine if these compounds display any anti-cancer activity in human lung cancers.

The role of α7-nAChR in non-neuronal systems has been predominantly studied using tobacco components like nicotine and NNK (Schuller, 2007; Schuller et al., 2000; Schuller et al., 2003; Schuller & Orloff, 1998; Zoli et al., 2018). Studies with genetically modified mice reveal that α7-nAChRs play a vital role in susceptibility and onset of lung diseases (like cancer and fibrosis) upon chronic exposure to cigarette smoke (Gahring, Myers, Dunn, Weiss, & Rogers, 2017). The binding of nicotine to α7-nAChR leads to the recruitment of the adapter protein β-arrestin, which in turn induces the activation of Src kinase. The activation of Src kinase triggers a mitogenic signaling cascade comprising of the Rb-Raf pathway, PI-3 kinase/Akt and the MAP kinase pathways which ultimately causes cell proliferation (Dasgupta & Chellappan, 2006; Dasgupta, Rastogi, et al., 2006; C Heeschen et al., 2001; C. Heeschen et al., 2002; Mucchietto et al., 2018; Schuller et al., 2000; West et al., 2003; Zhang et al., 2017a; Zheng, Ritzenthaler, Roman, & Han, 2007). Apart from cytoplasmic mitogenic signaling pathways, chronic nicotine and NNK exposure elevates the levels of α7-nAChR (Al-Wadei, Al-Wadei, Masi, & Schuller, 2010; Schaal & Chellappan, 2016). A similar upregulation of α7-nAChR is observed when electronic cigarette extracts are used instead of nicotine (Alasmari et al., 2017; Schaal & Chellappan, 2016). Unlike neuronal cells, nicotine-induced upregulation of α7-nAChR is a transcriptional event. The α7-nAChR promoter is controlled by many regulatory elements and transcription factors. Recent studies show that the transcription factor E2F1 activates α7-nAChR transcription, whereas STAT1 represses the promoter (Schaal & Chellappan, 2016). The activation of α7-nAChR transcription by E2F1 recruits a complex signaling network, such as the Src, MEK, PI-3 kinase/Akt and CDK4/6 which in turn form an autoregulatory feed-forward loop to further increase the expression of α7-nAChRs (Schaal & Chellappan, 2016).

The α7-nAChR-signaling network intermingles with other growth factor and nicotinic receptor-signaling pathways (Krais et al., 2011; Mucchietto et al., 2018). Chernyavsky, Shchepotin, Galitovkiy, & Grando (2015a) showed that nAChRs synergize with growth factors like EGF and IGF-1 to elevate the proliferation of BEP2D BECs. The synergistic effect of nicotine (used as a nAChR agonist) and EGF was reversed by α-bungarotoxin indicating that the combinatorial mitogenic effects of nicotine and EGF required α7-nAChR function. These experiments were repeated in NNK-transformed BEP2D BECs and similar results were obtained (Chernyavsky, Shchepotin, & Grando, 2015b). Fan & Wang (2017) have shown an interaction between the nicotinic receptor and the prostanoid receptor signaling pathway. The prostanoid receptor EP4 is overexpressed in human lung cancer cells relative to normal lung cells (Fan & Wang, 2017). The α7-nAChR was found to play a vital role in nicotine-induced upregulation of EP4 expression and proliferation of human LAC cells. Nicotine-induced proliferation of A549 and H1838 human LAC cells was mediated by α7-nAChR-induced activation of PI-3 kinase, JNK and protein kinase C pathways. Nicotine-induced EP4 expression was found to be a transcriptional event and correlates with the decreased binding of AP2α on the EP4 promoter (Fan & Wang, 2017).

Bordas et al. (2017) examined the α7-nAChR expression of 40 SCC-L tissues, 38 LAC tissues and adjacent normal tissues isolated from patients. All tumors showed dysregulation of the 15q25 chromosomal locus, which contains the CHRNA3, CHRNA5 and CHRNAB4 genes. Both SCC-Ls and LACs showed decreased CHRFAM7A (dupa α7-nAChR subunit) expression relative to normal tissue (Bordas et al., 2017; Gault et al., 1998). The dupa α7-nAChR subunit negatively regulates α7-nAChR activity in *Xenopus* oocytes (de Lucas-Cerrillo et al., 2011). However, SCC-Ls expressed higher levels of α3-, α5-, α7-, α9-, β2- and β4-nAChR mRNA relative to adjacent normal tissue. The levels of dupa α7-nAChR mRNA was decreased in SCC-L tumors relative to adjacent normal tissue. LAC tumors showed elevated expression of α3-, α5-, α7-, and β4-nAChR mRNA, compared to adjacent normal tissue. Expression analysis revealed α4-nAChR and dupa α7-nAChR mRNA was decreased in LAC tumors when compared to normal lung tissue. The ratio of α7-nAChR/dupa α7-nAChR mRNA was higher for SCC-L than LACs tissue (Bordas et al., 2017).

Bordas et al. (2017) also compared the expression of nAChR mRNA in 35 SCC-Ls tumors originating from never smokers and non-smokers exposed to second hand smoke versus SCC-L patients who were active smokers. They found that increased expression of α5- and α7-nAChRs correlated with a poor prognosis and decreased survival in SCC-L patients. No trends were found in the human LAC tumor samples (Bordas et al., 2017). Clinical studies show that SCC-L shows a stronger correlation with smoking than LACs (Gandara, Hammerman, Sos, Lara, & Hirsch, 2015; Heist, Sequist, & Engelman, 2012). Such genetic studies may provide novel insights into the differential correlation of smoking with histological subtypes of lung cancers.

Recent studies have revealed that functional nAChRs are not only localized on the outer cell plasma membrane but are also expressed on outer mitochondrial membranes of lung tissues (Gergalova et al., 2012; Gergalova et al., 2014; S. A. Grando, Kawashima, Kirkpatrick, Kummer, & Wessler, 2015; Kalashnyk, Gergalova, Komisarenko, & Skok, 2012; Skok et al., 2016). Experiments involving C57BL/6J mice show that α3-, α4-, α7-, β2- and β4-nAChRs are robustly expressed

Table 11
Anti-cancer drugs targeting muscarinic receptor subtypes in lung cancer

Name of drug	Specificity	Cell line	Activity	Model	References
Pirenzepine	M1R	A549	Inhibition of TGF-β1 and carbachol-induced EMT	Cell culture	Hua et al., 2012 Yang et al., 2014
Methoctramine	M2R	A549, PC-9	Inhibition of EMT, invasion, migration	Cell culture athymic mice	Zhao et al., 2015a Zhao et al., 2015b
Darifenacin	M3R	H520, H82	Inhibition of cell growth	Cell culture athymic mice	Ami et al., 2011 Spindel, 2012, Song et al., 2008 Song et al., 2007
Darifenacin	M3R	H1299	Inhibition of cell growth	Cell culture	Hua et al., 2012
4-DAMP	M3R	H82, H1694, SBC3	Inhibition of proliferation, viability, angiogenesis and carbachol-induced EMT	Cell culture, athymic mice	Caihong and Shuxiang, 2017 Song et al., 2007 Spindel, 2016 Yang et al., 2014 Zhang et al., 2017
Tiotropium	M3R	H520	Inhibition of cell growth	Cell culture, athymic mice	Song et al., 2009 Song et al., 2010b
R2BHJJ and R2PHC	M3R	A549, H1299, H157, H460	Inhibition of viability, cell cycle progression	Cell culture	Hua et al., 2012
AFDX-116	M2R/M4R	H1299	Inhibition of viability	Cell culture	Hua et al., 2012
P-F-HHSiD	M3R	H82	Inhibition of viability	Cell culture	Song et al., 2007
J-115311	M3R	H82	Inhibition of viability, cell growth	Cell culture, orthotopic mouse model	Ami et al., 2011

on the outer mitochondrial membranes of the lungs of these mice (Lykhmus et al., 2014). Chernyavsky, Shchepotin, Galitovkiy, and Grando (2015a) compared the tumor promoting activities of cell membrane-associated nAChRs (cm-nAChRs) and mitochondrial-nAChRs (mt-nAChRs) in the human SCC-L cell line SW900. They observed that the treatment of SW900 cells with a combination of nicotine and growth factors, namely VEGF and EGF, resulted in a synergistic increase in cell proliferation relative to SW900 cells treated with either agent alone. The combinatorial proliferative effects of nicotine and VEGF or EGF were correlated with concomitant increase in the functional activity of cyclin D1 and ERK1/2. Subsequently, the authors analyzed the molecular mechanisms underlying the synergistic activity of nicotine and the abovementioned growth factors in SW900 human SCC-L cells. Co-immunoprecipitation western blotting experiments showed that the activation of (cm)-nAChRs (by ACh or nicotine) induces their direct binding with VEGFR and EGFR via the α7- and β2-(cm)-nAChRs on SW900 human lung cancer cells (Chernyavsky, Shchepotin, & Grando, 2015b). The (mt)-nAChRs were primarily found to be responsible for the ability of nicotine to protect SW900 human SCC-Ls against apoptotic agents like hydrogen peroxide and staurosporine. The mt-nAChRs mediated the anti-apoptotic effects of nicotine via regulation of the of the mitochondria permeability transition pore (mPTP), which required the functional activity of both α7-(mt) and non-α7-(mt)-nAChRs. Most interestingly, the ACh-induced activation of (mt)-nAChRs induced the association of (mt)-nAChRs with Src and PI-3 intramitochondrial kinases (Chernyavsky, Shchepotin, & Grando, 2015b).

The same research group performed a parallel study where they showed that nicotine displayed synergistic growth-stimulatory activity with EGF in normal BECs, BEP2D immortalized BECs, NNK-transformed BEP2D cells and SW900 human SCC-L cells via the activation of α7- and α9-(cm)-nAChRs. Similarly, the synergistic proliferative effect of nicotine and insulin growth factor (IGF) was observed in BEP2D cells, NNK-transformed BEP2D cells and SW900 human SCC-L cells via α4- and α9-(cm)-nAChRs. The authors noted that nicotine and VEGF only displayed synergistic growth-promoting activity in SW900 human SCC-L cells and this required the α4- and α9-(cm)-nAChRs (Chernyavsky, Shchepotin, & Grando, 2015b). An interesting data obtained in this research paper was that the treatment of BEP2D BECs with the tobacco carcinogen NNK altered the gamut of (mt)-nAChRs on these cells. Furthermore, NNK-induced malignant transformation (of BEP2D cells) increased the magnitude of (mt)-nAChRs coupled to inhibition of mPTP activity, indicating that NNK treatment could elevate the anti-apoptotic effects of (mt)-nAChRs. Therefore, agents which can lower (mt)-nAChR-induced inhibition of mPTP activity could revive mitochondrial apoptotic pathways and arrest the progression of human lung cancers (Chernyavsky, Shchepotin, Galitovkiy, & Grando, 2015a).

Innovative studies by Schaal and Chellappan (2016) show that the overexpression of α5- and α3-nAChR by all histological types of NSCLC in men (who were smokers) correlated with decreased survival probability. On the other hand, the overexpression of α7-nAChR in NSCLC correlated with increased survival probability (Schaal & Chellappan, 2016). Kaplan-Meir plots from their study demonstrate that the survival probability of NSCLC patients whose tumors contained high levels of α7-nAChR mRNA was greater than NSCLC patients who expressed low levels of α7-nAChR mRNA in their tumors. This is indeed a surprising observation because a plethora of research papers have indicated that the α7-nAChR plays a pivotal role in accelerating the growth, angiogenesis and distant metastasis of human NSCLCs. Although, further research is required to fully explain the findings of Schaal and Chellappan (2016), it is probable that there are multiple cellular events underlying their observations. A somewhat analogous result was also obtained by Medjber et al. (2015), who studied the differential subcellular localization of nAChRs in primary cultures established from NSCLC tumors isolated from patients. They observed that α5-, α7-, β2- and β4-nAChR subunits were expressed by all LAC tumors and SCC-L tumors. These nAChR subunits were localized in the glandular structures of human LAC cells. Most interestingly α5-, β2- and β4-nAChR subunit were expressed on the invasive front of human SCC-L tumors, (Medjber et al., 2015). The unexpected result was that α7-nAChR was not expressed at the invasive front of these tumors.

As discussed in their elegant study, Schaal and Chellappan (2016) suggest that it may be possible that human NSCLC tumors express high magnitude of the α7-nicotinic receptors at early phases of tumorigenesis (enabling the tumor to grow rapidly, acquire angiogenic phenotype and propensity for metastasis) and that the expression of α7-nAChRs decline as the tumor progresses to an advanced stage, where other signaling pathways take over and control tumor growth (Schaal & Chellappan, 2016). This idea is supported (at least, in part) by the studies of Medjeber et al., (2015) who observed that well differentiated human SCC-L tumors express higher levels of α7-nAChR, relative to poorly differentiated SCC-L (Medjber et al., 2015). Conventionally, welldifferentiated SCC-Ls tend to be slow-growing and associated with a relatively good prognosis. On the other hand, poorly differentiated SCC-Ls are the very aggressive tumors, associated with dismal prognosis (Doroshow & Herbst, 2018; Herbst et al., 2018). These results lend support to the hypothesis of Schaal and Chellappan (2016) that the expression/functional activity of α7-nAChR may decline as the tumor

progresses to advanced stages. A reason for the decline in the functional activity of α7-nAChR in NSCLCs could be due to receptor desensitization which would attenuate its tumor-promoting ability (Schaal & Chellappan, 2016). Support for their hypothesis comes from the experiments of Medjeber et al., (2015) who observed an inverse correlation between the levels of the cell proliferation marker Ki-67 and α7-nAChR expression in the poorly differentiated NSLC tumors, (isolated from patients) used in their experiments (Medjber et al., 2015).

Apart from α7-nAChR desensitization, variations in the subcellular localization of α7- and other nAChR subtypes (cell membrane versus mitochondrial membrane) could possibly contribute to increase the survival of human NSCLCs patients. The mitochondrial (mt)-nAChRs mediate the pro-survival functions of nAChRs in human lung cancer cells. Several congruent studies reveal that the pro-survival activity of (mt)-nAChRs are mediated by a combination α7-(mt) and non α7-(α3-, β2- and β4-)-nAChRs (mt). The upregulation of α7-nAChR may be accompanied by concomitant decrease in mitochondrial membrane bound α3-, β2- and β4-nAChRs, which would enable the NSCLC tumors to respond better to chemotherapy and radiation.

Clinical studies show that the acquisition of chemoresistance is primarily responsible for the dismal survival rates observed in NSCLC patients. NSCLC patients initially respond well to chemotherapy; however, they inevitably relapse and subsequently the NSCLC tumors become unresponsive to chemotherapeutic drugs and radiation (Doroshow & Herbst, 2018; Herbst et al., 2018). The α3/β2-nAChR subunits (not α7-nAChR) confer resistance on human NSCLC cells against the apoptotic activity of chemotherapeutic drugs (Dasgupta & Chellappan, 2006). The data of Schaal and Chellappan (2016) show that high expression of α3-nAChR significantly correlates with decreased survival probability in all histological subtypes and variants of NSCLC. The seminal findings of Schaal and Chellappan (2016) emphasize the need for patient-oriented studies to precisely identify the role of α7-nAChR and other nAChR subtypes in the progression of human NSCLCs.

Small molecule antagonists of α7-nAChR have been extensively investigated for their anti-cancer activity in human lung cancer. The suppression of α7-nAChR expression by short-hairpin RNA (shRNA) suppresses nicotine-induced growth of H1299 human LACs in both cell culture and athymic mouse models (Zhang, Ding, et al., 2016a; Zhang, Yu, et al., 2017a). Nicotine elevated the levels of the extracellular matrix protein vimentin in H1299 human NSCLCs *in vitro* and *in vivo*. Vimentin is a vital regulator of EMT, a process which facilitates the invasion and metastasis of neoplastic cells (Kidd, Shumaker, & Ridge, 2014; C. Y. Liu, Lin, Tang, & Wang, 2015). The α7-nAChR-shRNA inhibited nicotine-induced vimentin expression via the MEK pathway in H1299 cells. The α7-nAChR is also required for nicotine-induced invasion and EMT of human NSCLC cells (Dasgupta et al., 2009; Zhang, Ding, et al., 2016a). Apart from α7- and α5-nAChR, the α9-nAChR has emerged as a novel molecular target in lung cancer therapy. Depletion of α7-nAChR or α9-nAChR by siRNA methodology ablated nicotine-induced proliferation on A549 and H1975 human LAC cells. The α9-nAChR antagonist RGIA4 (**Fig.11A**) and α7-nAChR antagonist ArIB (V11L:V16D) peptide (**Fig. 11B**) abolished nicotine-induced proliferation of nicotine-treated A549 cells (Mucchietto et al., 2016; Mucchietto et al., 2018). The design and synthesis of these compounds is detailed in Romero et al. (2017) and Whiteaker et al. (2007). The growth-inhibitory effects of RGIA4 or ArIB (V11L:V16D) peptide involved the Akt pathway. Dual α7-nAChR and α9-nAChR antagonists like α-bungarotoxin (α-BT; **Fig. 12A**), methyllycaconitine (MLA; **Fig. 12B**) and MG624 (**Fig. 12C**) displayed greater growth-suppressive activity than RGIA4 or ArIB (V11L:V16D) peptides (Mucchietto et al., 2016; Mucchietto et al., 2018). These observations seem to suggest that both of these receptors interact in a synergistic manner to mediate nicotine-induced proliferation of human LAC cells. MLA, α-BT and MG624 inhibited the activation of both ERK and Akt in A549 human LAC cells. Moreover, MG624 also induced oxidative stress in A549 cells (Mucchietto et al., 2018). Natural compounds like β-Cryptoxanthin (BCX; **Fig. 12D**) suppress α7-nAChR expression at both mRNA and protein levels in A549 human LAC and BEAS-2B immortalized human lung epithelial cells. BCX inhibited α7-nAChR-induced proliferative and pro-survival pathways like PI-3 kinase/Akt pathway, ERK pathway and its downstream effectors (Iskandar et al., 2016). Furthermore, BCX also suppressed cell migration, actin remodeling and lamellipodia formation in immortalized human lung epithelial cells. The anti-migratory effects of BCX were mediated by the MMP-2 pathway (Iskandar et al., 2016).

Radiolabeled nicotinic receptor ligands have been extensively used to visualize multiple regions of the brain (Lotfipour, Mandelkern, & Brody, 2011; Rotering et al., 2014). The potential of nAChR ligands for imaging lung tumors was examined using the radiolabeled α4-nAChR ligand ^{18}F-Nifene (**Fig. 13A**) in an A/J mice NNK lung carcinogenesis model (Galitovskiy et al., 2013). Western blot analysis and immunofluorescence experiments of the excised tumors revealed that the tumors nodules of NNK-treated A/J mice displayed higher expression of α4-nAChRs than the lungs of tumor-free A/J mice. Subsequently, the authors performed positron emission tomography (PET)/CT scans using ^{18}F-Nifene over a period of eight months after NNK administration. The uptake of ^{18}F-Nifene in the lungs of NNK-treated A/J mice (which developed lung tumors) was higher than the uptake of ^{18}F-Nifene in lungs of untreated A/J mice (Galitovskiy et al., 2013). Furthermore, ^{18}F-Nifene demonstrated a higher lung tumor to non-tumor uptake ratio than radiolabeled fluorodeoxyglucose (^{18}F-FDG), which is the conventional ligand for all PET/CT scans in the detection of lung cancers in patients. These results may pave the way for a novel application of nAChR ligands involving the detection and imaging of human lung cancers (Galitovskiy et al., 2013).

Apart from nicotine, the tobacco nitrosamine NNK is a high-affinity ligand of nAChRs. However, a subset of the tumorigenic activities of NNK is also mediated by the β-adrenergic receptor on lung cancer cells (Schuller, 2002; Schuller, Tithof, Williams, & Plummer, 1999). NNK-induced activation of insulin growth factor-1 receptor (IGF-1R) is mediated by β-adrenergic receptor pathway (Boo et al., 2016). On the other hand, NNK-induced exocytosis of insulin growth factor-2 (IGF-2) was mediated by the nAChRs via elevation of intracellular calcium in human lung epithelial cells. Calcium channel blockers like amlodipine (0.5 mg/kg bodyweight/day) and nifedipine (10 mg/kg bodyweight/day) ablated NNK-induced lung carcinogenesis in FVB mice (Boo et al., 2016).

An innovative study by Mei et al. (2018) has used the α7-nAChR ligand α-conotoxin lml (α-CT; **Fig. 13B**) as a molecular target for delivery of the chemotherapeutic drug docetaxel to α7-nAChR overexpressing human lung cancers (Mei et al., 2018). As a "proof-of-concept" the authors used the human LAC cell line A549 which robustly expresses α7-nAChR. They synthesized α-CT-poly(ethylene glycol)-(distearoyl-sn-glycero-3-phosphoethanolaminen) (PEG-DSPE) based micelles and loaded these micelles with docetaxel. The micelles were characterized and their growth inhibitory effect was measured in A549 human LAC cells by the Sulforhodamine B assay (Mei et al., 2018). α-CT-PEG-DSPE-docetaxel displayed significantly improved growth-suppressive activity than docetaxel alone in A549 cells (Mei et al., 2018). Such data indicate that α7-nAChR is not only a drug target in human lung cancers but can also be used to improve the intracellular delivery of chemotherapeutic drugs in α7-nAChR-positive lung cancers.

6. Lymphocyte Antigen 6 (Ly-6) Proteins as allosteric modulators of Nicotinic Acetylcholine Receptor (nAChR): Lynx and Secreted ly6/urokinase-type plasminogen activator receptor related peptide (SLURP)

The three-dimensional structure of the Ly-6 family of proteins resembles three finger snake venom toxins, like α-bungarotoxin (V. Tsetlin, 1999; V. I. Tsetlin, 2015). The Ly-6 protein family is comprised of six members; lynx1, lynx2, SLURP-1, SLURP-2, PSCA and

Pate-B (Fu, Song, & Spindel, 2015). All of these proteins have been found to be allosteric and orthosteric modulators of nAChRs (Arredondo, Chernyavsky, Jolkovsky, Webber, & Grando, 2006; Horiguchi et al., 2009; Hruska et al., 2009; Levitin et al., 2008; Miwa et al., 1999; Tekinay et al., 2009). Studies in pre- and post-natal monkey lungs has indicated a role for lynx1 (ly from ly-6 and nx for neurotoxin) in fetal lung development. Sekhon et al. (2005) have shown that prenatal nicotine exposure alters the development of pulmonary structures and functions in the lungs of fetal monkeys by regulation of lynx1 expression. (Sekhon et al., 2005). Furthermore, experiments in human BECs have revealed a role for lynx1 in airway physiology, mucus production, development of asthma and COPD (Fu et al., 2015; Fu, Rekow, & Spindel, 2012).

The proteins lynx1 and lynx2 are negative allosteric regulators of multiple nAChR subunits (Ibanez-Tallon et al., 2002; Miwa et al., 1999; Tekinay et al., 2009). As described in Section 5, the α7-nAChR is primarily responsible for the proliferative, pro-angiogenic and pro-metastatic activity of nicotine in lung cancer (S. A. Grando, 2008; Sergei A. Grando, 2014; Pillai & Chellappan, 2012; Schuller, 2012; Spindel, 2016; S. Wang & Hu, 2018; Zoli et al., 2018). Immunofluorescence experiments showed that lynx1 and lynx2 were expressed in the airways and co-localized with α7-nAChR (Fu et al., 2012). Several congruent studies indicate that lynx1 and lynx2 are negative allosteric regulators of α7-nAChR (Fu et al., 2012; Ibanez-Tallon et al., 2002; V. Tsetlin, Utkin, & Kasheverov, 2009). Therefore, lynx1 expression should be decreased in lung cancer relative to normal adjacent tissue. This is in alignment with data showing that human SCC-L tissue (isolated from patients) contained lower levels of lynx1 relative to adjacent normal lung tissue. The levels of lynx1 correlated with the degree of differentiation of the SCC-Ls; poorly differentiated SCC-Ls showed lower expression of lynx1 than moderately or well differentiated tumors (Fu et al., 2015; Song et al., 2008). Electrophysiology experiments suggest that the binding pocket for α7-nAChR lies in the extracellular domain of lynx1 (Lyukmanova et al., 2013). Lynx2 is also a negative modulator of α7-nAChR (Fu et al., 2015). However, no studies have addressed the potential role of lynx2 in the cell biology of lung cancer.

The effect of lynx1 on cell proliferation was studied using siRNA techniques (Fu et al., 2015). Lynx1-siRNA stimulated the proliferation of A549 human lung cancer cells. Conversely, the overexpression of lynx1 (using lentiviral vectors) suppressed the growth of A549 cells. The precise molecular mechanisms underlying the growth-inhibitory activity of lynx1 has yet to be identified. It is tempting to speculate that the growth-suppressive actions of lynx1 are mediated via inhibition of α7-nAChR (Fu et al., 2012; Fu et al., 2015). However, lynx1 also interacts with α4β2-nAChR and α6-nAChR (O'Neill et al., 2012). Studies have shown that NSCLC cells and tumors overexpress α6-nAChR (Lam et al., 2007). Similarly, CHRNA4 is one of the genes located on the 15q25 chromosomal region of the human genome, which has been shown to confer susceptibility to lung cancer in European populations (Amos et al., 2008; Hung et al., 2008; P. Liu et al., 2008; Thorgeirsson et al., 2008a). Such observations reveal that lynx1 modulates multiple nAChR subunits involved in the progression of lung cancer.

SLURP-1 is a member of Ly-6 family of proteins and functions as a positive allosteric regulator of α7-nAChR (Arredondo, Chernyavsky, Webber, & Grando, 2005; Favre et al., 2007; Lyukmanova et al., 2018). SLURP-1 is robustly expressed in the ciliated bronchial epithelium of the lungs (Horiguchi et al., 2009). SLURP-1 displays potent anti-inflammatory activity and regulates ciliary beat frequency in the airways (Narumoto et al., 2013). SLURP-1 also is profoundly downregulated in inflammatory lung diseases like asthma (Narumoto et al., 2010; Narumoto et al., 2013).

There are very few research papers which have examined the role of SLURP-1 in lung cancer. SLURP-1 suppresses NNK-induced transformation of human BECs. Kalantari-Dehagi et al., (2012) treated BEP2D immortalized human BECs with 1 μM of the tobacco carcinogen NNK in the presence or absence of 1 μg/ml recombinant SLURP-1 for 24 hours (Kalantari-Dehaghi, Parnell, Armand, Bernard, & Grando, 2015). Subsequently, they performed a PCR-array analysis for oncogenes and tumor suppressor genes. NNK upregulated the expression of the EGF, the pro-survival gene RB1, CTNBB1 gene (encoding for the EMT gene β-catenin), and oncogenes MYB, PIK3A, AKT and KIT in BEP2D cells. The expression of pro-apoptotic genes, namely Bax and caspase-8, were downregulated by NNK. The presence of recombinant SLURP-1 (along with NNK) completely or partially abrogated of the expression pattern of the above-mentioned genes (Kalantari-Dehaghi et al., 2015). The gene expression signature obtained with NNK facilitated the malignant transformation of BECs, whereas SLURP-1 had a reciprocal effect on NNK-induced gene expression (Kalantari-Dehaghi et al., 2015). Several convergent studies show that the α7-nAChR is responsible for the proliferative effects of nicotine and NNK (Sergei A. Grando, 2014; Schuller, 2012; Spindel, 2016; S. Wang & Hu, 2018; Zoli et al., 2018). Therefore, SLURP-1 (a positive allosteric modulator of α7-nAChR) should amplify NNK-induced changes in gene expression, not reverse them (Kalantari-Dehaghi et al., 2015). The authors speculate that perhaps there is a common binding site for NNK and SLURP-1 on α7-nAChR. The presence of SLURP-1 knocks out NNK from its cognate binding site on α7-nAChR, thereby abolishing the NNK-induced gene expression profile (Kalantari-Dehaghi et al., 2015; Lyukmanova, Shulepko, Kudryavtsev, et al., 2016b). Durek et al. (2017) generated synthetic SLURP-1 using solid phase peptide synthesis methodology. Synthetic SLURP-1 was found to potently inhibit the functional activity of rat and human α3β4-nAChR (and to a lesser extent α3β2- α4β4-nAChRs) at high concentrations of ACh (Durek et al., 2017). Similarly, SLURP-1 suppressed ACh-induced current traces in rat and human α9-nAChRs and α10-nAChRs (Lyukmanova, Shulepko, Kudryavtsev, et al., 2016a). However, these experiments were performed by transfecting the indicated nAChRs in *Xenopus* oocytes, not in human lung cells (Durek et al., 2017). Published data show that the S442 naturally occurring variant of α9-nAChR promotes the growth of BEP2D BECs. Most importantly, the S442 variant stimulated spontaneous as well as NNK-induced transformation of BEP2D cells (Chikova & Grando, 2011). It may be probable that the reciprocal effects of SLURP-1 on NNK-induced gene expression is mediated by its interaction with α9-nAChRs in BECs. Lyukmanova et al. (2018) observed that SLURP-1 suppresses the viability of A549 human NSCLC cells over 24 hours. WST cell viability assays reveal that SLURP-1 had a low IC_{50} (1.8± 0.7 nM) in A549 cells (Lyukmanova et al., 2018). In contrast, SLURP-2 stimulated the growth of A549 cells (1.5-fold increase compared to the untreated cells) over 24 hours (Lyukmanova et al., 2018). The role of SLURP-2 in cell proliferation is not very well understood. SLURP-2-transfected Het-1A human oral keratinocytes suppressed NNK-induced tumorigenesis (Arredondo, Chernyavsky, & Grando, 2007), whereas exogenous SLURP-2 induced proliferation of Het-1A cells (Lyukmanova, Shulepko, Kudryavtsev, et al., 2016a). Based on such observations, further studies are required to clarify the role of SLURP-2 in the cholinergic signaling pathway in human lung cancer. Studies involving transfected Chinese hamster ovary (CHO) cells show that SLURP-2 interacts with M1R and M3R (Lyukmanova, Shulepko, Kudryavtsev, et al., 2016a). It may be probable that some of the biological effects of SLURP-2 are mediated its interaction with muscarinic receptors on target cells. SLURP-2 interacts with several nAChRs and muscarinic receptor subtypes, so it is probable that such oligomeric complexes are responsible for the spectrum of downstream signaling pathways in neoplastic cells.

Swamynathan et al. (2015) observed that the treatment of HUVECs with exogenous histidine-tagged SLURP-1 suppressed TNF-α-induced angiogenic tubule formation in Matrigel models (Swamynathan et al., 2015). Similarly, SLURP-1 decreased the adhesion of HUVECs to a panel of extracellular matrix proteins namely collagen1, collagen4, vitronectin and fibronectin. Although, these studies were performed in the context of corneal angiogenesis, they may have potential applications in the angiogenesis of lung cancers (Swamynathan et al., 2015).

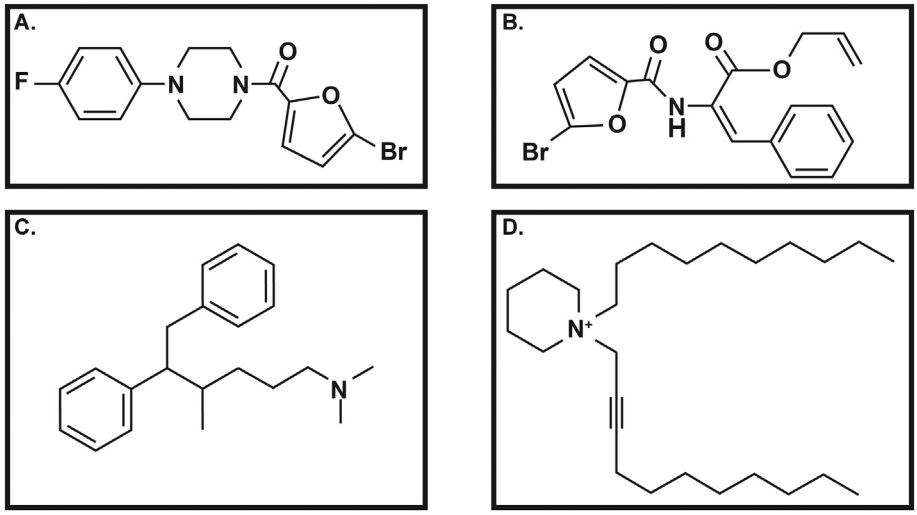

Fig. 10. Structures of nicotinic receptor antagonists selective for α5-nAChR and α5-D398N-nAChR. A. AK-968/12117231: 1-(5-bromo-2-furoyl)-4-(4-fluorophenyl)piperazine B. AN-038/15563010: allyl 2-[(5-bromo-2-furoyl)amino]-3-phenylacrylate C. AE-641/30177001: N,N,4-trimethyl-5,6-diphenyl-1-hexanamine D. AK-968/40218701: 1-decyl-1-(2-undecynyl) piperidium.

7. Cholinesterases (ChEs)

A survey of literature shows that vertebrates express two types of ChEs (Barman et al., 2016; Pohanka, 2011). The first is AChE (also called acetylcholineacetylhydrolase) and the second is BChE (also called acylcholine acylhydrolase; Barman et al., 2016; Colovic, Krstic, Lazarevic-Pasti, Bondzic, & Vasic, 2013; Soreq & Seidman, 2001). Classically, the central function of AChE is the hydrolysis of ACh, terminating cholinergic signaling (Barman et al., 2016). The substrate for AChE is A-Ch. The identification of ChEs in non-neuronal tissue, blood and body fluids indicates that these proteins have cellular functions which are independent of its ACh-hydrolytic activity (Jiang & Zhang, 2008; Pickett, Dush, & Nascone-Yoder, 2017; Pohanka, 2011; Soreq & Seidman, 2001; Xi et al., 2015). HUVECs express enzymatically active AChE (Carvalho, Graca, Martins-Silva, & Saldanha, 2005). The expression of AChE in HUVECs is higher than other cholinesterases. The functions of endothelial AChE are yet to be fully understood. Yeast two-hybrid experiments have revealed that AChE interacts with extracellular matrix protein like laminin and heparin, suggesting a role for this protein in cell-cell recognition and transmembrane-receptor-mediated cytoplasmic signaling pathways (Paraoanu & Layer, 2008).

The presence of multiple AChE-isoforms supports the notion that these proteins have functions independent of their role in the cholinergic signaling pathway (Zimmermann, 2013). These isoforms are generated by alternate mRNA splicing, post-translational modifications, assembly of catalytic domains and the binding of non-catalytic structural domains (Lockridge, Norgren, Johnson, & Blake, 2016; Nazim et al., 2017; Soreq & Seidman, 2001). We will be discussing the isoforms AChE-T (tailed), AChE-R (readthrough) and AChE-S (synaptic) in this review because they have been studied in human lung cancers. Three alternative exons 4, 5, 6 (E4, E5 and E6) constitute the C-terminal motif of AChE subunits. AChE-S is a tetrameric protein containing a C-terminal collagen tail or a hydrophobic domain which enables it to anchor to the membrane (Meshorer et al., 2004; Meshorer & Soreq, 2006; Taylor & Radic, 1994). AChE-T is generated by alternate splicing of E4 to E6. The AChE-E (erythrocyte) is a dimeric protein that is produced by joining E4 to E5. It is also called AChE-H (hydrophobic) and contains a glycosylphosphoinositol (GPI)-anchor (Paraoanu & Layer, 2008). Additional alternative splicing leads to the generation of the monomeric AChE-R protein. The effect of AChE in cellular survival is varied in an isoform-dependent manner (Meshorer et al., 2004; Meshorer & Soreq, 2006). AChE-S is highly expressed in apoptotic cells and tissues (Jiang & Zhang, 2008). Overexpression of the N-terminally extended form of AChE-S (N-AChE-S) induces cell death in a diverse array of cells and tissues (Toiber et al., 2008; Toiber, Greenberg, & Soreq, 2009). In contrast, AChE-R promotes cell proliferation and confers protection against injurious stimuli (Deutsch et al., 2002; Dori & Soreq, 2006; Grisaru, Keidar, Schreiber, Lessing, & Deutsch, 2007; Jiang & Zhang, 2008; Perry, Sklan, & Soreq, 2004).

A survey of literature shows that BChE is encoded by a single gene (Allderdice et al., 1991; Arpagaus et al., 1990). It is a glycoprotein found in both the central and peripheral nervous system. The structure of BChE bears a 65% homology to AChE (Nicolet, Lockridge, Masson, Fontecilla-Camps, & Nachon, 2003). Both of these proteins contain a catalytic active site, a deep gorge and a peripheral anionic site (PAS; Brus et al., 2014; Shafferman et al., 1992). It is a pseudocholinesterase or serum cholinesterase, hydrolyzing choline, butryl- and succinylcholine and aliphatic esters (Q. Li, Yang, Chen, & Sun, 2017). Studies have indicated that BChE can replace AChE in degrading ACh, when AChE is absent or inhibited (Chatonnet & Lockridge, 1989).

The role of AChE in human lung cancers is not very well defined. Human SCLC, SCC-L and LAC cell lines express AChE (**Table 12**). Our laboratory has compared the AChE expression and activity in a panel of human SCC-L cell lines and normal lung cells. We results revealed that the expression (and activity) of AChE in normal lung cells is higher than in SCC-L cell lines (Dasgupta et al., 2016; Dasgupta et al., 2018). We also observed that treatment with nicotine decreased the levels of AChE in human LACs relative to untreated cells (Lau et al., 2013). However, Zhang et al., (2014) performed immunoblotting and found that the levels of AChE in H520 human SCC-Ls cells and H460 human LAC cells was higher than immortalized lung cell lines (B. Zhang et al., 2014). Depletion of AChE by siRNA did not affect the viability of H520 human SCC-L cells. However, the transfection of AChE-siRNA abrogated the apoptotic activity of cisplatin in human SCC-L and LAC cells (B. Zhang et al., 2014). Such observations may be explained by the fact that several anti-cancer drugs increase AChE to trigger apoptosis (Steinritz et al., 2007; Ye, Zhang, Chen, & Zhou, 2015; X. J. Zhang & Greenberg, 2012). Another fact to note was that Zhang et al., (2014) cultured cells in fetal bovine serum-containing media (B. Zhang et al., 2014). Fetal bovine serum contains substantial amounts of AChE (Ralston, Rush, Doctor, & Wolfe, 1985). Our laboratory conducted studies involving measurement of AChE expression and activity in serum-free medium containing bovine serum albumin (BSA) and insulin-transferrin-selenium supplement (as described by Song et al., (2003a)), and measured the levels of intracellular AChE (Song, Sekhon, Jia, et al., 2003). Additionally, we used primary SAEC and NHBE for our experiments. Zhang et al.

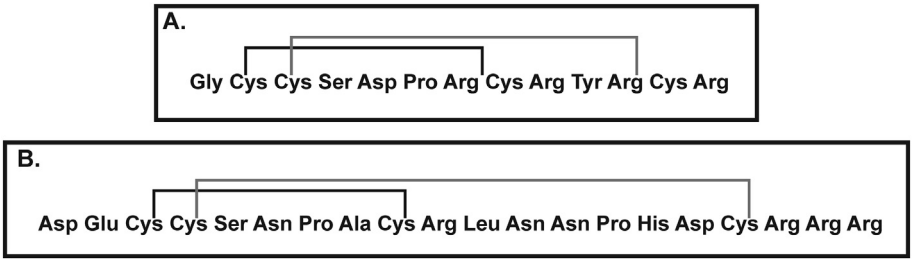

Fig. 11. Structure of nAChR subunit antagonists. A. RGIA4 B. ArIB (V11L;V16D). The disulfide bridges are formed between the cysteine residues of the peptides depicted in A and B.

(2014) used the immortalized BEAS-2B cells for their studies (B. Zhang et al., 2014). Such differences in the experimental design may at least in part, explain the divergent results obtained by different research groups.

Clinical studies in NSCLC patients show that the biological activity of AChE in human lung cancers varies according to the histological type of lung tumors (**Table 13**). Martinez-Moreno et al. (2005) compared the levels of AChE expression and functional activity in a spectrum of NSCLC and adjacent normal lung tissue isolated from patients. They found that total AChE enzyme activity in SCC-L tissue was significantly lower than adjacent normal tissue (Martinez-Moreno et al., 2005; Martinez-Moreno et al., 2006). The AChE activity in human SCC-L tissue is represented by the symbol "+", whereas the AChE activity of normal lung tissue is represented by the symbol "++" to indicate that the enzymatic activity of AChE is higher in normal lung tissue relative to human SCC-Ls (**Table 13**; rows 1-5). The AChE expression (and activity) of human SCLCs, LACs and LCCs was comparable to normal lung tissue. A similar result was obtained from bronchial aspirates of human SCC-L patients. The AChE activity of bronchial aspirates of SCC-L patients was found to be about half of that in non-cancerous patients (Martinez-Lopez de Castro et al., 2008). A similar representation schema has been used to describe the data involving bronchial aspirates of SCC-L patients versus those of normal individuals (using the symbol * in **Table 13**; rows 6-9). The data from Song et al., (2008) show that the levels of AChE in human SCC-L tissues is dependent on the extent of differentiation of the tumor (Song et al., 2008). The well differentiated SCC-Ls did not display a significant difference of AChE levels compared to adjacent normal tissue. In contrast, AChE levels in moderately to poorly differentiated SCC-Ls showed substantially lower AChE expression relative to normal lung tissue (Song et al., 2008). The role of BChE in lung cancer has been not been studied extensively. Martinez-Moreno et al. (2005) detected BChE activity in normal lung tissue, SCLC and NSCLC tissues (Martinez-Moreno et al., 2005). The activity of BChE is significantly decreased in human SCC-L, LAC and LCC (isolated from patients) relative adjacent normal non-cancerous lung tissue (Martinez-Moreno et al., 2005; Martinez-Moreno et al., 2006; Song

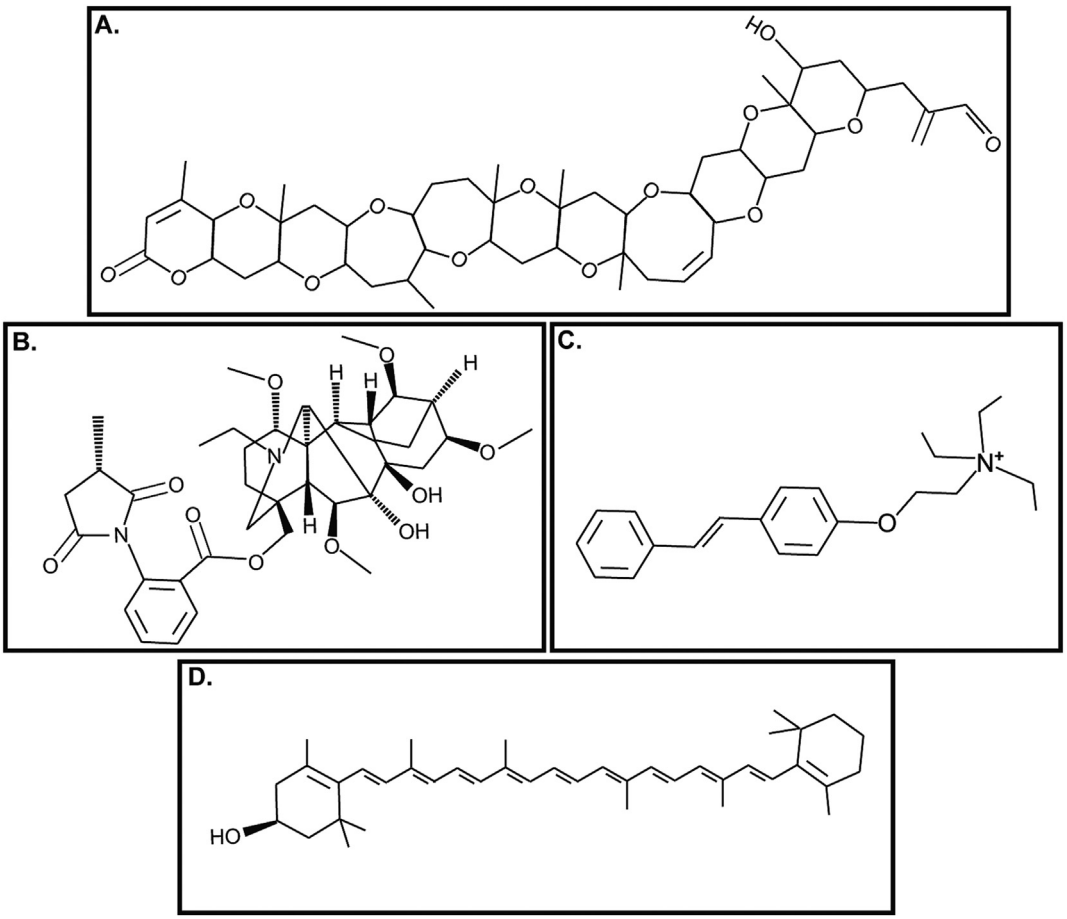

Fig. 12. Nicotinic Receptor Antagonists targeting both α7-nAChR and α9-nAChR or α7-nAChR alone. A. α-bungarotoxin B. Methyllycaconitine C. MG624 D. β-cryptoxanthine.

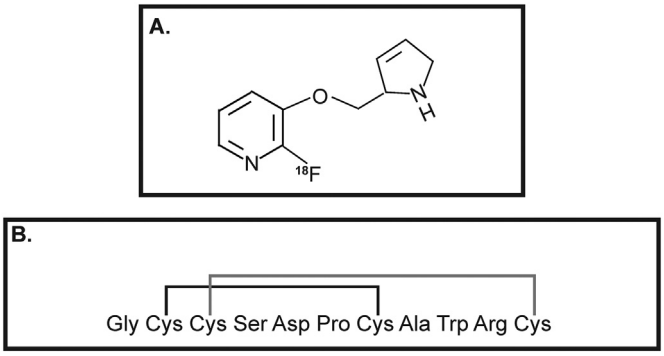

Fig. 13. Novel applications of nicotinic receptor ligands in human lung cancer. A. ^{18}F-Nifene B. α-conotoxin lml. The disulfide bridges are formed between the cysteine residues of the peptides depicted in **B.**

et al., 2008). The decrease in functional activity of AChE and BChE in human lung cancer tissue in may be a mechanism to maximize the levels of the growth factor ACh, thereby promoting the proliferation of human lung cancer cells.

Zakut et al. (1988) compared the total serum cholinesterase activity of 88 patients with 21 healthy volunteers who served as controls. Out of the 88 patients enrolled in the study, seven patients suffered from lung cancer. They found that total serum cholinesterase (sChE) activity of human lung patients was significantly ($P<0.05$) higher than normal controls (**Table 13**; Zakut et al., 1988). The total serum cholinesterase activity of lung cancer patients (denoted by xx) was higher than the cholinesterase activity of normal individuals (shown by the symbol "x" in **Table 13**, rows 10-11). An analogous study by Zanini et al. (2013) measured AChE activity in whole blood of NSCLC patients receiving gemcitabine or cisplatin-based chemotherapy (Zanini et al., 2013). For normal controls, they isolated the whole blood from healthy individuals and treated them with a range of cisplatin or gemcitabine concentrations *in vitro*. The concentration ranges of cisplatin and gemcitabine were based on pharmacokinetic data and reflected the amount of these drugs found in the serum of patients (Zanini et al., 2013). The enzymatic activity AChE in the whole blood of NSCLC patients showed a slight but significant ($P<0.05$) elevation (231 ± 12.81 μmol ACh/mg protein), relative to normal controls (191.4 ± 10.89 μmol ACh/mg protein). The levels of BChE in serum remained constant across all whole blood samples (Zanini et al., 2013).

ACh acts as a growth factor for human SCLC and NSCLCs. The regulation of AChE activity by small molecules has been investigated as a strategy for lung cancer therapy (**Table 14**). It may be envisaged that drugs which elevate the levels of AChE in human lung cancer cells, will cause increased degradation of the ACh and thereby decrease the concentration of ACh in the cellular milieu (**Fig. 14**). The downregulation of ACh production will suppress its proliferative, pro-invasive and EMT-promoting activity and block the growth and survival of human lung cancers. Lu et al. (2013) identified the synaptic microRNA-212 (miR-212) as a negative regulator of AChE expression. They also observed that miR-212 acts as a tumor suppressor in human NSCLC by targeting AChE. Furthermore, they generated AChE-S-overexpressing H520 human SCC-L cells (hereby referred to as H520-AChE cells). Experiments in athymic mouse models revealed that the growth rate of H520-AChE cells was decreased relative to mock transfected H520 tumors (L. Lu et al., 2013). This is in alignment with data from our laboratory and those of other laboratories showing that AChE expression (or activity) in normal lung tissue is greater than that of NSCLCs (Dasgupta et al., 2016; Dasgupta et al., 2018; Martinez-Lopez de Castro et al., 2008; Martinez-Moreno et al., 2005; Song et al., 2008). Athymic mice studies by Lu et al. (2013) suggest that elevation of AChE expression may suppresses the growth of human SCC-Ls (L. Lu et al., 2013). The inhibition of AChE (by eserine) stimulated the growth of rat mammary tumors (Calaf, Parra, & Garrido, 2007). Although this study was not performed in human lung cancer cells, it seems to provide the important proof-of-concept that elevation of AChE activity could attenuate cell growth and trigger apoptosis. Studies in neuronal systems have shown that the amyloid-beta peptide 1-40 fragment inhibits the release of ACh via stimulation of AChE activity (W. Hu, Gray, & Brimijoin, 2003; Kar et al., 1998). We tested the growth-inhibitory activity of amyloid beta (Aβ) peptide fragment 1-40 and Aβ peptide fragment 1-28 in a panel of human SCC-L cell lines (Dasgupta et al., 2018). We observed that both Aβ peptide fragment 1-40 and 1-28 elevated AChE activity in H520, H226 and SK-MES human SCC-L cells (Dasgupta et al., 2018). Furthermore, MTT assays revealed that these peptides suppressed the viability of aforementioned SCC-L cell lines.

An interesting observation is that AChE inhibitors have also been investigated for their growth-inhibitory activity in human lung cancer cell lines. Several natural products potently inhibit AChE activity (Patel, Raghuwanshi, Masood, Acharya, & Jain, 2018). Methanolic extracts of the medicinal plant belonging *Annonaceae* genus were tested for their ability to inhibit AChE and cell growth in five human cancer cells (Formagio et al., 2015). Eleven *Annonaceae* extracts were tested for their AChE-inhibitory/growth-suppressive activity in parent NCI-H460 and Adriamycin-resistant NCI-H460 cells. The extracts isolated from *Annonaceae coriacea* and *Annonaceae crassiflora* seeds displayed the maximal cytostatic activity in both NCI-H60 and Adriamycin-resistant NCI-H460 cell lines (Formagio et al., 2015). A panel of compounds isolated from the plant *Sonneratia ovata Backer* were tested for their ability to inhibit the enzyme activity of AChE. One of the compounds, namely Rhodolatouchol (**Fig. 15A**) suppressed AChE activity and the growth of NCI-H460 cells in cell culture models (Nguyen et al., 2015). 3-alkyl pyridium polymers (poly-APS), a class of compounds isolated from marine sponge extracts displayed potent AChE-inhibitory activity (Zovko, Specici, & Turk, 2009). Polymers comprising of 3-octylpyridinium units isolated from the marine sponge *Haliclona (reniera) sarai* displayed the greatest inhibition of AChE activity. These poly-APS compounds displayed decreased viability of A549 human NSCLC cells, as well as primary human NSCLC cell lines (isolated from NSCLC patients). These results were confirmed in colony formation assays and similar results were obtained. Poly-APS compounds induced robust apoptosis in lung cancer cells, as measured by TUNEL assays, via the activation of caspases and loss of mitochondrial membrane potential (Zovko et al., 2009). These compounds also decreased the growth rate of A549 tumors xenografted in athymic mice. These poly-APS compounds were selective towards tumor cells and displayed minimal growth-inhibitory activity in healthy lymphocytes. In addition, these compounds displayed no toxicity (or inflammation) in the liver, heart or kidney of C57BL/6N mice (Zovko et al., 2009).

Synthetic phenylcinnamide based compounds are potent inhibitors of both AChE and BChE (Saeed et al., 2014). These compounds displayed robust growth-inhibitory activity in H157 human SCC-L cells *in vitro*. Enzyme activity and molecular docking experiments demonstrate AChE-inhibitory and BChE- inhibitory activity of azomethine-dihydroquinazolinone conjugates. These compounds also displayed cytotoxic activity in H157 human lung cancer cells, as determined by Sulfarhodamine B assay (Iqbal, Saeed, Shah, al-Rashida, & Shams-ul, 2016). The 2-O-methoxy and 4-bromo derivatives of these azomethine-dihydroquinazolinone conjugates displayed the greatest selectivity for AChE over BChE. However, the growth-inhibitory activity of 2-O-methoxy-derivative was greatest (out of all the compounds tested) in H157 human lung cancer cells (Iqbal et al., 2016). The natural alkaloid dehydrocorydaline is a potent inhibitor of AChE activity (**Fig. 15B**). Dehydrocorydaline abrogated migration and invasion of H1299 human LAC cells, as measured by wound-healing assays (Lee et al., 2017). However, the caveat of these studies is that none of them have used overexpression or siRNA-based experiments to definitively prove that these compounds are mediating their growth-inhibitory activity via the AChE pathway. Natural compounds have many pleotropic biological effects apart from suppressing AChE activity. This is clearly

Table 12
Expression of AChE in normal human bronchial epithelial cells and lung cancer cell lines

Cell Line	Nature of lung cells	Expression of AChE	References
BEAS-2B	Immortalized human bronchial epithelial cells	+	Lu et al., 2013 Zhang et al., 2014
NHBE	Normal human bronchial epithelium	+	Dasgupta et al., 2018
SAEC	Small airway epithelial cells	+	Dasgupta et al., 2018
HPAEpics	Human pulmonary alveolar epithelial cells	+	Lau et al., 2013
H82, H69, H187, N417	Small cell lung cancer	+	Hua et al., 2012
H157, H520, H226, SK-MES-1	Squamous cell carcinoma of the lung	+	Dasgupta et al., 2018 Dasgupta et al., 2016 Lau et al., 2013 Hua et al., 2012
A549, H358, H650, H2228, H1975, H1563, H1650, H23, H460, H1944, H1355	Lung adenocarcinoma	+	Dasgupta et al., 2018 Dasgupta et al., 2016 Lau et al., 2013 Hua et al., 2012

exemplified by the data of Zhang et al., (2014) who identified AChE as a potential target of microRNA-132 (hsa-miR-132). Subsequently, they observed that hsa-miR-132 showed potent apoptotic activity in a panel of human NSCLC cell lines. However, the apoptotic activity of hsa-miR-132 was independent of its inhibition of AChE (B. Zhang et al., 2014).

8. Conclusions and future directions

Lung cancer is the leading cause of cancer-related deaths in the United States. The mortality due to lung cancer exceeds the combined deaths from breast, prostate and colon cancers (Dela Cruz, Tanoue, & Matthay, 2011). Recent studies have shed light on molecular events contributing to LAC, leading to the development of targeted therapies (Chan & Hughes, 2015; Rolfo et al., 2015). A major challenge with these targeted therapies is that they are minimally effective in lung cancer patients exposed to tobacco smoke. For example, targeted treatments based on EGFR and anaplastic lymphoma kinase are most effective in LAC patients who are never-smokers (Han et al., 2012; Waller, Miller, & Petty, 2010; Y. Zhang et al., 2015). Apart from LAC, the quest for molecular targets, suitable for lung cancer therapy has proved to be elusive. Anti-angiogenic therapies have proved problematic in NSCLC (especially SCC-L) due to severe pulmonary hemorrhage in patients and discontinuation of treatment (Johnson et al., 2004; Piperdi, Merla, & Perez-Soler, 2014). Similarly, clinical trials involving tyrosine kinase inhibitors like sunitinib have been associated with severe toxicity in SCLC patients (H. Lu & Jiang, 2017; Ready et al., 2015). Such sobering statistics define the arena where novel molecular targets and anti-cancer drugs are urgently required to combat this lethal malignancy.

The cholinergic pathway is functional in both SCLC and NSCLCs. Different components of the cholinergic pathway have altered functional activity between normal lung cells and lung cancer cells. Such differences have proved to the basis of investigating cholinergic ligands for treatment of lung cancers (S. A. Grando, 2008; Sergei A. Grando, 2014; Mucchietto et al., 2016; Song & Spindel, 2008; Spindel, 2012, 2016). An important concern may be that these cholinergic ligands may display unwanted adverse side effects on the brain, muscles and the peripheral nervous system. The aim of cholinergic anti-cancer therapies is not to perturb the elements of the acetylcholine-signaling pathway but to restore them to normal levels. For example, SCC-Ls display decreased AChE activity, so AChE modulators would suppress the growth of SCC-Ls by bringing the AChE levels to those observed in normal lung tissue (Martinez-Moreno et al., 2005; Martinez-Moreno et al., 2006). Moreover, studies in athymic mice models and orthotopic mice models have demonstrated that the administration of these cholinergic compounds was not associated with any adverse cognitive or behavioral side effects (Akers et al., 2017; Ami et al., 2011; Song, Olivas, et al., 2010; Song et al., 2008). An advantage with a few of these compounds like BW813U (ChAT antagonist) is that they have been extensively studied in neuronal models. Therefore, their effects on the brain and peripheral nervous system are well established. BW813U does not induce any adverse cognitive or behavioral effects in rats (Meck, 2006; Wenk et al., 1986). Similarly, muscarinic receptor antagonists are being used in the clinic to treat asthma and COPD in patients. The therapeutic potential of these drugs in lung cancer is has shown promising results in preclinical model systems (Song, Sekhon, Lu, et al., 2007; Song & Spindel, 2008; Spindel, 2012, 2016). The re-purposing of these anti-asthma (or anti-

Table 13
Expression of AChE, BChE and total ChE activity in lung cancer tissue, bronchial aspirates, serum and adjacent normal tissue (isolated from normal individuals and patients)

	Type of lung tissue	Expression of AChE	AChE Activity	BChE Expression/ Activity	Total ChE Activity	References
1.	Normal lung tissue	++	++	++		Martinez-Moreno et al., 2005 Martinez-Moreno et al., 2006 Song et al., 2008
2.	Small cell lung cancer tissue	++	++	++		Martinez-Moreno et al., 2005 Martinez-Moreno et al., 2006
3.	Squamous cell lung cancer tissue	+	+	+		Martinez-Moreno et al., 2005 Martinez-Moreno et al., 2006 Song et al., 2008
4.	Lung adenocarcinoma tissue	++	++	++		Martinez-Moreno et al., 2005 Martinez-Moreno et al., 2006
5.	Large cell lung carcinoma	++	++	++		Martinez-Moreno et al., 2005 Martinez-Moreno et al., 2006
6	Bronchial aspirates associated with normal lung tissue	**	**			Hua et al., 2012
7	Bronchial aspirates from SCLC patients	**	**			Hua et al., 2012
8	Bronchial aspirates from SCC-L patients	*	*			Hua et al., 2012
9	Bronchial aspirates from LAC patients	**	**			Hua et al., 2012
10	Serum from normal individuals	x	x	x	x	Zakut et al., 1988 Zanini et al., 2013
11	Serum from lung cancer (all histological subtypes)	x	x	x	xx	Zakut et al., 1988 Zanini et al., 2013

Blanks indicate Not Determined (ND).

Table 14
AChE-based anti-cancer drugs tested in human lung cancer cells in cell culture model.
The term ADR denotes for Adriamycin-resistant cell line

Name of drug	Cell line	Nature of cells	References
Poly APS polymers from *Halicona (reniera) sarai*	A549, Primary NSCLC cell lines (isolated from patients)	NSCLC	Zovko et al., 2009
Aβ1-40, Aβ1-28	H520, H226, SK-MES	Squamous cell lung cancer	Dasgupta et al., 2018
miRNA-132	H520	Squamous cell lung carcinoma	Zhang et al., 2014
miRNA-212	H520	Squamous cell lung carcinoma	Lu et al., 2013
Methoxy azomethine-dihydroquinazolinone	H157	Squamous cell lung carcinoma	Iqbal et al., 2016
Rhodolactouchal	NCI-H460	Lung adenocarcinoma	Nguyen et al., 2015
Methanolic extracts of *Anonaceae*	NCI-H460, NCI-H460/ADR	Lung adenocarcinoma	Formagio et al., 2015
Dehydrocordaline	H1299	Lung adenocarcinoma	Lee et al., 2017

COPD) drugs for lung cancer will accelerate their eventual clinical development in the treatment of human lung cancer.

An exciting new development in the field of ACh signaling pathway in lung cancer has been the design and synthesis if selective ligands suitable for imaging tumors. Selective ligands of ChAT, ChT1, nAChRs and nAChR subunits may prove useful for early detection and diagnosis of lung cancers in which these cholinergic proteins are overexpressed relative to normal tissue (Challapalli & Aboagye, 2016; Galitovskiy et al., 2013; Gilissen et al., 2003; Kumar et al., 2017; M. Li et al., 2013; Ramirez de Molina et al., 2007; C. Ray et al., 2017). In addition, α7-nAChR ligands have also been used as a strategy to improve the targeting and delivery of chemotherapeutic drugs to lung tumors (Mei et al., 2018). It is hoped that selective ligands to other nAChR subunits (like the α5-nAChR and α5-nAChR-D398N) will improve the targeting efficacy of conventional anti-cancer drugs and minimize unwanted side effects (C. Ray et al., 2017).

The nAChR-signaling pathway has transpired as an important drug target in the management and treatment of human lung cancer. Emerging studies have shown that competitive α9-nAChR and α7-nAChR antagonists may be useful for lung cancer therapy (Mucchietto et al., 2016; Mucchietto et al., 2018). An alternate promising drug-design strategy is to design allosteric modulators of nAChRs, which do not suppress the physiological function of nAChRs. Endogenous allosteric modulators of nAChRs namely to the Ly-6 family of proteins have been detected in the bronchial epithelium. The mechanisms by which these Ly-6 proteins regulate nAChRs are poorly understood (Fu et al., 2015; Lyukmanova, Shulepko, Kudryavtsev, et al., 2016a; Song et al., 2008; Spindel, 2016). The identification of molecular targets downstream of the lynx and SLURP proteins will foster the design of allosteric nAChR ligands with an improved biological activity and side effect profile (Arredondo et al., 2007; Chernyavsky et al., 2010; Durek et al., 2017; Horiguchi et al., 2009; Kalantari-Dehaghi, Bernard, & Grando, 2012; Lyukmanova et al., 2018; Lyukmanova, Shulepko, Kudryavtsev, et al., 2016; Lyukmanova, Shulepko, Shenkarev, et al., 2016).

The signaling proteins like CTL1-5, OCTs and OCTNs are promising drug targets for lung cancer (Inazu, 2014; Pochini, Scalise, Galluccio, & Indiveri, 2012a; Pochini et al., 2013; Pochini, Scalise, Galluccio, & Indiveri, 2012a; Song, Mark, et al., 2010; Song et al., 2013; Spindel, 2016; Tamai, 2013; Traiffort et al., 2013; Volk, 2014). Choline is also a

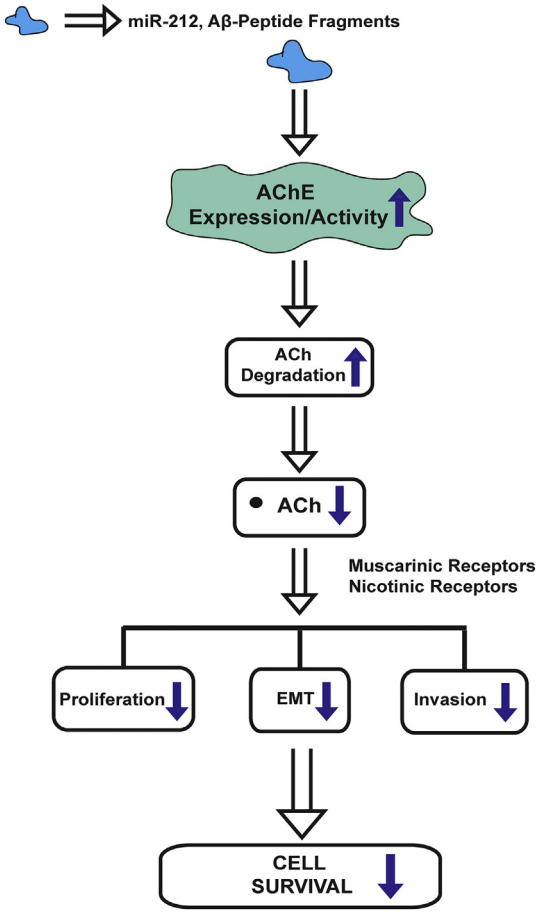

Fig. 14. Pharmacological manipulation of AChE expression/activity may have potential applications in lung cancer therapy. AChE modulators like miR-212 and Aβ-peptide fragments increase the expression (and activity) of AChE, which in turn accelerates the degradation of ACh in human lung cancer cells. Such a decline in ACh levels decreases its tumor promoting activities like proliferation, induction of EMT and invasion potentially suppressing the progression of human lung cancers.

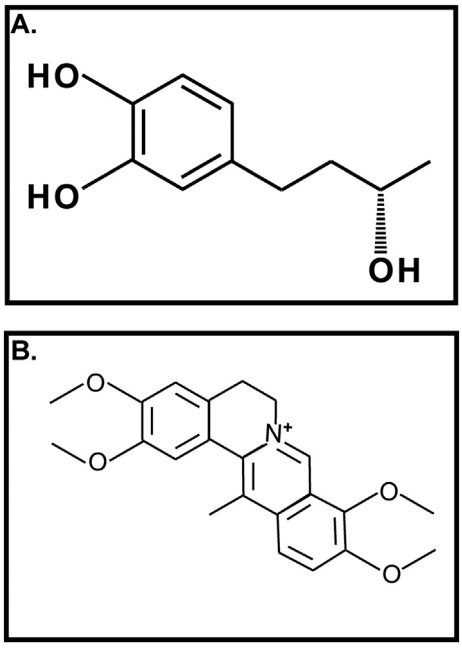

Fig. 15. Structure of AChE inhibitors investigated for their growth inhibitory activity. **A.** Rhodolatouchol **B.** Dehydrocorydaline.

vital component of phospholipid synthesis (Inazu, 2014; Traiffort et al., 2013). Therefore, drugs targeting choline uptake will suppress the growth of lung cancer cells by two mechanisms; first, by blocking the production of the growth factor ACh and second by suppressing cell membrane synthesis in lung cancer cells.

Future research will clarify the signaling pathways regulating the production of ACh in human lung cancer cells. An interesting observation is that human microvascular endothelial cells secrete, transport and degrade ACh (Carvalho et al., 2005; Haberberger et al., 2000; Kirkpatrick et al., 2001; Kirkpatrick et al., 2003). Endothelial ACh has been implicated in the transport of intracellular calcium, relaxation of arteries and protecting the endothelium from hypoxia/reoxygenation injury (Chataigneau et al., 1999; Wilson et al., 2016; Zhao, He, et al., 2015a). The pro-angiogenic activity of nAChRs in human lung cancers has been extensively studied (Cooke, 2007; Cooke & Ghebremariam, 2008; J. C. Wu et al., 2009). Similarly, the M3R receptor antagonist 4-DAMP displayed robust anti-angiogenic activity in athymic mouse models xenografted with human SCLC (C. Hu & Zhang, 2017). However, the potential role of other cholinergic proteins namely ChAT, CTLs, VAChT and AChE in neovascularization of lung tumors is yet to be understood. A few cholinergic proteins like ChAT, CTLs and AChE exist in multiple isoforms. AChE and CTL isoforms have been detected in human lung cancer tissues, as well as bronchial aspirates of lung cancer patients (Martinez-Moreno et al., 2005; Martinez-Moreno et al., 2006). The function of these cholinergic protein isoforms in the pathophysiology of lung cancer is unknown. Studies in human lung, oral cancers and colon cancers have revealed the existence of two types of nAChRs, one localized on the cell membranes and the other on mitochondrial membranes (Chernyavsky, Shchepotin, Galitovkiy, & Grando, 2015a; Chernyavsky, Shchepotin, & Grando, 2015b; S. A. Grando et al., 2015). The proliferative activity of nAChRs are controlled by membrane-bound nAChRs, whereas the pro-survival effects of nAChRs are mediated via the mitochondrial nAChRs. The development of selective high affinity cholinergic modulators will provide valuable insights into non-neuronal ACh-mediated-signaling pathways and will foster the hope of novel acetylcholine pathway-based therapies in lung cancer.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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