Foundation review: α7-Nicotinic receptor antagonists at the beginning of a clinical era for NSCLC and Mesothelioma?

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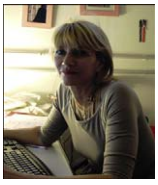
Of the human solid cancers, Non-Small Cell Lung Cancer (NSCLC) and Malignant Pleural Mesothelioma (MPM) display a natural history supporting the concept that they develop from multiple preneoplastic pathways. Recently, new evidence suggested that nicotinic Acetylcholine Receptors (nAChRs) play a significant role in lung cancer predisposition and natural history. This review is based on some translational research aimed at evaluating the potential therapeutic effect of nAChR antagonists on NSCLC and MPM. The background and rationale of this approach are based on the experimental observations that: (a) NSCLC and MPM cells express nAChRs and (b) the activation of these receptors by agonists, namely nicotine, inhibits apoptosis, whereas receptor antagonists have a pro-apoptotic effect.



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Patrizia Russo is in charge of the Lung Cancer Unit at the National Institute for Research on Cancer, Genoa, Italy. In 2006 she became a member of the Italian Consensus Board for the definition of Italian lung cancer guidelines. Since 1987, she has been working in the field of Molecular Pharmacology and in 1987, 1991 and 1997–1998 she attended the Laboratory of Molecular Pharmacology at NCI, NIH (Bethesda, USA). She studied the molecular mechanism of action of caffeine, then the association of antitumor drugs with cytokines. Parts of the latter studies were funded by AIRC (Milan, Italy), CNR (Rome, Italy) and NATO (Bruxelles, Belgium). Since 1995, she has been studying the relationship of sensitivity or resistance to classical and new experimental targeted antineoplastic drugs, receiving a fellowship from FIRC (Milan, Italy) to join the Laboratory of Molecular Pharmacology at NCI, NIH. These studies were supported by EC (Ispra, Italy). Since 2000, she has been studying lung cancer, looking at early detection and the development of new drugs. These studies were supported by the Italian Health Ministry, Fondazione Compagnia di San Paolo (Turin, Italy), Fondazione CARIGE (Genoa, Italy) and Liguria Local Government (Genoa). Starting in 2003, she has been investigating the role of nAChRs in lung cancer biology.

Introduction

Non-Small Cell Lung Cancer (NSCLC) and Malignant Pleural Mesothelioma (MPM) demonstrate great molecular heterogeneity in which several pathways are believed simultaneously and actively to lead to tumorigenesis [1]. Thus, their natural history supported the concept that they develop from multiple preneoplastic pathways. MPM is an aggressive neoplasm of mesothelial cell origin that arises mainly from the pleura and is strongly associated with asbestos exposure [2]. Conventional therapies, such as surgery, radiotherapy and chemotherapy, do not necessarily improve overall survival [3–4]. Lung cancer consists of several histological types in which NSCLC represents 75–85% of the total, it is subclassified into: adenocarcinoma (AD, including the noninvasive type of bronchioloalveolar carcinoma), squamous cell carcinoma (SQ), epidermoid and large cell carcinoma [5]. In AD at least two pathways have been identified: (i) smoking-related and (ii) nonsmoking-related [6]. Recently, genetic variations in a region of chromosome 15 that encompasses a gene implicated in nicotine dependence had been linked to the risk of lung cancer in genome-wide association studies, but data were not definitive as to whether the variants were linked to lung cancer *per se* or to nicotine dependence [7–12]. A recent work [12] reported that the increased risk of lung cancer conferred by the genetic variants might be explained by an increased

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likelihood of nicotine dependence, although some of the results of this study, and a previous study of the variant in subjects who reported that they had never smoked, suggest that the variants may also have a direct role in lung carcinogenesis.

Pharmacology and signaling of nAChRs

nAChRs belong to the ligand-gated ion channel (LGIC) family that includes the excitatory 5HT-3 receptor and the inhibitory receptors for glycine and γ-aminobutyric acid ($GABA_A$ and $GABA_C$) [13]. The nAChR consists of either a homo or heteropentamer composed of the various subunits that have been identified so far (α1–α10; β1–β4) [14,15] and that are arranged symmetrically around an axis perpendicular to the membrane, thus delineating the ionic pore. The composition and stoichiometry of the subunits constituting the pentamer may have a profound impact on receptor pharmacology, cation selectivity, desensitization kinetics and spatial distribution. All nAChR subunits share a homologous structure, with a large extracellular domain, four transmembrane regions (M1–M4) structured in α-helices, a large cytoplasmic domain between M3 and M4 and, finally, a short extracellular C-terminal tail [16].

The cognate ligand for the nAChRs is nicotine, an agonist interacting with various affinities, from 1 to 130 μM, depending upon the different neuronal receptor subtypes [17,18]. The presence of β2 and β4 subunits in the receptor pentamer seems to be correlated with high and low affinity for nicotine, respectively.

On the contrary, as an allosteric receptor (see the below paragraph), nAChR may undergo rapid conformational transitions from a resting basal state to an active or desensitized state. Application of nicotine initially provokes the stabilization of the receptor in a high affinity, open state followed by a progressive stabilization of a closed, desensitized state [19]. In the case of smoking behavior, long exposure to a low concentration of nicotine favors receptor desensitization.

Mutational and photo-affinity labeling experiments identified the agonist binding site at the interface of the extracellular regions of the principal α and the complementary non-α subunits, whereas the transmembrane segment M2 is the major contributor to the pore domain [13]. The structural coupling between the extracellular and the pore domains provides efficient transduction between agonist binding and the ion channel gating. Recently, the resolution of the crystallographic structures of a protein homologous to the extracellular domain of nAChR, the acetylcholine binding protein (AChBP) either alone or complexed with various ligands, in addition to numerous biochemical studies on the ligand binding sites, allowed better understanding of how various ligands interact with different nAChRs and revealed, at the molecular level, the fundamental events underlying the receptor activation [20–24].

The nicotine-binding site was initially studied using structural models of the extracellular domain of nicotinic receptors and then the crystallographic structure of the AChBP–nicotine complex [25]. Even if the nicotine-binding pocket is similar to those determined for acetylcholine or epibatidine, involving mainly aromatic and hydrophobic contacts, the specific binding of nicotine is due to additional hydrogen bonds with the receptor and a closer packing of the aromatic groups [25]. These subtleties in nicotine interaction compared to other agonists were confirmed by a physical chemistry approach using unnatural amino acid mutagenesis combined with computational modeling studies [26].

Nicotine activates different subtypes of nAChR, inducing a complex pattern of mixed sympathetic and parasympathetic responses. The stimulation, desensitization and upregulation of these receptors by nicotine seem to be responsible for diverse physiological effects targeting the cardiovascular [27,28], pulmonary (as we will see in the next chapters), endocrine [29] and central nervous systems [16]. Of course, one of the most studied effects of nicotine is its smoking-related addictive property [30–32].

Allosteric modulation

Introduced by Wyman and colleagues in 1965 [33], the allosteric concept refers to the assumption that proteins could exist in multiple conformational states and that binding of allosteric ligands alters the energy barriers or 'isomerization coefficients' between various states, preferentially stabilizing the protein in a given conformation. The site occupied by the natural ligand, which is typically at the interface between subunit protomers, is called 'the orthosteric site'. Allosteric sites are distinct from the orthosteric site and can be localized elsewhere on the protein. Binding of the ligand at the orthosteric site stabilizes the protein in the active state, whereas binding of an effector at an allosteric site alters the overall properties by modifying the energy barriers, represented by isomerization coefficients, between one or more states. In the specific case of the nAChR, agonists are ligands that preferentially stabilize the receptor in the active open state, whereas competitive antagonists are ligands that stabilize the protein in a closed conformational state. Thus, endogenous ligands, such as acetylcholine, bind at the orthosteric site, whereas all the molecules that bind elsewhere on the nAChR subunit(s) act via allosteric interactions. Bertrand and Gopalakrishnan, in a recent review, outlined extensively the principles of the allosteric concept and summarized the profiles of novel compounds that are emerging as allosteric modulators at the α7- and α4β2-nAChR subtypes [34].

Ca^{2+} permeability of nAChR

The nAChR channels are permeable to cations, including Ca^{2+}. Ca^{2+} entry through nAChR channels modulates several Ca^{2+}-dependent cellular processes, such as neurotransmitter release, synaptic plasticity and cell motility. Two different classes of neuronal nAChR may be identified according to their Ca^{2+} permeability, which correlates with other pharmacological and structural properties: (i) neuronal nAChRs containing subunits (α7–α9) able to bind α-bungarotoxin (α-BTX) and form homopentameric channels (α-BTX nAChR), exhibiting the highest measured Ca^{2+} permeability values [35]; (ii) heteropentameric, non-α-BTX-sensitive nAChRs (non-α-BTX nAChR), always comprising at least one α (out of α2–α6) and one β (out of β2–β4) subunits, with lower measured Ca^{2+} permeability [36]. Studies indicate a functional correlation between the activation of α7-nAChR and Ca^{2+}-dependent cellular processes, such as neurotransmitter release, synaptic plasticity, cell growth, migration and survival.

A recent study by Gilbert *et al.* [37] showed that the Ca^{2+} transients were predominantly due to the opening of plasma membrane α7-nAChR, because the signals were (a) evoked by nicotine, (b) sensitive to two α7-specific nAChR inhibitors

TABLE 1

nAChR expression on different normal airways epithelial cells or Mesothelial cells

Cells	nAChR subtype												
	α1	α2	α3	α4	α5	α6	α7	α9	α10	β2	β3	β4	Reference
Airways epithelial cells													
BAC1													[116]
BAC2													[116]
BAC3													[116]
BEAS-2B	+		–	–	+	+	++	–	++	–	–	++	[130]
BEC	–	–	–	+	–	–	+	–	–	+	–		[55]
BEC			+				+						[47]
BEP2D*	+	–	+	–	+	–	+	+	+	+	–	+	[131]
HBEC			+	–	+					+		–	[57]
HBEC		–				–					–	–	[57]
HBEC-KT1	+		+	–	+	+/–	–	–	+	–	+/–	+	[47]
HBEC-KT2	+/–		–	–	+	–	–	–	+/–	–	–	+	[47]
HBEC-KT3	–		–	–	+	–	–	–	–	–	–	–	[47]
HBEC-KT4	–		–	–	+	–	–	–	–	–	–	–	[47]
NHBE	–	–	+ +	–	+	–	+ +	+	+	+ +	–	+	[55,60,132]
NHBE							+						[79]
SAEC	–	+	–	+	–	–	+	+	+	+	–	+	[60,79]
SAEC				–	+/–	+	–	–		+/–			[44]
SAEC	+		–	–	+/–	–	–	–	+	+/–	–	+	[47]
SAEC							+						[79,133]
Pleural mesothelial cells													
Mes-1							+						[109,116]
Mes-2							+						[109,116]

* This cell line is an established clonal population of HPV-18-immortalized human BEC. The cells have an epithelial morphology in culture, near diploid karyotype, and relatively stable genotype. They are anchorage-dependent and do not form tumors in immunosuppressed host animals.

(methyllycaconitine and α-BTX), (c) increased by a known α7-nAChR allosteric modulator (PNU-120596) and (d) absent when Ca^{2+} was omitted from the bathing medium.

Calcium imaging, combined with whole-cell patch-clamp recordings, has been used previously to determine the Ca^{2+} permeability of native or expressed nAChRs [35]. These studies provided important clues to the physiological role of the different nAChR subtypes and to the structural determinants of their Ca^{2+} permeability (reviewed in [38]), but did not provide any spatial information about the underlying Ca^{2+} signals. This study clearly demonstrated that the α7-nAChR is able to generate large Ca^{2+} signals in neuronal cells stimulated with relevant agonists. Consequently, significant signals are expected to be generated by these agents in cells expressing high levels of the α7-nAChR, such as neurons [16]. In neurons, Ca^{2+} elevations can trigger the secretion of neurotransmitters, open membrane channels to modulate the cell's excitability, and activate the transcription of several genes [16]. Thus, the activation of the α7-nAChR is expected to impact on neuronal activity, both in the central and in the peripheral nervous system. The same prediction can be made for non-neuronal cells known to express the α7-nAChR, such as monocytes and macrophages [39,40]. In these cells, central for innate immunity, Ca^{2+} elevation is involved with the control cell migration, bacterial killing, antigen presentation and cytokine release [41].

Expression of nAChR in airways cells

nAChR genes are also expressed in different epithelial cells, including normal and lung cancer cells [42–50].

In this section we outline the differential distribution of nAChR subunits in normal and/or unaffected airway epithelial cells, in NSCLC and pleural Mesothelioma cell lines, as well as in tissue cancer specimens. The data are summarized in Tables 1–3. From Table 1 it is manifest that normal airway epithelial cells show a different pattern of nAChR subtypes. Even so, looking at the normal, nonimmortalized bronchial epithelial cells, the α7 subtype receptor seems to be the most predominantly expressed receptor. This is a regular feature of cancer cell lines (Table 2) and cancer tissues (Table 3).

Comparison of the expression of nAChR subunits between tumor and matched normal tissue revealed a significant upregulation of the β4 subunit and a concomitant decrease in α4 levels [47]. In addition, NSCLC tumors from nonsmokers showed elevated expression of the α6β3 receptor, compared with smokers, in a gender adjusted manner [47]. Recent data suggested that α7-nAChRs in NSCLC are significantly more expressed in squamous

TABLE 2

nAChR expression on different NSCLC or Mesothelioma cell lines

Cells	nAChR subtype												
	α1	α2	α3	α4	α5	α6	α7	α9	α10	β2	β3	β4	Reference
NSCLC adenocarcinoma													
201T	+		+		+		**+**		+				[134]
A427							**+**						[135]
A549	– +	– –	– –	+ –	+ +	–	**+** –	+	–	+ +	+	–	[55,79–81,112,113,132,134]
H1299	–	–	+		+	+	**+**		+				[79,80]
H1355	–	–	+	+	+	–	**+**	–	–	+	–	–	[133]
H1437	–		+	+/–	+	–	**+**	+	+	+	+/–	–	[47]
H1648	+/–		–	–	+	+/–	**+**	–	+	–	+/–	+	[47]
H1650							**+**						[116]
H1703	–	–	+	+	+	–	**+**	+	–	+	–	–	[132]
H1770	+/–		–	–	+	++	**+**	–	+	–	+	–	[47]
H1819	+/–		–	–	+	+/–	**+**	–	+	–	+	+	[47]
H1993	+/–		–	–	+	–	**+/–**	–	+	–	+	+	[47]
H2009	+		–	–	+	–	**+**	+/–	+	–	+	+	[47]
H2087	+/–		–	–	+	+/–	**+**	–	+	–	+/–	+	[47]
H2122							**+**						[80,135]
H2122	+/–		+/–	–	+	+/–	**+**	+/–	+	–	+	+	[47]
H23	–	–	+		+	+	**+**		+				[79,80]
H2347	+/–		–	–	+	+/–	**+**	+/–	+	–	+/–	+	[47]
H322							**+**						[47]
H441							–						[79,80,133]
HKULC1	+		+/–	+/–	+	–	**+**	–	+	–	+	+	[47]
HKULC2	+		–	–	+	+/–	**+/–**	–	+	–	+/–	+	[47]
HKULC3	+		+	++	++	++	**++**	+	+	+	–	+	[47]
HKULC4	+/–		+/–	–	+	–	**+/–**	–	+	+	–	–	[47]
LT1							**++**						[116]
LT2							**+**						[116]
NSCLC squamous cell carcinoma													
273T	+		+		+		**+**			+			[132,134]
H157	–	–	–	+	+	+	**+**	+	–	+	–	–	[132]
H2170							**+**						[133]
H226	–	–	+		+	+	**+**		+				[133]
H520			+	+			**+**			+	+	+	[133]
SK-MES							**+**						[48]
LT3							**+**						[116]
LT4							**++**						[116]
NSCLC large cell carcinoma													
COR-L23							**+**						Our unpublished data
H1155	–	–	+	+	+	–	**+**	–	–	+	–	+	[132]
MPM													
MSTO-211H							**+**						[109]
MPP-89							**+**						[78,109]
IST-MES1							**+**						[78,109]
IST-MES2							**+**						[78]

Primary NSCLCs [LT1, LT2 (adenocarcinoma), LT3, LT4 (squamous carcinoma)].

TABLE 3

nAChR expression on different NSCLC or Mesothelioma tissue samples

No. of samples	nAChR subtype												
	α1	α2	α3	α4	α5	α6	α7	α9	α10	β2	β3	β4	Reference
NSCLC adenocarcinoma													
2			+				**+**						[77]
54	++		+	+	++	++	**++**	+	++	+	++	++	[47]
19							**+**						[48]
NSCLC squamous cell carcinoma													
2			+				**+**						[42]
31			+ (45%)	+ (87%)	+ (71%)		**+ (90%)**			+ (100%)	+ (87%)	+ (90%)	[133]
6	++		++	+	++	++	**++**	+	++	+	++	++	[47]
28							**++**						
NSCLC large cell carcinoma													
5	++		+	+	++	++	**++**	+	++	+	++	++	[47]
28							**++**						[48]
MPM													
4							**+**						[78,109]

Carlisle *et al.* [97] found that none of the lung tissues that they examined were positively stained for α7-nAChR. By contrast, we found that α1 and β1 were highly expressed in NSCLC tissue (12 adeno 4 active smokers, 5 exsmokers, 1 nonsmoker, 2 unknown and 3 squamous IH, 2 exsmokers, 1 unknown) (Paleari, *Am. J. Respir. Crit Care*).

carcinoma than in adenocarcinoma. Among this histological subtype, smokers showed the highest upregulation. Interestingly, all NSCLC female patients, either smokers or nonsmokers, expressed less mRNA and protein for the α7-nAChR than males, suggesting a different response to nicotine between females and males [48]. As a consequence of the presence of nAChRs on lung cells, the potential role of cholinergic activation in the development and growth of lung cancer has been intensely studied in recent years [49,50].

α7-nAChR activation and subcellular signaling in lung cells

Effects on normal epithelial lung cells

In human bronchial tissues and in cultures of human BEC, the nAChRs were visualized on the cell membranes, predominantly at the sites of cell-to-cell contacts using subunit-specific antibodies. The epithelial cells of submucosal glands abundantly expressed α7-nAChR. The function of the nicotinic cholinergic signaling pathway in airway bronchial epithelium is highly likely to be affected by nicotine in smokers. In smokers, plasma nicotine levels peak around 200 nM during the day and drop to 5–10 nM during sleep. Nicotine levels in lung airways directly exposed to smoke may be five to tenfold higher, and peaks and troughs are much sharper [51]. These levels are high enough to activate α4β2-nAChRs [51,52] and may either inhibit [53] or activate [54] α7-nAChR. It is also interesting that the expression of nAChR appears highly expressed at the apical regions of cells, where they are more exposed to airway nicotine. Smoking significantly ($P < 0.05$) increased the relative numbers of nAChRs and these effects could be reproduced in cultures of BEC exposed to 10 μM nicotine [55]. Whole-cell patch-clamp studies of cultured human BECs demonstrated the presence of fast-desensitizing currents activated by choline and nicotine that were blocked reversibly by methyllycaconitine (1 nM) and irreversibly by α-BTX (100 nM), consistent with the expression of functional α7-nAChR [55].

Recently Lam *et al.* [47], after having exposed human BEC lines to 100 nmol/L nicotine and then harvested RNA at 72 and 144 hours, showed a significant increase in the expression levels of α1-, α5- and α7-nAChRs at 72 hours, with return to baseline levels of expression upon nicotine removal.

To gain insights into the molecular mechanism underpinning such nicotine-induced effects, microarray-bioinformatics analysis was carried out to explore the gene expression profiles in human BEC treated with nicotine at 5 μM for 4, 8 and 10 hours [56]. Of 1800 assessed genes overall, 260 (14.4%) were upregulated and 17 (0.9%) downregulated significantly. Membrane array analysis suggested that both extracellular signal-regulated kinase (ERK) 1/2 and c-Jun-NH_2-terminal kinase (JNK) signaling, but not p38 MAPK signaling, were activated in response to nicotine. Thus, pretreatment of human bronchial epithelial cells (BECs) with specific inhibitors against ERK 1/2 and JNK, but not p38, significantly inhibited nicotine-induced interleukin-8 production, suggesting that MAPK pathway may mediate the effect of nicotine through ERK 1/2 and JNK, but not p38 in HBECs treated with nicotine.

The biological roles of epithelial nAChRs apparently involve the regulation of cell-to-cell communications, adhesion and motility, because Mec caused rapid and profound changes in these cell functions that were reversed by nicotine. An over-exposure of BECs to nicotine, however, produced an antagonist-like effect, suggesting that the pathobiological effects of nicotine toxicity might result from both activation of nAChR channels and nAChR desensitization [57]. It has been reported, using an animal model of nicotine infusion [58], that the tissue-to-blood ratio of nicotine is 3.0 for brain and 2.0 for lung. Therefore, lung tissues can reach nicotine levels that approximate those achieved in the brain. As such, the duration and persistence of nicotine administration over time becomes an important pharmacological variable in the use and interpretation of this drug's actions. The treatment of rat lung epithelial cells with nicotine for various periods differentially mobilizes multiple intracellular pathways [59]. Protein kinase C

and PI3-OH-kinase are transiently activated after the treatment. Also, Ras and its downstream effector ERK1/2 are activated after long-term exposure to nicotine. The activation of Ras by nicotine treatment is responsible for the subsequent perturbation of the methotrexate (MTX)-mediated G_1 cell cycle restriction, as well as an increase in production of reactive oxygen species [59]. These data suggest that persistent exposure to nicotine perturbs the G_1 checkpoint and causes DNA damage through the increase of the production of reactive oxygen species. A third element rendered by loss of p53 is, however, required for the initiation of the process of gene amplification. Under p53-deficient conditions, the establishment of a full oncogenic transformation, in response to long-term nicotine exposure, is achieved through the cooperation of multiple signaling pathways.

Through activation of separate nAChR α-subunits, nicotine activates one of the best-characterized signaling pathways that promote cellular survival: the PI3K/Akt pathway. Activation of Akt by nicotine occurred within minutes, but peaked at 45–60 min, and is maintained for hours. The presence of phosphorylated Akt in human lung cancers from smokers may support the hypothesis that nicotinic activation of Akt is not limited to cultured primary cells. Once activated by nicotine, Akt increased phosphorylation of multiple downstream components that control cellular cell cycle and protein translation, such as glycogen synthase kinase-3 (GSK-3), binding protein for eukaryotic translation initiation factor 4E (4EBP-1) and ribosomal kinase p70S6K [60].

Pulmonary neuroendocrine cells (PNEC) are a highly specialized population of airway epithelial cells which contain and secrete biogenic amines, in particular 5-hydroxytryptamine (5-HT) and various peptides [61,62]. By studying the release of 5-HT from isolated rabbit tracheae it was shown that the secretory activity of PNEC is stimulated via nicotinic receptors, but not modulated by muscarinic mechanisms [63]. A nicotinic receptor-induced release of 5-HT could also be observed from PNEC in culture [64].

To sum up in BEC exposed to nicotine, for short time, nAChR mediated activation of the serine/threonine kinase Akt and/or ERK 1/2 resulting in the phosphorylation of several downstream substrates. This was associated with the a transformed cellular phenotype manifested as loss of contact inhibition, loss of dependence on exogenous growth factors and attenuated apoptosis induced by various pro-apoptotic stimuli.

Effects on normal fibroblast lung cells

In primary murine lung fibroblasts, nicotine stimulates the expression of fibronectin via the activation of intracellular signals that lead to increased fibronectin gene transcription. It was observed that the stimulatory effect of nicotine was associated with the activation of protein kinase C and mitogen-activated protein kinases, increased levels of intracellular cAMP and phosphorylation and DNA binding of the transcription factor CREB. Increased transcription of the gene was dependent on cAMP-response elements (CREs) present on the 5′ end of its gene promoter. The stimulatory effect of nicotine on fibronectin expression was abolished by α-BTX. Of note, nicotine increased the expression of α7-AChR on fibroblasts. To assess the relevance of these *in vitro* observations to the situation *in vivo*, Roman *et al.* [54] examining fibronectin expression in the lungs of nicotine-exposed mice observed an increased expression of fibronectin mRNA and protein when compared with control animals. Immunohistochemical analysis revealed that nicotine exposure was also associated with increased fibronectin protein in alveolar *septae* as well as in airway epithelial cells and in vascular structures. These data suggest that nicotine induces lung fibroblasts to produce fibronectin by stimulating α7-nAChR-dependent signals that regulate the transcription of the fibronectin gene. This may result in alterations in the composition of the lung matrix. In doing so, nicotine might promote increased tissue remodeling around the airways and within the lung parenchyma and this is likely to represent one mechanism by which tobacco results in abnormal lung function. In addition, the newly deposited fibronectin-containing matrix primes lung resident and incoming cells to response to injurious agents in an exaggerated manner. Further delineation of the factors and conditions that regulate nicotine-induced fibronectin expression *in vivo* will be needed before a full understanding can be obtained of the true consequences this process has in lung as well as other organs.

Effects on embryonic lung cells

Maternal smoking during pregnancy has various adverse effects that are well known and documented [65]. In our point of view, it is interesting that in the WI38 human embryonic lung fibroblast cell line, which displays α3- and α7-nAChR subunits, nicotine disrupted the specific paracrine signaling pathway that caused pulmonary transdifferentiation of interstitial lipofibroblast (LIF)-to-myofibroblast (MYF), resulting in altered pulmonary development and function [66]. This effect was counteracted by the use of nonspecific (D-tubocurarine) and specific (α-BTX and Mec) antagonists of nAChR. Alveolar interstitial LIF to MYF transdifferentiation results in failed alveolarization in the developing lung, which leads to an arrest in pulmonary growth and development, the hallmarks of in uterus nicotine-induced lung damage. Specifically, the interstitial LIF phenotype is of functional importance, because it provides cytoprotection against oxygen free radicals [67], traffics neutral lipid substrate to alveolar type II cells for surfactant phospholipids synthesis [68] and causes alveolar type II cell proliferation [69]. Although MYF also seems to be important for normal lung development, these cells are also the hallmarks of chronic lung diseases in both the neonate and adult. In the developing lung, MYF are fewer in number and are predominantly located at the periphery of the alveolar septa, where they probably participate in the formation of new septa [70,71]. According to Sekhon *et al.* [72] nicotine, after having passed across the placenta, interacts with nAChRs of the fetal monkey lung, thus maternal nicotine exposure upregulated nAChR expression in fetal lung. In parallel with changes in nAChR expression, prenatal nicotine exposure downregulated the surface complexity of the parenchyma, increased collagen accumulation, upregulated surfactant protein gene expression and induced neuroendocrine cell hyperplasia in fetal lungs and altered pulmonary function [73,74]. lynx1, an evolutionary precursor to snake venom toxins, is expressed during the early development of lung, gradually increased through the prenatal period, and then persisted throughout adulthood. Spatial differences occur between lynx1 expression in the proximal and distal parts of the lung suggesting a possible role in cellular differentiation and/or function. lynx1 upregulation following prenatal nicotine exposure suggested that it plays a role in

regulating the interaction of exogenous agonist (nicotine) or endogenous ligand (ACh) in lung cells that express nAChR [75].

Effects on lung cancer cells

As reported above, from biological, histopathologic and clinical perspectives, lung cancer is a highly complex neoplasia, probably having multiple preneoplastic pathways [1,76]. The histopathologic heterogeneity is the major confounding factor in lung cancer diagnosis and treatment. Cigarette smoking has been linked to all histological subtypes; however, the proportion of smokers is highest in those who develop SQ.

In 1990, Maneckjee and Minna [42] have described the presence of α7-nAChR on small and NSCLC cell lines. Afterwards different authors reported the presence of nAChRs in lung cancer cell lines and in tissues obtained from human biopsies taken from patients suffering from lung cancer (see Tables 1 and 2).

More recently, Lam *et al.* [47] showed the expression of nAChR subunit genes in 66 resected primary NSCLCs; those derived from nonsmokers demonstrated higher expression of α6- and β3-nAChR subunit genes than those from smokers, adjusted for gender. In addition, nAChR α4- and β4-subunit gene expression showed significant difference between NSCLC and normal lung. Using Affymetrix GeneChip U133 Sets, 65 differentially expressed genes associated with a NSCLC nonsmoking α6β3-nAChR phenotype were identified, which gave high sensitivity and specificity of prediction. The authors concluded that between NSCLC from smokers and nonsmokers, different nAChR subunit gene expression patterns were found, and a 65-gene expression signature was associated with nonsmoking α6β3-nAChR expression. Furthermore, an important study of Song *et al.* [77] presented data that SCLC express a cholinergic autocrine loop that can regulate cell growth.

Such a study demonstrates that:

(a) genes for all components of an ACh autocrine loop, including choline acetyltransferase (ChAT), vesicular ACh transporter (CHT1), nAChR and muscarinic AChR (mAChR) are expressed in SCLC cells, as well as in neuronal cells;
(b) ChAT is present in biopsies of SCLC and in SCLC cell lines;
(c) SCLC cells are able to synthesize, secrete and degrade ACh;
(d) SCLC cell growth is modulated by endogenous ACh synthesis.

Such work is probably the first study that demonstrates that SCLC cells have a cholinergic phenotype and that ACh exerts an autocrine growth factor in human lung tumors. Thus, the identification of a cholinergic autocrine loop by SCLC now provides a framework and rationale for the many studies, in the literature, that nicotine and related compounds stimulate SCLC growth.

The major effects of nicotine in lung cancer cells (SCLC and NSCLC) are

(a) Enhancement of cancer cell growth.
(b) Inhibition of drug-induced apoptosis.

Recently, it has been shown that nicotine stimulated the tumoral growth of A549 cells orthotopically implanted in BALB/c in *NOD/SCID* mice [48].

It was suggested that:

(i) Bax may be an essential component in the nicotine survival signaling pathway, through a mechanism involving activation of PI3K/AKT that directly phosphorylates and inactivates the pro-apoptotic function of Bax [78–81].
(ii) Survivin and XIAP played a key role in the antiapoptotic activity of nicotine [79].
(iii) Nicotine exerted its role of antiapoptotic inducer through NF-κB upregulation [80].
(iv) The mitogenic effects of nicotine resulted in enhanced recruitment of E2F1 and Raf-1, causing dissociation of Rb from these promoters. Proliferative signaling via nAChR required the scaffolding protein β-arrestin since ablation of β-arrestin or disruption of the Rb–Raf-1 interaction blocked nicotine-induced proliferation; thus, nicotine induces cell proliferation by β-arrestin-mediated activation of the Src and Rb–Raf-1 pathways [82].

α7-nAChR activation and effects on neoangiogenesis

Heeschen *et al.* [83] provided anatomic and functional evidence that nicotine-induced angiogenesis. They also showed that nicotine accelerates the growth of tumor and atheroma in association with increased neovascularization. Nicotine increased endothelial cell growth and tube formation *in vitro* and accelerated fibrovascular growth *in vivo*. In a mouse model of hind-limb ischemia, nicotine increased capillary and collateral growth, and enhanced tissue perfusion. In a Lewis lung tumor model, nicotine enhanced lesion growth in association with an increase in lesion vascularity. These effects of nicotine were mediated through nAChR at nicotine concentrations that are pathophysiologically relevant. The endothelial production of nitric oxide, prostacyclin and vascular endothelial growth factor might have a role in these effects.

Interestingly, it was shown that the second hand smoke (SHS), where nicotine is one of the major components, is associated with increases in plasma levels of angiogenic cytokines such as VEGF and MCP-1. Fascinatingly the angiogenic effects of SHS can be blocked by inhibition of the endothelial nAChR [84].

In spite of its importance, the neoangiogenetic effect, however, is not directly associated with a specific effect of nAChR activation on lung cells and is beyond the intention of this review.

α7-nAChR activation and inflammation

In 2005, an important article by Ulloa [41] reviewed different studies indicating that the vagus nerve (which is the longest of the cranial nerves and innervates most of the peripheral organs) can modulate the immune response and control inflammation through a 'nicotinic anti-inflammatory pathway', depending on the α7-nAChR. This article reported all the advances supporting the therapeutic potential of selective nicotinic agonists in several diseases. The author suggested that, similar to the development of α- and β-agonists for adrenoceptors, selective agonists for α7-nAChR could represent a promising pharmacological strategy against infectious and inflammatory diseases. Specifically, he proposed that the vagus nerve could modulate the innate immune response and prevent inflammation through a physiological mechanism that can be translated into a pharmacological strategy. Acetylcholine, the principal neurotransmitter of the vagus nerve, that signals through either muscarinic or nicotinic receptors; selective agonists (atropine, α-conotoxin or mecamylamine) were used to identify the receptors involved in the control of macrophages. This mechanism has been called the 'nicotinic anti-inflammatory pathway' because acetylcholine can inhibit the production of proinflammatory cytokines from macrophages

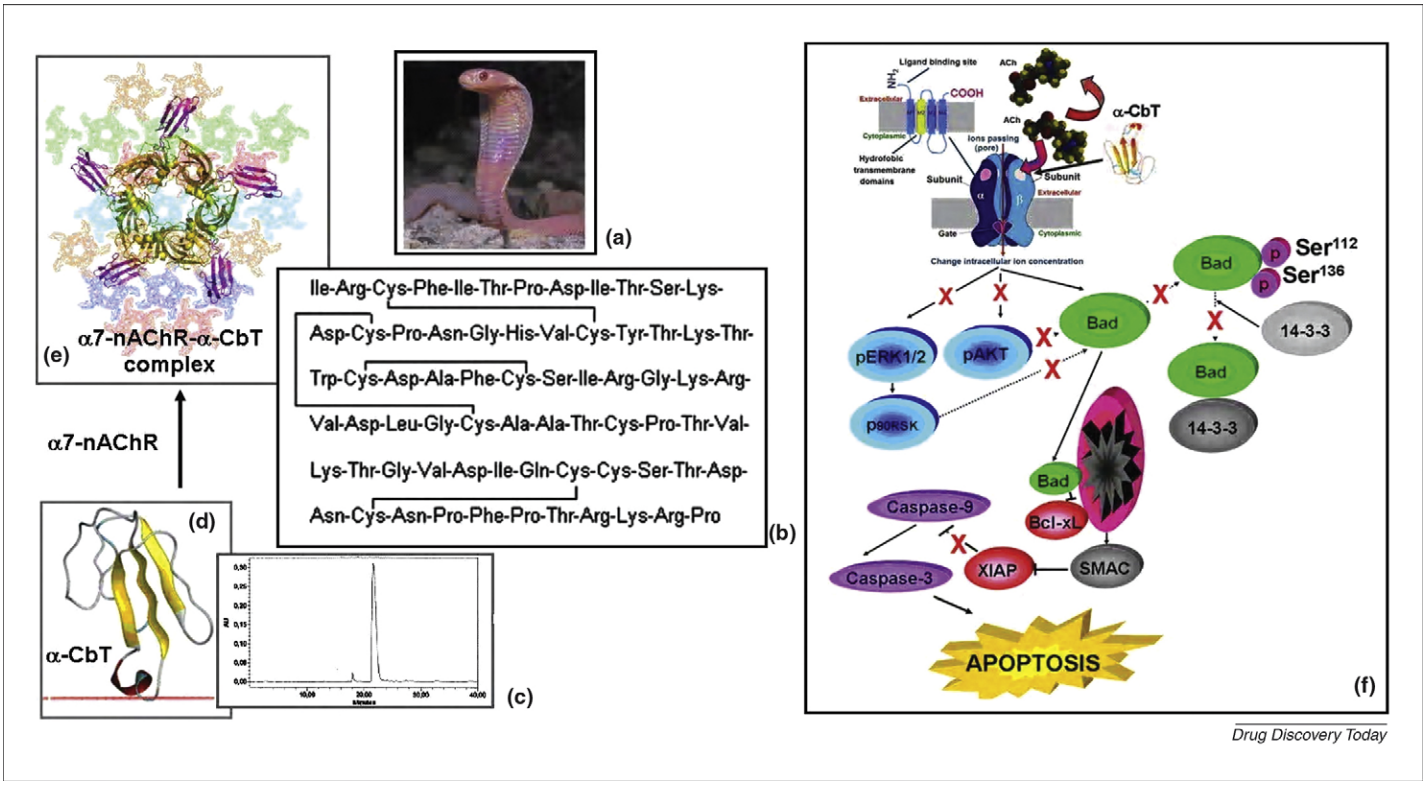

FIGURE 1

Structure of the quaternary alkaloids (+)-tubocurarine, on the top, from the South American vine *Chondodendron tomentosum*, on the bottom.

through a nicotinic acetylcholine receptor. Nicotine, a more selective cholinergic agonist, is more efficient than acetylcholine in inhibiting the production of proinflammatory cytokines from macrophages through a mechanism that is dependent on the α7-nAChR.

Efferent vagus nerve cholinergic signaling inhibits TNF and other proinflammatory cytokine levels through α7-nAChR-mediated mechanisms. Experimental evidence indicates that activation of α7-nAChR on immune cells by nicotine can prevent NF-κB nuclear translocation. α7-nAChR-mediated activation of the JAK2/STAT3 pathway has also been demonstrated in response to nicotine. These signaling pathways play a central role in transmitting vagal or nicotine-induced cholinergic anti-inflammatory signaling leading to inhibition of immune cell activation and suppression of TNF, HMGB1, macrophage inflammatory protein-2 (MIP-2) and IL-6 synthesis. Brain muscarinic acetylcholine receptor (mAChR) signaling mechanisms also modulate vagal immunoregulatory and anti-inflammatory output [85].

Experiments with α7-nAChR knockout mice revealed that in the absence of the α7-nAChR, vagus nerve stimulation was ineffective at preventing TNF release indicating that the α7-nAChR is essential for the effectiveness of the cholinergic anti-inflammatory pathway [39].

A recent work of Rosas-Ballina *et al.* [86] suggested that the cholinergic anti-inflammatory pathway regulates TNF production in discrete macrophage populations via two serially connected neurons: one preganglionic, originating in the dorsal motor nucleus of the vagus nerve, and the second, postganglionic, originating in the coeliacsuperior mesenteric plexus, and projecting in the splenic nerve.

Interestingly, from our point of view, the potential role of the α7-nAChR on acid-induced acute lung injury was studied in a rodent model. Specifically it was found that nicotine, choline, and PNU-282,987 (a specific α7-nAChR agonist) decreased excess lung exudates and lung vascular permeability via reduction of proinflammatory cytokines (macrophage inflammatory protein-2 and TNF-α) through suppression of NF-κB activation in alveolar macrophages [87].

In spite of its importance, the inflammatory effect, however, is not directly associated with a specific effect of on lung cancer cells and is beyond the intention of this review.

Antagonists of nAChRs

There are a limited number of potent, competitive nicotinic antagonists. Quaternary alkaloids, such as (+)-tubocurarine, are competitive antagonists for nicotinic receptors, but are relatively nonselective. (+)-Tubocurarine, from the South American vine *Chondodendron tomentosum*, is known as *Curare* (Fig. 1), one of the names coined by South American Indians to describe the plant-derived poisons that they used to coat the tips of their hunting arrows or blow-pipe darts. The antagonism by (+)-tubocurarine had both competitive and noncompetitive components [88].

One of the most abundant sources of natural ligands interacting on cholinergic receptors is, however, the venom of animals like cone snails and snakes. A huge diversity of peptidic toxins have been isolated from these venoms over the past 40 years and used in

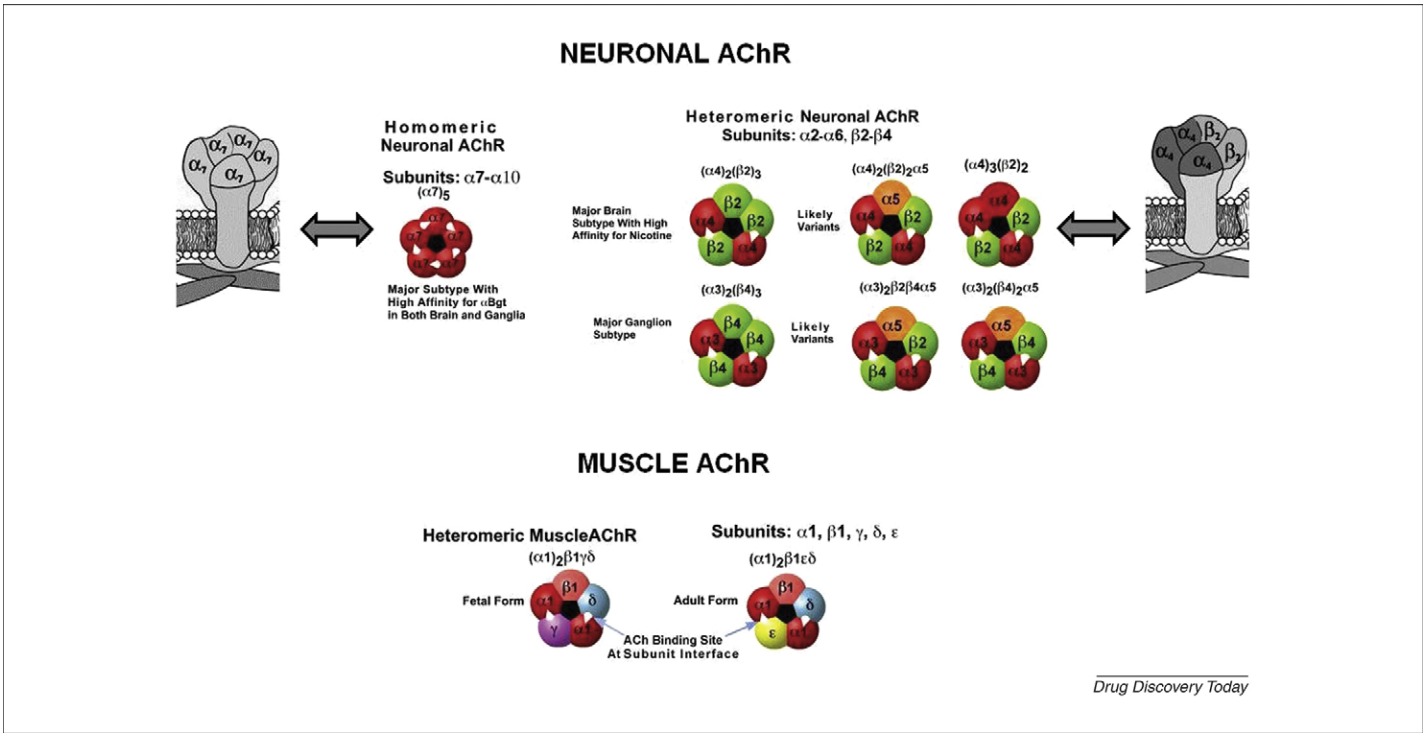

FIGURE 2

Three-finger α-CbT toxin. **(a)** *Naja kaouthia* snake, **(b)** α-CbT chemical structure Must be corrected for the Cys–Cys pattern, **(c)** HPLC profile of the venom-purified toxin, **(d)** α-CbT 3D structure with the β-sheet in yellow and the helical turn in red, **(e)** Structural model of the α-CbT–α7-nicotinic receptor complex (from 51), **(f)** hypothetical mechanism of action of the pro-apoptotic property of α-Cbt on A549 cells grafted *NOD/SCID*-treated mice.

the isolation, purification, subtypes classification or pharmacological and functional characterization of nAChR [57,89,90].

Three-finger toxins interacting on nAChR

The three-finger toxin family includes a vast diversity of neurotoxins from *Elapid* and *Hydrophiid* snakes' venoms that are characterized by a common structural fold. These toxins of 60–74 residues are rather flat molecules with three adjacent loops forming a large β-pleated sheet that emerge from a small globular core where four invariant disulfide bonds are embedded [91,92]. Local structural deviations may occur on this common scaffold, as the presence of an additional disulfide bond or variation in the size of the loops or the C-terminal tail. The structural plasticity of these three-finger fold toxins is correlated with their various functional diversity and selectivity toward their different molecular targets as, for example, the nicotinic and the muscarinic ACh receptors or the acetylcholinesterase [91,93]. Depending on their amino acid sequence and/or tertiary structures, α-neurotoxins (NT) interacting with the nAChR can be classified into (a) short-chain αNT, (b) long-chain α/κNT, (c) long-chain κNT and (d) nonconventional or weak αNT. Short αNT have four core disulfide bonds, whereas the long NT and nonconventional αNT have an additional fifth disulfide at the tip of the loop II and I, respectively [94–97]. Furthermore, each family can be associated with a particular pharmacological profile. Thus, short-chain αNT bind selectively with high affinity (subnanomolar range) to muscular-type nAChR only whereas long α/κNT interact efficiently with both muscular-type and neuronal α7-nAChR receptors. The long-chain κNT bind specifically to neuronal receptor subtypes (α3β2, α7) and the nonconventional αNT interact with relatively low affinity to α7- and/or muscular-nAChR [91,92].

Interestingly, Cobra's (*Naja* species) nAChRs exhibit resistance to Erabu sea snake short-chain αNT [98]. This effect correlates with the variations in αNT sensitivity of different species and, importantly, reflects the evolutionary conservation of the binding site on the nAChR polypeptide backbone *per se*. Phylogenetic analysis of αNT resistance suggests that αNT-resistant nAChR evolved first, which permitted the evolution of snake venom αNT.

α-CbT, a long α-NT, purified from the venom of *Naja kaouthia* has 71 amino acid residues (Fig. 2). This toxin can be expressed recombinantly in *E. coli* and the recombinant wild-type toxin is chemically, functionally and structurally indistinguishable from the venom toxin and is produced with a yield of approximately 1.2 mg/l of culture. By mutational analyses, the residues through which α-CbT interacts with the muscular-type or neuronal α7 receptor were identified [95,96]. The use of r-CbT will avoid conflicting results due to the contamination of 'venom-purified' drug as previously observed in the case of κ-bungarotoxin [99]. Erabutoxin-α (ETX-α) from the Erabu sea snake *Laticauda semifasciata* (isoform Eb) is a short-chain α-NT of 62 amino acids [100]. The long α-CbT and the short Erabutoxin have been used as selective ligands for studying nAChR [94,101,102]. These peptidic toxins appear unique among the ligands, because of their distinctive binding kinetics and remarkably high affinity and selectivity for the various nAChR subtypes. Hence, understanding their mode of interaction and defining the interface of the toxin–receptor complexes have been areas of substantial interest in neurobiology for four decades [92].

A lot of site-directed mutagenesis experiments have been performed to identify the functional determinants involved in the interaction of various three-finger fold toxins on their respective targets. These studies demonstrate that these toxins utilize, on the one hand, a common binding core to interact with key invariant residues on nAChR and, on the other hand, toxin-specific residues crucial in their selective recognition toward one receptor subtype [103]. Furthermore, using first cycle-mutant methodology and modelization of the α7 receptor extracellular domain [94] and by the resolution of the crystallographic structure of the Cobra-toxin pentameric acetylcholine-binding protein (AChBP) complex [104], the molecular origin of the antagonistic property of αNT on nAChR had been elucidated. Indeed, the tip of the central loop of the toxin plugs at the interface between two receptor subunits, preventing the ACh to reach its binding site.

More precisely, the crystallographic structure unambiguously revealed the positions and orientations of all five three-fingered toxin molecules inserted at the AChBP subunit interfaces and the conformational changes associated with toxin binding. AChBP loops C and F, which border the ligand binding pocket, moved markedly from their original positions to wrap around the tips of the toxin first and second fingers and part of its C-terminus, while rearrangements also occurred in the toxin fingers. At the interface of the complex, major interactions involved aromatic and aliphatic side chains within the AChBP binding pocket and, at the buried tip of the toxin second finger, conserved Phe and Arg residues that partially mimic a bound agonist molecule. Hence, this structure provides a lead template resembling a resting state conformation of the nAChR and for understanding selectivity of curare-mimetic αNT for the various receptor species [104].

Very recently [105] it was reported that the toxin-bound form was relatively stable. However, in the *apo* form, one subunit spontaneously moved away from the conformation of the other four subunits. This motion resembles what has been proposed for leading to channel opening. The molecular dynamics (MD) simulation results suggested a mechanistic model in which the *apo* form, although predominantly sampling the 'closed' state, can make excursions into the 'open' state. The open state has high affinity for agonists, leading to channel activation, whereas the closed state upon distortion has high affinity for antagonists, leading to inhibition.

With all of this knowledge [104–106] it might be possible to start to imagine how to design smaller inhibitors, or perhaps even agonists [107], that act selectively at different nAChRs.

As a final point, it must be highlighted that snake venoms are not the exclusive source of αNTs interacting with nAChRs; cone snail venoms also represent an important source of potent ligands [108].

α7-nAChR as a valuable molecular target for therapy of NSCLC and MPM

A direct connection between the activation of the α7-nAChR, induced by nicotine, and the proliferation of mesothelial and epithelial lung cells (normal and cancer cells) has been demonstrated [48,50,109–112]. On the basis of these results, it was therefore proposed that D-tubocurarine or α-CbT should be considered as potential anticancer agents [48,50,109–117].

To support this opinion it has been demonstrated that α-CbT caused *in vitro* (in A549 NSCLC–adenocarcinoma- or MPP89–MPM-cell line): (i) clear concentration-dependent cell growth decrease; (ii) mitochondrial apoptosis characterized in A549 cells by (a) inhibition of BAD phosphorylation at Ser^{112} and Ser^{136}, (b) BAD dissociation from 14-3-3, (c) BAD association with BCL-XL and (d) cleavage of caspase-9 while in MPP89 cells by: (e) changing in mitochondrial potential, (f) cleavage of caspase-3, (g) downregulation of mRNA and protein for Survivin, XIAP, IAP1, IAP2 and Bcl-XL, (h) inhibition by caspase-3 inhibitor; and finally (iii) down-regulation of basal high levels of activated NF-κB. As a result of these processes, α-CbT treatment produced a significant reduction of tumor growth in nude mice orthotopically grafted with A549–luciferase cells or in *NOD/SCID* mice orthotopically grafted with MPP89–luciferase without any signs of significant toxicity [113–117].

In vivo, the α-CbT acute LD_{50} was 0.15 μg/kg (LD_{10} = 0.12; LD_{90} = 0.20 μg/kg). The LD_{100} [0.24 μg/kg] induced fatal respiratory failure and massive kidney necrosis characterized by tubular necrosis associated with hemorrhage as the main histomorphological finding. Indeed, the adrenal gland showed necrosis and hemorrhage, involving both cortex and medulla, with the presence of some apoptotic cells. Liver showed only diffuse dilatation of the central veins, while lung had focal alveolar hemorrhage and perivascular and interstitial transudate, with edematous widening of alveolar *septa*. In antitumoral experiments, animals receiving intravenous injection of 0.12 ng/kg α-CbT (1/1000 of LD_{10}) three times a week for two months showed no signs of lethality or toxicity such as: (a) histological alterations (lung, kidney, liver, brain, spleen, heart and pancreas), (b) body and organ weight loss, (c) serum chemistry alterations, (d) hematological, (e) serum kidney and (f) liver enzymes alterations. Moreover, mice did not show any alteration in neurological behavior when assessed using tests involving autonomic, convulsive, excitability, neuromuscular, sensory-motor and general motor activity domains [113,115].

Furthermore, it has been reported that the expression of α7-nAChR in human NSCLC tissues was higher in smoker patients affected by SQ than in those affected by AD and among male smoker patients more than in females [48]. The entire set of results supported the hypothesis that a major expression of α7-nAChR is related to a major activation of the Rb–Raf-1/phospho-ERK/phospho-p90RSK pathway [48].

To demonstrate that α-CbT might be an 'efficacious adjuvant therapy' for NSCLC and MPM, these observations were expanded to a panel of NSCLC or MPM cell lines and primary cultures of different histological subtypes and reported consistent outlines of the expression patterns and level of this receptor. The final endpoint of the experiment was the recording of the survival periods of each of the NSCLC-bearing *NOD/SCID* mice instead of the reduction of tumor masses as evaluated previously. An optimized schedule for multiple intravenous injections of Cisplatin [0.5 mg/kg once a week for three weeks (G. Sozzi, National Cancer Institute, Milan, Italy, pers. commun.)] was used to mimic current chemotherapy in clinic. Treatment started seven days after cell implantation, when cells were well implanted and tumors were moderately grown, as evaluated by bioluminescence and/or histopathologic examination. In this model the optimal chemother-

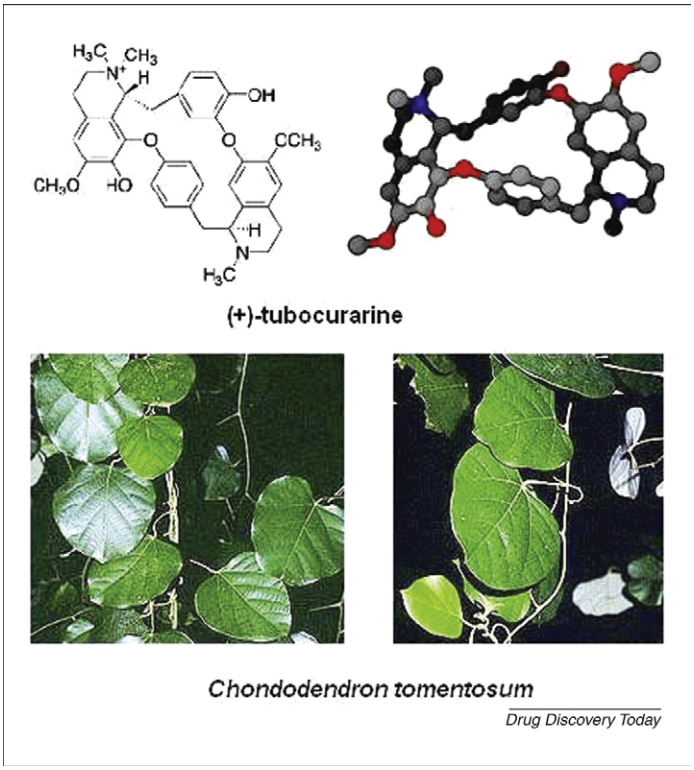

FIGURE 3

nAChR are ligand-gated cation channels that are composed of five subunits, and can exist either as homopentamers or heteropentamers (i.e. five identical subunits or five nonidentical subunits, respectively) [18–20]. Currently, 12 neuronal and 5 muscle nAChR subunits have been identified (neuronal: α2–α10, β2–β4; muscle: α1, β1, γ, ε and δ). Subunits capable of forming homomeric nAChR are α7–α9, whereas α10 forms a heteromer with α9).

apy by i.v. Cisplatin had limited improvement of median survival of mice by 16% versus no treatment control. α-CbT significantly increased the median survival by 1.7- and 2.1-fold compared with Cisplatin treatment and the no treatment control, respectively. The increased life span of α-CbT was 80% and 93% higher than that of Cisplatin and no treatment groups. In this model the number of cells positive to Ki67, a marker of cell proliferation, or CD31, a marker of angiogenesis decreased in α-CbT-treated mice. Captivatingly, Western-blotting experiments revealed that cells obtained from α-CbT-treated animals displayed a marked reduction of the level of Snail protein expression and consequently a reduction of fibronectin and an increase in E-cadherin proteins expression suggesting that α-CbT treatment could encourage an epithelial phenotype restoration [48,116].

Information on the relationship between quantitative expression of the receptor and target inhibition by the α-CbT have been clearly reported along with the above-mentioned evidence [48,109,113,115,116]. This is a crucially relevant point for the provision of any clinical application because (a) patients' stratification according to the level of receptor expression might be useful in the classification of the pathology and (b) because α7-nAChR seems to be ubiquitously expressed (positively detected in all unaffected lung and tumor pairs examined [48,109,113,115,116]. Finally, it has been shown that α-CbT did not affect normal cells [109,113,115,116]. Little is known on the relationship between the amount and the number of these receptors and responses to their antagonists. In a preliminary set of experiments, it has been reported that human unaffected mesothelial cells and human proliferating T-lymphocytes were not affected by α-CbT [115]. Consequently, an important issue to be resolved is why α-CbT did not affect the proliferation of unaffected (normal) cells. A possible explanation is that the lack of effect might be correlated to the different number of binding sites that are effectively present in unaffected or tumoral cells. Indeed, it has been shown that, independent of the histological subtype, NSCLC cells expressed more α7-nAChR receptors than 'normal' bronchoalveolar cells, which were almost insensitive to α-CbT effect. Among NSCLC cells, those that displayed a major number of receptors appeared more responsive to α-CbT. Similar experiments performed on other unaffected cells, namely: human mesothelial, aortic endothelial, bladder epithelium and oral keratinocytes confirmed these results. These data tend to support the hypothesis of a correlation between the amount of the number of α7-nAChR and sensitivity to α-CbT [116]. This outcome is not completely unexpected, since a previous study reported a significant relationship among higher levels of expression of the α7-nAChR and increased response to stimuli [118].

To check the specificity of α7-nAChR/α-CbT, the effect on survival in *NOD/SCID* mice orthotopically grafted with A549 cells treated with (i) recombinant-α-CbT (rα-CbT), and (ii) weakly active mutated α-CbT (CbT-R33E) as compared to wild-type α-CbT [116] has been measured. Grafted mice treated with rα-CbT survived longer than mice treated with the vehicle alone or CbT-R33E. This experiment supported the hypothesis that the antitumor activity is related specifically to α-CbT and not to some unknown substances present on the purified natural toxin, and reinforced prior results showing that α-CbT did not cause any cell death in A549-si-mRNA (α7-nAChR) cells [114], sustaining the postulation that its antitumor activity passes through α7-nAChR inhibition.

Conclusions and future prospects

In conclusion, the results of these studies showed that α-CbT, a powerful high affinity α7-nAChR inhibitor, induces antitumor activity against NSCLC and MPM by triggering apoptosis. The prolonged survival of α-CbT-treated animals in a mouse model of NSCLC and MPM is most probably the result of multiple

FIGURE 4

Three finger toxin Erabutoxin Erabutoxin-a (ETX-a) chemical structure, on the left, from the Erabu sea snake Laticauda semifasciata, on the right.

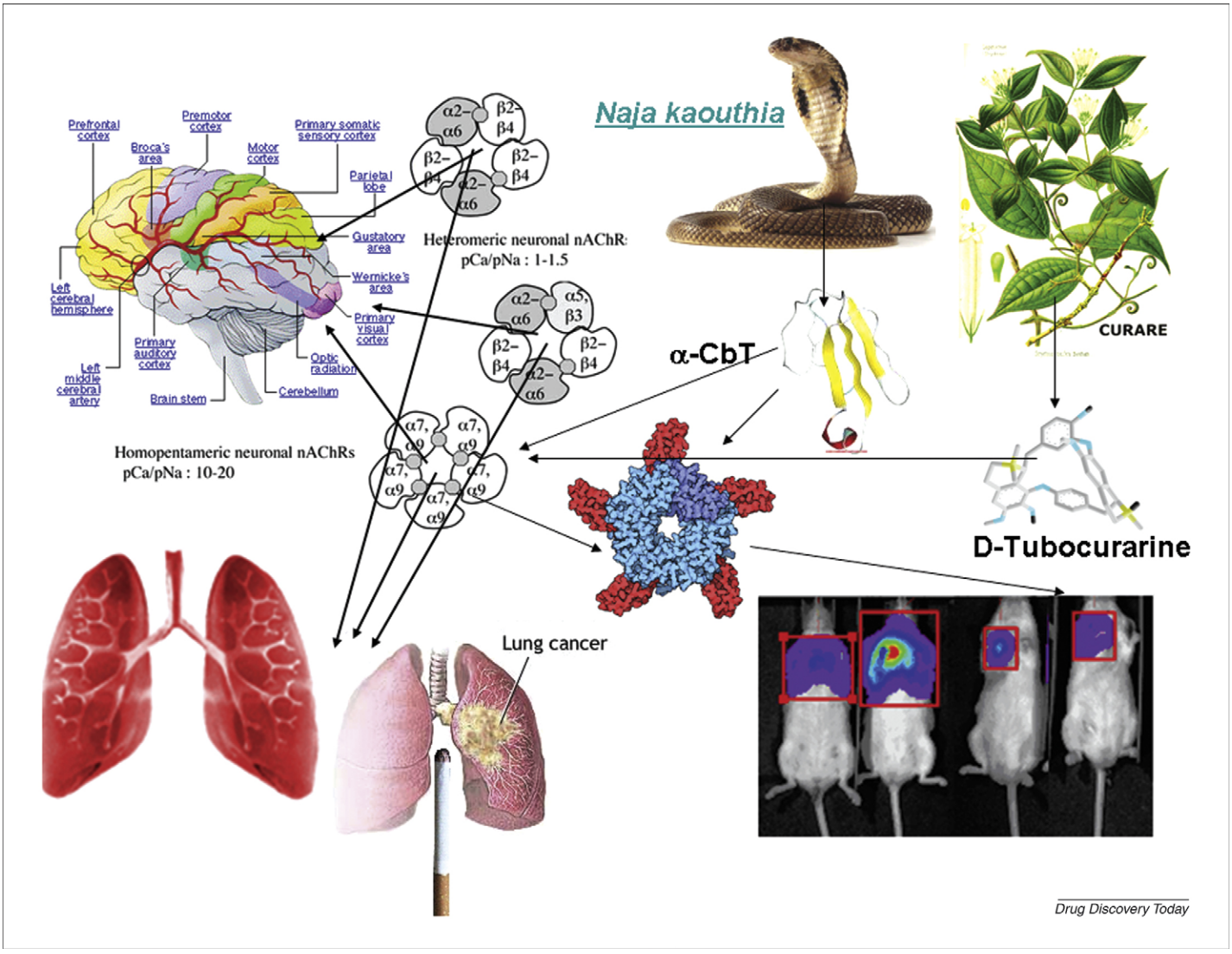

FIGURE 5

Our working hypothesis, considering a7nAChR antagonists as possible anticancer agents.

mechanisms, including different antiproliferative and antiangiogenic effects.

On the contrary, we are aware that in the *Nature* reports, referred above [7–9] none of the groups reported an association with the *CHRNA7* gene. Indeed, the region where the identified SNPs lie is known to contain the genes for the nAChR subunits *CHRNA3*, *CHRNA4* and *CHRNA5*. At this point we have to remember again that neuronal nAChR are assembled from five αβ transmembrane subunits and the combinations include α2–α6 and β2–β4 [16]. Subunits capable of forming homomeric nAChR are α7–α9, whereas α10 forms a heteromer with α9 (Fig. 3). However, subunits forming heteromers or homomers are not completely distinct because the subunits from these separate classes are also capable of combining to form nAChR [16].

Additionally, we have to consider that nicotine could also determine an effect at a post-transcriptional level in a functional manner, as reported by different studies [55,97,119–123]. Importantly, one of these studies [123] showed that if α7 receptors are intensively desensitized, the loss of function may not be fully compensated by other coexpressed Ca^{2+} channels. This last observation confirmed the previous one reporting that α7-nAChR has unique pharmacological properties, compared with other nAChRs [44]. Additionally, Lam *et al.* [47] have identified differential gene expression in NSCLC with class predictive significance: short-term nicotine exposure corresponded with an upregulation of *CHRNA1*, *CHRNA5* and *CHRNA7* subunit genes. These subunit genes could play an important role in the pathogenesis of bronchogenic carcinoma and may mediate the effects of nicotine addiction in lung cancer patients of different sex.

Some important results obtained in schizophrenia (SZ) disease might be of help in understanding the role of α7 receptor at a post-transcriptional level. As reported above the gene encoding the α7 subunit resides in a chromosomal region (15q13–14) that has repeatedly demonstrated positive genetic linkage to SZ [124]. Negligible differences in α7 mRNA levels between disease and control states have led to conclusions that cholinergic dysfunction in (SZ) must occur post-transcriptionally [125]. Alternatively, it was, recently, proposed that the dysregulation of splice variants of the α7 receptor could account for cholinergic deficiencies observed

in this disease [126]. These data demonstrated that α7 transcription is altered in several ways in SZ, suggesting that transcription-level mechanisms could account, in part, for the impaired cholinergic neurotransmission observed in this disease. These observations imply that if some α7 isoforms are functional, certain variants, as well as others associated with both the full-length and duplicated α7 loci, may translate as novel subunits, whereas others may also function as antisense regulators of α7 expression. Additionally, they may be regulatory in nature or may contribute to altered ligand-gated ion channel activity.

All of these findings supported the 'special' role played by α7 nicotinic receptors and reinforced our hypothesis that the inhibition of α7-nAChR by antagonists might represent a strategy to treat NSCLC or MPM (Fig. 4). Various animal venom toxins, antagonists of AChR such as α-bungarotoxin from the *Bungarus multicinctus* snake and conotoxins purified from the venom of *Conus* marine snails have previously been used as molecular tools for the characterization of neuronal and non-neuronal nAChR in cells [16]. A direct cytotoxic effect of venom components against cancer cells has, however, to the best of our knowledge, never has been investigated and demonstrated. Several very early reports, that did not include any mechanistic aspect [127,128], or naïve (at best) reports described some anticancer activity associated with the snake venoms. Our group, following a policy of independent research, has a timely established interest in the characterization of the therapeutic (anticancer) potential of some natural compounds coming from libraries in Europe, financed exclusively by National Research Institutes and Universities, among these plant and snake venoms. In this setting, we have clearly demonstrated that α-CbT as a result of multiple mechanisms, including different antiproliferative and antiangiogenic effects, has a potent antitumor effect against NSCLC or MPM (Fig. 5).

The idea to use venom's peptides as therapeutic agents in the treatment of human diseases is not new but, only recently, has become a reality. In this setting, it has to be borne in mind that snake venom's derived compounds (peptides, proteins) have been used as lead molecules to develop novel strategies in antithrombotics, antihemorrhagic, antihypertensive or defibrinogenating therapy [129]. As a consequence of their high selectivity, venom peptides have proved particularly useful for *in vitro* and *in vivo* proof-of-concept studies. For therapeutic applications, however, several issues associated with safety, pharmacokinetics and delivery need to be addressed.

Acknowledgements

This review is devoted to the memory of Dr André Ménez, President of the French Natural History Museum, a world renowned expert in the molecular and structural biology of nicotinic and muscarinic receptors and three-finger fold toxins; and to the memory of Dr Cassian Bon, President of SFET (*Société Francaise pour l'Etude des Toxines*).

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