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Ciclo XXIX

Pharmacological and functional characterisation of neuronal nicotinic acetylcholine receptors in lung cancer cell lines: a challenge for new therapeutical strategies?

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ABSTRACT

Lung cancer is the leading cause of cancer-related deaths worldwide and cigarette smoking is related to 90% of all deaths due to lung cancer.

Tobacco smoke contains many classes of carcinogens and, although nicotine, the addictive and most active component of tobacco smoke, is unable to initiate tumourigenesis in humans and rodents, it promotes tumour growth and metastasis by inducing cell-cycle progression, cell migration, angiogenesis and the evasion of apoptosis in a variety of systems.

Nicotine and its metabolites are highly lipophilic compounds that bind and activate a family of ligand-gated cation channels (the neuronal nicotinic acetylcholine receptors, nAChRs) that are widely expressed in the central and peripheral nervous systems.

Over the last few decades, the extra-neuronal localisation of nAChRs has been demonstrated in a large number of cell types including endothelial cells, glia, immune cells, lung tissue and cancer cells, indicating that they might have functions well beyond simple neurotransmission.

Recent studies have shown that most of the tumour-promoting effects of nicotine are primarily due to the binding and the activation of nAChRs, which lead to downstream intracellular signalling cascades. However, identifying the receptor subtypes expressed in lung tumour cells and their signalling pathways is still in its early stage.

For these reasons, we investigated the pathophysiological role of nAChRs in lung cancer cells.

We found that different non-small cell lung cancer (NSCLC) cells express distinctive nicotinic receptor subtypes and that this variety affects nicotine-induced proliferation and migration. In the A549 adenocarcinoma cell line, nAChRs containing the α7, α9 and α5 subunits regulate not only the nicotine-induced cell proliferation and migration but also the activation of anti-apoptotic and proliferative pathways. Blocking nAChRs containing the α7 or α9 or α5 subunits with specific toxins or silencing their expression by means of subunit-specific siRNAs abolishes the nicotine-induced proliferation, migration and signalling.

Prompted by these results, we also studied oxystylbene compounds previously characterised by our group and started to syntehesise some new oxystylbene/resveratrol derivatives with specific modifications. We found that these 4-oxystilbene derivates act

on both α7 and α9-containing receptors and block NSCLC cell proliferation and viability in a dose-dependent manner.

These results highlight the pathophysiological role of specific nAChR subtypes in promoting NSCLC cell growth and migration and raise the possibility of targeting them in order to treat tobacco related cancer.

RIASSUNTO

Il cancro al polmone è la principale causa di decessi correlati al cancro in tutto il mondo e il 90% è legato al fumo di sigaretta.

Il fumo di tabacco contiene molte classi di sostanze cancerogene e anche se la nicotina, l'elemento principale e più attivo del fumo di tabacco, non è in grado di avviare tumorigenesi nell'uomo e nei roditori, essa è però in grado di promuovere la crescita tumorale, indurre la progressione del ciclo cellulare, la migrazione, l'angiogenesi e l'evasione dall'apoptosi in una varietà di sistemi.

La nicotina e i suoi metaboliti sono composti lipofili che legano e attivano una famiglia di canali cationici ligando-dipendenti (i recettori neuronali nicotinici, nAChRs) ampiamente espressi nel sistema nervoso centrale e periferico.

Nel corso degli ultimi decenni, la localizzazione extra-neuronale dei nAChRs è stata dimostrata in un gran numero di tipi cellulari, comprese le cellule endoteliali, gliali, cellule del sistema immunitario, il tessuto polmonare e le cellule tumorali, indicando che essi potrebbero svolgere funzioni diverse, oltre la semplice neurotrasmissione.

Recenti studi hanno dimostrato che la maggior parte degli effetti pro-tumorigenici indotti dalla nicotina sono principalmente dovuti al legame e alla conseguente attivazione dei nAChRs, che stimolano diversi *signalling* intracellulari. Tuttavia, l'identificazione dei sottotipi recettoriali nicotinici espressi nelle cellule tumorali del polmone e le loro vie di segnalazione sono ancora in una fase di studio primordiale.

Per queste ragioni, abbiamo studiato il ruolo fisiopatologico dei nAChRs in linee cellulari di tumore al polmone.

Abbiamo scoperto che diverse linee di carcinoma polmonare non a piccole cellule (NSCLC) esprimono distinti sottotipi recettoriali nicotinici e che questa varietà influenza la proliferazione e la migrazione cellulare indotta dalla nicotina. Nella linea cellulare di adenocarcinoma A549 sono espresse le subunità nicotiniche α7, α9 e α5, che regolano non solo la crescita cellulare e la migrazione indotte dalla nicotina, ma anche l'attivazione di vie intracellulari anti-apoptotiche e proliferative. Bloccando i nAChRs contenenti tali subunità con tossine specifiche o silenziandone l'espressione mediante siRNA specifici è possibile inibire gli effetti proliferazione, migrazione e attivazione di cascate intracellulari indotte dalla nicotina.

Spinti da questi risultati, abbiamo anche studiato i composti oxy-stilbenici, in precedenza caratterizzati nel nostro gruppo, e abbiamo iniziato a sintetizzare alcuni nuovi derivati

oxy-stilbenici o ricavati dal resveratrolo apportando modifiche specifiche. Abbiamo osservato che questi derivati 4-oxystilbenici agiscono sia sui recettori α7 sia α9 espressi in cellule di NSCLC e sono in grado di bloccare la proliferazione cellulare e la vitalità in maniera concentrazione-dipendente.

Questi risultati evidenziano il ruolo fisiopatologico di specifici sottotipi di nAChRs nel promuovere la crescita e la migrazione di cellule tumorali polmonari e forniscono la possibilità di poter migliorare la caratterizzazione di specifici composti al fine di poterli utilizzare per i casi di cancro legati al tabacco.

ABBREVIATIONS

α-Bgtx- α-Bungarotoxin

Abs- antibodies

AKT- serine/threonine protein kinase AKT

ALK- anaplastic lymphoma kinase

A549- human alveolar adenocarcinoma cell line

β–AR- β–arrestin

BAD- Bcl-2-associated death promoter

BAX- Bcl-2-associated X protein

CDK- cyclin-dependent kinase

CNS- central nervous system

CREB- cAMP response element-binding protein

DA- dopamine

ECM- extracellular matrix

E-cig- e-cigarette

EGF- epidermal growth factor

ERK- extracellular signal-regulated protein kinase 1 and 2

FBS- fetal bovine serum

GABA- gamma-Aminobutyric acid (γ-Aminobutyric acid)

GWAS- genome-wide association studies

ID1- DNA-binding protein inhibitor

H1975- human adenocarcinoma cell line

JAK- Janus kinase

LTP- long-term potentiation

MAPK- mitogen activated protein kinase

MLA- methyllycaconitine

NA- noradrenaline

NAc- nucleus accumbens

nAChR- nicotinic acethylcoline receptor

NAM- negative allosteric modulators

NNK- 4-(methylnitrosamino)-1-(3-pyridyl)1-butanone

NNN- N-nitrosonornicotine

NSCLC- non-small cell lung cancer

PAM- positive allosteric modulators

PFC- prefrontal cortex

PI3K- phosphatidylinositol-4,5-bisphosphate 3-kinase

PKC- protein kinase C

PNEC- pulmonary neuroendocrine cells

PSN- peripheral nervous system

RAF- proto-oncogene serine/threonine-protein kinase

ROS-reactive oxigen species

RT- room temperature

SCLC- small cell lung cancer

siRNA- small interfering RNA

SNP- single nucleotide polymorphism

SRC- tyrosine-protein kinase Src

STAT- signal Transducer and Activator of Transcription

VTA- ventral tegmental area

WHO- world health organisation

XIAP- X-linked inhibitor of apoptosis protein

INTRODUCTION

Foreword

Tobacco smoke is a complex and reactive mixture of about 5000 molecules; a toxic and carcinogenic combination that represents one of the most significant causes of dangerous chemical exposure and chemically mediated diseases in humans (Fowles J et al., 2003). The World Health Organisation (WHO) has stated that 5.4 million of preventable deaths a year are due to tobacco smoking worldwide (WHO, 2008) and it has been estimated that this number double in 2025 (Hatsukami et al., 2008).

ISS/Doxa data showed that in Italy 11.5 million people are smokers (6.9 million men and 4.6 million women) with the highest prevalence occurring among those aged 25-44 years of both sexes (24.1% of women and 31.9% of men). The prevalence of male smokers is in the Central regions of Italy (30.4%) while the prevalence of female smokers is in the North (19.9%).

Every smoker smokes about 6.5 kg of tobacco per year with an average consumption of 1600 cigarettes. These numbers are increasing not only in the industrialised world but also in the third world where, since 1970, the consumption of tobacco increased by 67%.

Given these data, it is clear how smoking is a health and social problem that must be confirmed by public health agencies throughout the world.

Many smokers have recently switched to e-cigarettes (E-cigs) as an alternative mean of nicotine delivery. E-cigs are devices that produce an aerosol by heating a liquid that contains a solvent (vegetable glycerine, propylene glycol, or a mixture of both), one or more flavourings and nicotine. The inhalation of this aerosol leads to peak serum nicotine concentrations within five minutes. This rapidity of systemic delivery, and the fact that E-cigs look, feel and taste like traditional cigarettes, but do not contain toxic combustion products, had led them being increasingly used. Dawkins et al. (2012; 2013) found that smokers using E-cigs had less desire to smoke, fewer symptoms associated with abstinence from tobacco, improved prospective and working memory. Furthermore E-cigs, as are less harmful product, are increasingly used as a mean of reducing or stopping smoking despite the contrary recommendation of WHO (World Health Organization, 2008). However, as reported by the Food and Drug Administration (USFAD), many studies have shown the harmfulness of E-cigs, which contain carcinogens and toxic compounds leading to the idea that more scientific studies are necessary to define their safety and their real benefits.

Statistical analyses have shown that in Italy the E-cig consumption is variable among the population being from 4% in the 2014, 1,1% in 2015 and increasing in this year (3,9% in 2016).

The harmful effects of tobacco do not end with smoking because every year thousands of non-smokers die from heart disease and lung cancer and many children suffer from respiratory infections caused by the exposure to second-hand smoke (Rockville, MD, Public Health Service, Office of Surgeon General, 2010).

Tobacco is responsible for the onset of the 25% of cardiovascular diseases (including hypertension, stroke, coronary aortic aneurysms), 80% of chronic respiratory disease (bronchitis and emphysema) and 90% of lung cancers.

Moreover, it is also associated with reproductive disorders, increased likelihood of developing cataracts and in women it contributes to decrease the bone density that leads to an increase in the percentage of fractures (HHS, 2004).

We can conclude that there is no risk-free level of exposure to tobacco smoke, and there is no safe tobacco product.

1. The addiction to smoke

Tobacco addiction represents one of the most social and medical problem worldwide. The percentage of people that try smoking and then becomes smoker is very high, as is high the difficulty to quit smoking and the facility to relapse.

The age of smoking onset and the cigarette consumption are two important elements, which can lead to smoke addiction.

The long-term exposure to nicotine produces biological adaptations, which can be translated in tolerance and need of higher nicotine doses to feel the same effects.

1.1 Tobacco components

Tobacco smoke is a complex and dynamic chemical mixture, composed by gas and particulate portions. Tobacco is so dangerous for human health because it is composed by several toxic components, which can lead to different toxic effects, as seen before.

Tobacco components include different classes of chemicals, gas and particulate portions responsible for tobacco harmfulness.

Carbon monoxide, an odourless and tasteless gas, has chemical affinity for haemoglobin over 200 times greater than oxygen. It binds the haemoglobin reducing the amount of oxygenated blood circulating in the body organs possible leading to coronary heart disease.

Cigarette smoke contains also relatively high levels of nitrogen oxide: this gas is known to cause lung damages in experimental animals similar to that found in smokers, and may be responsible for initiating lung damage leading to emphysema.

Moreover, there are many compounds that affect the cilia present in the respiratory tract (that filter out dust and other particles that enter to the nose with the breathed air), among which hydrogen cyanide, acrolein, ammonia, nitrogen dioxide and formaldehyde. In particular hydrogen cyanide has a direct interfering effect with the "cleaning" system that results in an accumulation of toxic agents in the lungs that increases the likelihood of developing diseases.

Tobacco smoke contains also more than thirty metals including cadmium, nickel, arsenic, chromium, which are associated with human cancers.

1.1.2 Nicotine and its derivatives

Nicotine, the addictive and most active component of tobacco smoke, was first isolated from the tobacco plant *Nicotiana tabacum* (Solanaceae) in 1828 by German chemists Posselt and Riemann.

Nicotine is a tertiary amine consisting of a pyridine and a pyrrolidine ring (3-(-1methyl-2-pyrrolidnyl) pyridine); in tobacco is largely present the levorotary (*S*)-isomer and only 0.1 to 0.6% of total nicotine content is (*R*)-nicotine (Figure 1).

It is a volatile and lipophilic molecule with an absorption and renal secretion highly pH dependent. Alkaline environment increases its absorption in the non-ionized state, which crosses more rapidly the lipid membranes (Benowitz N. 1999).

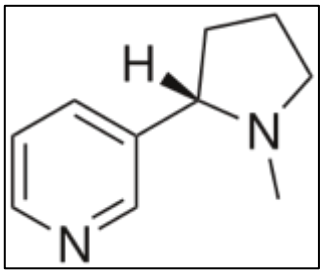


Figure 1. Nicotine structure

Tobacco smoking releases nicotine, which rapidly enters into the lungs, in the large surface area of the alveoli and small airways and undergoes dissolution in pulmonary fluid. It is then transported to the heart and immediately passes into the brain reaching a brain concentration of 100–500 nM, a concentration comparable to that found in animals that self-administer nicotine.

About the 70-80% of human nicotine is converted by the liver enzyme CYP26 in cotinine, an important metabolite used as marker of tobacco consumption. The half-life of nicotine is about two hours whereas that of cotinine's is much longer (Hukkanen et al., 2005).
Animal studies have demonstrated that cotinine is a psychoactive and behaviourally active (Hatsukami et al., 1997) compound whereas human clinical studies have shown the opposite effects, indicating that cotinine has an antagonist effect (Keenan et al., 1994).
The polycyclic aromatic nitrosamines N-Nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are two nicotine combustion derivatives very harmful and dangerous for human health. NNN and NNK are able to directly bind the DNA and induce modifications, which can lead to mutations and genotoxic effects.
In the brain nicotine stimulates different cerebral areas involved in nicotine addiction and reward through the activation of neuronal nicotinic cholinergic receptors (nAChRs)(Hatsukami et al., 2008). Brain imaging studies demonstrate that nicotine increases activity in the prefrontal cortex, thalamus and visual system consistent with the activation of corticobasal ganglia-thalamic brain circuits (Brody, 2006).
The nicotine-induced psychoactive effects, both positive effects, such as pleasure, decrease in stress, improvement in concentration and negative such as addiction, withdrawal, increase in irritability, depression, insomnia and anxiety are due to the nicotine modulation of the release of the neurotransmitters in the different brain areas (Hughes, 2006).

2. Neuronal nicotinic acetylcholine receptors

The cholinergic pathways in the central nervous system (CNS) of animals and humans have always been considered highly relevant to cognitive and behavioural functions. Until a few years ago the key molecules transducing the cholinergic message were thought to be the muscarinic receptors, the role of neuronal nicotinic receptors (nAChRs) has recently been re-evaluated.
nAChRs are a heterogeneous family of ion channels involved in a wide range of physiological functions both in the central nervous system (CNS) and in the peripheral nervous system (PNS).
nAChRs are widespread distributed in the brain with a preferential localisation at the presynaptic and/or preterminal sites, where they regulate the release of several neurotransmitters, either excitatory or inhibitory (Gotti et al., 2006). As a consequence

they can have opposite modulatory effects on the same circuit, depending on the inhibitory or excitatory nature of stimulated neurons. nAChRs are expressed also at the somatodendritic postsynaptic terminals, where they regulate neuron depolarisation, firing and long-term potentiation (LTP) (Hurst et al., 2013). Moreover these receptors are also involved in proliferation, differentiation and migration of neural progenitors (Liu et al., 2007; Campbell et al., 2010).

Much of our knowledge has been obtained by studying genetically modified mice in which nAChR subunit genes have been knock out (KO) or modified (knock-in). These studies have provided important knowledge on the physiological role of different nAChR subtypes. nAChRs contribute to cognitive function, and their decline or dysfunction has been observed in neurodegenerative diseases and mental illness. In addition genetic studies have linked nAChRs to epilepsy and schizophrenia, and studies of KO or knock-in mice have shown that they are involved in pain mechanisms, anxiety and depression (Gotti et al., 2004).

nAChRs are particularly important in two critical periods of brain life: early pre- and post-natal circuit formation, and age-related cell degeneration. They are involved in neuronal survival, as it has been shown that nicotinic agonists are neuroprotective in both *in vivo* and *in vitro* models (Quik et al., 2015; Kalappa et al., 2013). Furthermore, it is becoming evident that the perturbation of cholinergic nicotinic neurotransmission can lead to various diseases during development, adulthood and aging (Changeux and Edelstein, 2001).

2.1 Structure of the nAChRs

nAChRs belong to the superfamily of homologous receptors called Cys-loop ion channel receptors, which include muscle-type nAChR, $GABA_A$, $GABA_C$, glycine and serotonin 5HT3 receptors (Gotti et al., 2009).

nAChRs are a family of ligand-gated ion channels permeable to mono- and divalent cations. They are expressed in the CNS and PNS and share a common basic structure consisting of five subunits distributed around a central pore, permeable to cations. The nine α (α2–α10) and three β (β2–β4) subunits identified so far can assemble in a high variety of combinations, conferring specific functional and pharmacological features to receptor subtypes.

nAChRs are formed by the pentameric assembly of identical (homomeric receptors) or homologous subunits (heteromeric receptors). Two main classes have been identified: the α-Bungarotoxin (α–Bgtx)-sensitive receptors, which are made up of the α7, α8, α9

and/or α10 subunits and can form homomeric or heteromeric receptors, and α–Bgtx-insensitive receptors, which are heteromeric receptors that consist of α2-6 and β2-4 subunits, which bind nicotine and many other nicotinic agonists with high affinity, but not α–Bgtx.

All the different subunits have a common architecture consisting of a large N-terminal extracellular domain followed by four transmembrane (TM) domains and a large cytoplasmic loop between TM3 and TM4 followed by a short extracellular carboxyl domain (C-domain) (Zoli et al., 2015) (Figure 2).

The M1–M4 transmembrane domains are arranged in concentric layers around the central aqueous pore: the M2 domain lines the pore membrane, M1 and M3 shield M2 from the surrounding lipid bilayer, and M4 is in contact with lipids.

The subunits are arranged around a central pore and site-directed mutagenesis experiments have identified in the transmembrane M2 domain the important residues for the ion selectivity, permeability and channel gating of the receptors. The intracellular loop contributes most of the cytoplasmic domain of these receptors and includes multiple interaction sites for putative trafficking and postsynaptic scaffold proteins as well as phosphorylation sites for diverse serine/threonine and tyrosine kinases (Changeux, 2010). The α-subunits present adjacent cysteines (192 and 193) involved in the agonist binding specificity, whereas β subunits do not have such cysteines (Changeux and Edelstein, 1998).

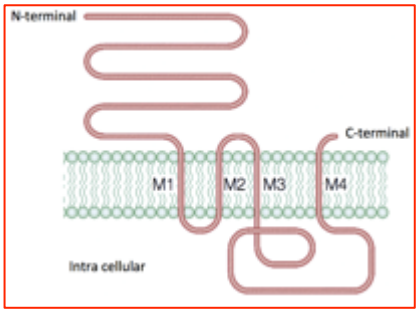


Figure 2. nAChR protein structure (Modified from Karlin, 2002)

Upon agonist binding to pentameric nAChRs, the domains of each subunit rearrange and open the central pore to allow ion flux through the channel for a few milliseconds, after which the receptor closes to a non-conducting state. All the subunits participate to channel kinetics, ion conductance and selectivity (Albuquerque et al., 2009) and their composition determines the specific functional and pharmacological features of the receptor.

Chronic exposure to a low nicotine concentration (such as that present in the blood of smokers) leads to consistent receptor desensitisation, which stabilises the receptor in a closed state that is unresponsive to agonist (Gotti et al., 2009).

2.1.1 nAChR subtypes

Earlier studies designed to characterise the nAChRs in different brain areas were based on binding assays using nicotinic radioligands, which demonstrated that the nervous system contains at least two classes of putative nAChRs: one class that binds nicotineand nicotinic agonists with high affinity but not α–Bungarotoxin (α–Bgtx), and the other that binds nicotine and nicotinic agonists with μM affinity and α–Bgtx with nM affinity (Lindstrom, 1997; Gotti, et al., 1997; Hogg, et al., 2003).

The pharmacological heterogeneity of nAChRs revealed by these ligand studies was later confirmed and extended by means of the molecular cloning of a family of genes encoding twelve subunits and by studies their expression in heterologous systems.

The class of α–Bgtx receptors is composed by homo- and hetero-meric subtypes (α7, α8, α9, α7–α8, α9–α10), whereas the class of high affinity receptors is composed only by heteromeric receptors made of α2–α6; β2–β4 subunits (Gotti et al., 2004). In the case of heteromeric high affinity receptors the heterogeneity is very elevated with nAChRs made up of four different subunits.

The homomeric receptors are composed by five identical α–subunits with five (orthosteric) binding sites per receptor molecule (Figure 3A) whereas the heteromeric receptors are composed by different nicotinic subunits and have two orthosteric binding sites (Figure 3B) at the interface between α and β subunits.

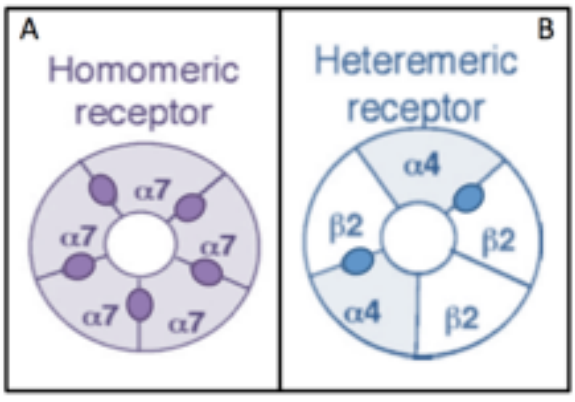


Figure 3. Representation of the structure in the homome ric α7 (A) and heteromeric α4β2 (B) subtypes and localisation of the ACh binding site (Modified from Gotti et al., 2004)

2.1.1.2 The α7 subunit variant CHRFAM7A

The α7 subunit is encoded by the 10-exon *CHRNA7* gene located on chromosome 15q14, which gives rise to a transcript that is translated into a protein of approximately 57 kDa. The neuronal α7 nicotinic receptor subunit gene (*CHRNA7*) is partially duplicated (α7dup) in the human genome and forms a hybrid gene with the novel *FAM7A* gene (*CHRFAM7A*, α7dup) whose transcript codes for a α7dup protein that has a molecular weight of 46.2 kDa and is unique to humans (Gault et al., 1998) (Figure 4 A and B).

The resulting subunit lacks the signal peptide and the ligand-binding domain for ACh.

The *CHRFAM7A* mRNA is expressed in the human brain at low level, while is abundant in peripheral lymphocyte cells and other tissues (Araud et al., 2011).

Several mutations have been mapped specifically on *CHRFAM7A* gene including a deletion of 2bp in exon 6 (*CHRFAM7A* Δ 2bp) (Figure 4C), which seems to be associated with schizophrenia (Sinkus et al., 2009). This particular mutation is more common in Caucasian populations than those of American and African.

Recent studies have shown that the CHRFAM7A cDNA injected in *Xenopus laevis* oocytes acts as a dominant regulator of α7 nAChR (de Lucas-Cerrillo et al., 2011; Araud et al., 2011). In oocytes, the co-expression of *CHRFAM7A* and α7 cDNAs reduces the ACh-evoked current by 53% and oocytes expressing the *CHRFAM7A* Δ 2bp form have a further decrease of 10% of the ACh-evoked current (Araud et al., 2011).

Pharmacological binding studies using the selective α7 ligand α–Bgtx in oocyte-expressing α7 subunit alone or α7 together with α7dup show a lower expression of surface binding sites, suggesting that the presence of the α7 dup might alter the level of the receptors inserted in the plasma membrane (Araud et al., 2011).

In the same year another group have shown that GH4CI cells transfected both with the α7- and α7 dup cDNAs have comparable protein expression level but a different localisation: α7 is primarily located at the plasma membrane while α7 dup in the endoplasmic reticulum (ER) (de Lucas-Cerrillo et al., 2011).

More recently, by using the Forster resonance energy transfer (FRET) technique it has been confirmed that the α7dup subunits are assembled and transported to the cell membrane together with full-length α7 subunits and these α7-α7dup receptors show altered receptor function (Wang et al., 2014).

Until now it is not clear how α7dup works and it has been hypothesised that it could interfere with the correct assembly of α7 receptors, leading to a decrease in the plasma

membrane insertion of them and/or the α7-α7dup receptors present in the plasma membrane have pharmacological properties different from that of the homomeric α7 receptors.

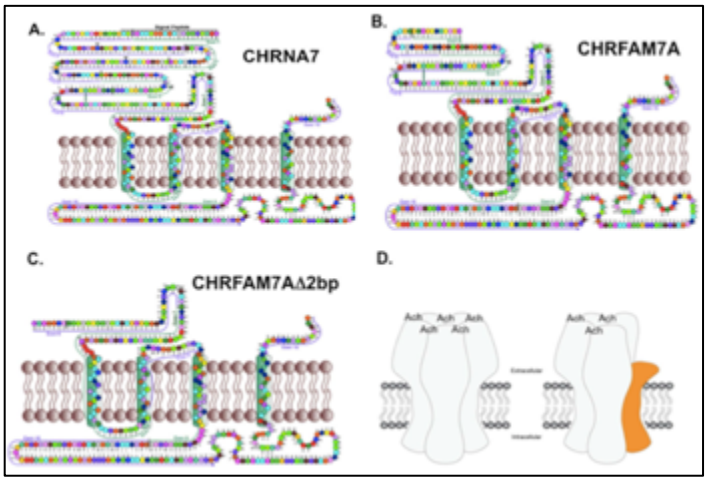


Figure 4. Structure of *CHRNA7,* CHRFAM7A and CHRFAM7AΔ2 bp gene products: (A) normal α7 subunit; (B) duplicated form (dupα7) and (C) *CHRFAM7A* gene with a deletion of 2bp in exon 6 (dupΔα7); (D) Pentameric structure of the normal α7nAChR (on the left) and a pentamer containing the peptide of the duplicated subunit (on the right) (Modified from Sinkus et al., 2015)

2.2 nAChR transition states

The difficulty in studying nAChR pharmacology is that in addition to structurally distinct subtypes, the nervous system has functionally distinct transition states for individual nAChRs.

Functionally, nAChRs can exist in three conformations: closed, open, and desensitised closed channel state (D or I). The agonists, by binding to the orthosteric site, determine a rapid transition of the receptor from the closed to the open state and stabilise the open state whereas the antagonists (competitive and non-competitive) stabilise the closed state. The competitive antagonists prevent the binding of agonists at the orthosteric binding site, whereas non-competitive antagonists interact at sites distinct from the orthosteric site (Hurst et al., 2013).

nAChRs have a transition from the agonist-bound open conformation, to a non-conducting desensitised state (D), either by dissociation of the agonist from the receptor, known as deactivation, or by an agonist bound conformational change to a high affinity (pM - nM), non-conducting state, called desensitisation. When this desensitisation state goes on for a long time the receptor enters in the inactive state (I) (Gotti et al., 2004).

Deactivation is the transition from the open state to the resting state associated with dissociation of the agonist from the ligand-binding domain.

The binding of ligands to the receptors at the neurotransmitter-binding site or in any of

the allosteric sites can modify the equilibrium between the different conformational states of the receptors.

The kinetics of the transition states depend on the different subtypes and the chemical nature of the agonist, which binds to the ligand-binding domain (Hurst et al., 2013), but is also regulated by the activity of kinases and phosphatases and/or allosteric modulators (Changeux and Edelstein, 1998; Quick and Lester, 2002).

2.3 Ligand-binding sites

2.3.1 The orthosteric achetylcholine binding site

Crystal structural studies of ACh binding proteins from the fresh water snail *Lymnaea stagnalis*, have given a significant contribution to the identification of the ligand binding site in nAChRs, allowed us to understand the molecular details of the nicotinic receptor binding site (Gotti et al., 2006). Snail glial cells produce this homopentameric soluble protein, which has an affinity spectrum resembling that of homomeric α7 or α9 receptors. Structural data concerning the crystallised ACh binding protein have revealed that the topology of the binding sites is very similar to that predicted by mutations and computer modelling.

The binding sites for the nAChR agonists are located in hydrophobic pockets that lie at the interface between two adjacent subunits. One subunit provides the main binding component, consisting of short non-contiguous sequences that form loops A, B and C and the other participate with the complementary component formed by loops D, E and F. In the case of homomeric receptors the same subunit contributes to both the principal and complementary component (Figure 5A). In the heteromeric nAChRs the principal component is carried by the α2, α3, α4 and α6 subunits while the complementary site by the β2 or β4 subunits (Figure 5B).

In order to fully activate homomeric nAChR, ACh needs to bind three binding sites at non-consecutive subunit interfaces whereas in the heteromeric receptors it binds two binding sites (Rayes et al. 2009).

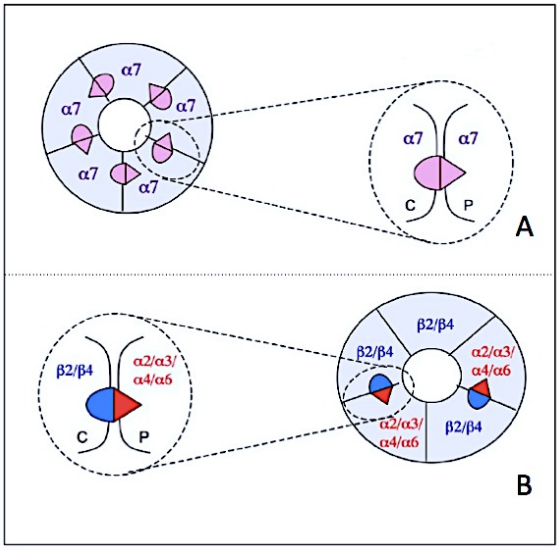


Figure 5. Localisation of the orthosteric binding sites in homomeric (A) and heteromeric (B) nicotinic receptors (Modified from Zoli et al., 2015)

The conformational changes that take place after ACh binding to nicotinic receptors have not yet been fully defined, but the binding site located in the extracellular portion of the α subunit is closely connected to the adjacent subunit. It is believed that the binding of the quaternary ammonium cationic head of ACh to a subsite present in loops A, B, C and D and the simultaneous attraction of negatively charged amino acids by quaternary ammonium residues of the adjacent subunits, close the two sites, and create a movement between the subunits that leads to the channel opening (Taly et al., 2009).

Interestingly, the α10 subunit, even if it is considered an α subunit, cannot act as principal component of the ACh binding site and operates only with the α9 subunit (Sgard et al., 2002).

Notwithstanding their initial classification in the α and β subunit list, α5 and β3 subunits carry neither the principal nor the complementary component of the ACh binding site but both form functional channels in the *Xenopus* expression system only when they are expressed with a ligand binding and a complementary subunit; for this reason they are known as non-ligand-binding (accessory) subunits (Lindstrom, 2000).

The accessory subunit α5 in the α4β2 receptors changes their pharmacological and biophysical properties with higher sensitivity to the allosteric modulator galantamine (Kuryatov et al., 2008).

It has been demonstrated that α5 is able to modulate ACh sensitivity in receptors composed by different subunits and in particular it increases the response of ACh in the α3β2 subtype but not if β2 is replaced with β4 in the α3β4 subtype, indicating a specific and selective activity of the α5 accessory subunit (Wang et al., 1996).

β3 seems to be important in the formation of α6β2* or α4α6β2* receptors, and its loss causes defects in nAChR assembly, degradation and/or trafficking (Gotti et al., 2006).
In conclusion the accessory subunits modulate and influence the pharmacological and functional properties of the receptors such as sensitivity to agonists, channel kinetics, permeability to calcium, correct assembly and membrane localization (Hurst et al., 2013; Taly et al., 2009).

2.3.2 Allosteric binding site

Allosteric binding sites are evolutionary less conserved and exhibit a greater structural diversity than ortostheric sites. Therefore, allosteric sites are more likely to allow selective targeting by synthetic compounds than the orthosteric ones (Yang et al., 2012).
Allosteric modulators are a pharmacological group of molecules that bind to the receptor at distinct allosteric ligand-binding site changing the free energy associated with transitions between functional states (Young et al., 2008).
These molecules have mainly low intrinsic activity and act as enhancer or inhibitor in the physiological activity of the nAChR in the presence of agonist. They are not able to induce the activation of the channel *per se* but co-operate with the agonist favouring or limiting the opening and the entry of ions.
The positive allosteric modulators (PAM) are molecules able to reduce the energy barrier from resting to activation state, increase the receptor affinity, the slope of the dose-response curve and the maximal effect of the responses. They can reduce the agonist concentration required to achieve channel opening and enhance the channel opening. When they are administered alone, they do not activate nicotinic receptors but they can increase the activation of endogenous nicotinic agonist, like choline or acetylcholine (Bertrand et al., 2007). On the contrary, the negative allosteric modulators (NAM) are compounds that increase the energy barrier between the resting and open states of the channel, increasing the concentration of agonist required for its activation (Hurst et al., 2013).
For the α7 nAChRs Daniel Bertrand and Murali Gopalakrishnan (2007) show the existence of two classes of positive allosteric modulators: called type I and type II. The first (i.e. NS-1738) predominantly affects the peak current response, whereas the second (i.e. PNU-120596) affects both peak current responses and the kinetics of agonist-evoked response.

2.4 Receptor stoichiometry

A further complexity in studying nAChR subtypes is due to the fact that in addition to the high level of subunit heterogeneity, nAChR subtypes, made by one type of α and 1 type of β subunit, can also show the same subunit composition but with different subunit stoichiometry.

The α4β2 and α3β4 subtypes can exist in two stoichiometries, made of either two or three copies of the α subunit in the channel pentamer. The two stoichiometries of the α4β2 subtype can be distinguished because the subtype with two α4 subunits ($(\alpha4)_2(\beta2)_3$) is activated at much lower ACh concentrations and is more sensitive to other agonists than the subtype with three α4 subunits ($(\alpha4)_3(\beta2)_2$) (Moroni, 2006). The two stoichiometries also have different Ca^{2+} permeability with the $(\alpha4)_3(\beta2)_2$ stoichiometry having a higher Ca2* permeability than the $(\alpha4)_2(\beta2)_3$ (Tapia et al., 2007).

Studies of transfected cells have shown that chronic exposure to nicotine up-regulates the expression of $(\alpha4)_2(\beta2)_3$ stoichiometry and normalises the intracellular subunit stoichiometry of nAChRs carrying mutations linked to autosomal dominant nocturnal frontal lobe epilepsy (Son et al., 2009).

In the case of the α3β4 stoichiometries the EC_{50} for ACh is not significant different, but they differ for the conductance and the single channel kinetic (Krashia et al., 2010).

Moreover only the $(\alpha3)_2(\beta4)_3$ subtype is susceptible to enhancement by low zinc concentrations.

2.5 Biosynthetic and secretory pathways of nAChRs

The assembly of nAChRs channels is a slow and inefficient process.

nAChR subunits cannot exit the ER until they have achieved their correctly folded conformation and the ER apparatus have evolved several mechanisms to regulate protein quality control. This quality control machinery prevents that dysfunctional subunits reach to the membrane and retro-translocated misfolded or unassembled proteins from ER to the cytoplasm for proteasomal degradation (Colombo et al., 2013).

Vertebrate neuronal nAChRs can assemble in many different combinations but it seems that native nAChRs have a restricted number of possible subunit combination (Millar and Gotti, 2009). For example in neurons the assembly depends mainly on the cell-type specificity and localisation.

Many chaperones proteins play a critical role in the folding, assembly and retention of immature nAChR subunits in the ER. This class of proteins are not only responsible for helping nascent polypeptides folding but they are also important in retaining the immature polypeptides within the ER. This retention increases the probability that the immature polypeptide will be correctly folded and that it does not prematurely enter to the secretory pathway. At the end, the prolonged retention of the misfolded or incompletely assembled polypeptide leads to its degradation.

In the case of nAChRs several chaperone proteins have been identified among which RIC-3, UBXD4 and VILIP-1. RIC-3 (Resistance to Inhibitors of Cholinesterase) protein interacts with a number of nAChR subtypes and enhances their functional response depending on the subtype or the host system (Millar, 2008). RIC3 increases the expression of certain nAChR subtype in particular the α7 subtype and in certain host cell types, in the absence of RIC-3 no functional receptors are expressed on the plasma membrane. RIC-3 probably associates with unassembled receptor subunits in the ER facilitating their receptor folding and assembly.

In two hybrid screening studies the calcium sensor protein VILIP-1 (visinin-like protein) (Lin, 2002) and UBXD4 (Rezvani et al., 2010) were found to interact with the intracellular loop between trasmembrane domains 3-4 of α3 and α4 subunits and these interactions significantly increase the α4 subunits steady-state levels. The greater availability of α4 subunit increases the formation of the α4β2 subtype and the surface expression α4β2 receptors.

UBXD4 is expressed both in neuronal and non-neuronal cells where it increases the amount of α3-containing receptors at the plasma membrane, possibly by preventing the ubiquitination and degradation of this subunit (Rezvani et al., 2010).

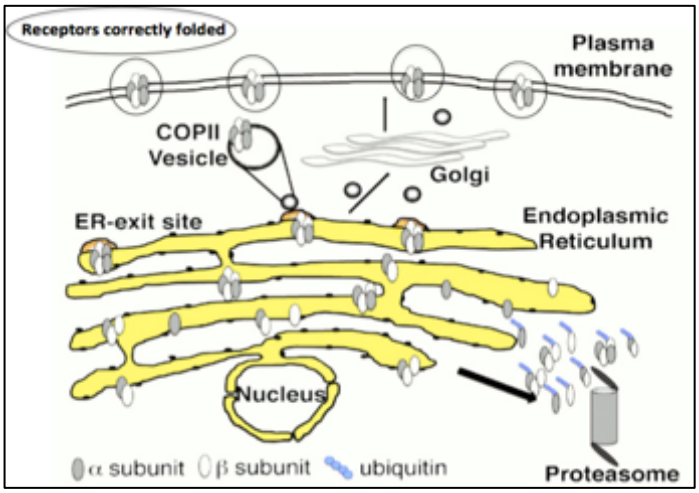


Figure 6. Schematic representation of the biosynthetic and trafficking pathways of nAChRs (Modified from Colombo et al., 2013)

The exit from the ER is the limiting step within the secretory pathway, pointing to an accumulation of receptors in the ER, as demonstrated for neuronal α4β2 AChRs (Sallette et al., 2005) (Figure 6). In the ER nAChRs that pass quality control are exported in coat protein complex II vesicles from the ER to Golgi, presumably after masking specific ER retention signals (Keller et al., 2001; Wang et al., 2002) and recognition of ER export motifs in properly folded and oligomerized nAChR subunits. After passing the Golgi the receptors are transported to the plasma membrane where depending on their localisation they carry specific functions: if they are expressed in the soma they influence the resting potential, whereas if they have a presynaptic localisation they can regulate the release of many neurotransmitters.

2.6 Native nAChR subtypes: localisation and composition

The nAChRs expression in the SNC is highly heterogeneous and their subunit composition is cell - and region-specific (Figure 7).

The identification of the nAChR subtypes expressed in different brain areas have been obtained using different techniques, which include *in situ* hybridisation, PCR, immunoprecipitation, receptor binding studies and autoradiography as well as functional assays (Zoli et al., 2015) using brain tissues from WT and KO mice.

The large majority of nAChRs in the nervous system contain one type of α and one type of β subunit. α4β2 receptors accounts for the 90% of the high affinity neuronal nAChRs in mammalian brain while the α3β4 subtype is expressed in the autonomic ganglia, adrenal medulla and in a subset of CNS neurons located in the medial habenula, dorsal medulla, pineal gland and retina (Gotti and Clementi, 2004).

Both the α4β2 and α3β4 subtypes may also contain the α5 subunit, whose presence is believed to increase the rate of channel desensitisation and calcium permeability and/or modify receptor localisation (Lindstrom, 2000).

mRNA distribution of the α3, α6, β3 and β4 subunits in brain shows that receptors containing these subunits have a more restricted localisation, with the α6 and β3 subunits expressed in the dopaminergic cells of the mesocorticolimbic and visual pathways and the α3β4 subunits in the habeno-interpeduncular pathway.

Biochemical and functional assays have characterised two principal nAChR subtypes (α6β2β3 and α6α4β2β3) in rodent striatum and retina containing the α6 subunit.

The distribution of sensitive α-Bgtx receptors was studied mostly by pharmacological binding experiments. These studies showed a high level of α7 in the cortex, hippocampus, subcortical limbic regions, while they are expressed at low level in the thalamic regions and in the basal ganglia (Gotti et al., 2006; Jones and Wonnacott, 2004).

Recent studies have identified a new α7-containing subtype containing the α7β2 subunits, which has been biochemically purified from mice and human basal forebrain, but not from human cerebellum (Moretti et al., 2014).

The receptors α9 and α10 are not present in the brain and they are only expressed in the cochlea and in certain ganglia where the α9 subunit forms homomeric channels, while the α10 subunit forms functional channels only when co-expressed with the α9 subunit.

α8 subunit is not expressed in mammals, but only in the chickens CNS where form homomeric and heteromeric α7- α8 receptors.

The α2–containing nAChR has a very restricted localisation in rodent brain (in the interpeduncolar nucleus) while in primates (human and monkey) this subtype is much more widely expressed (Han et al., 2000).

The physiological functions of nAChR subtype depend on their location on the plasma membrane: in pre- or postsynaptic domains, in the active zones of the synapse or extrasinaptic domains.

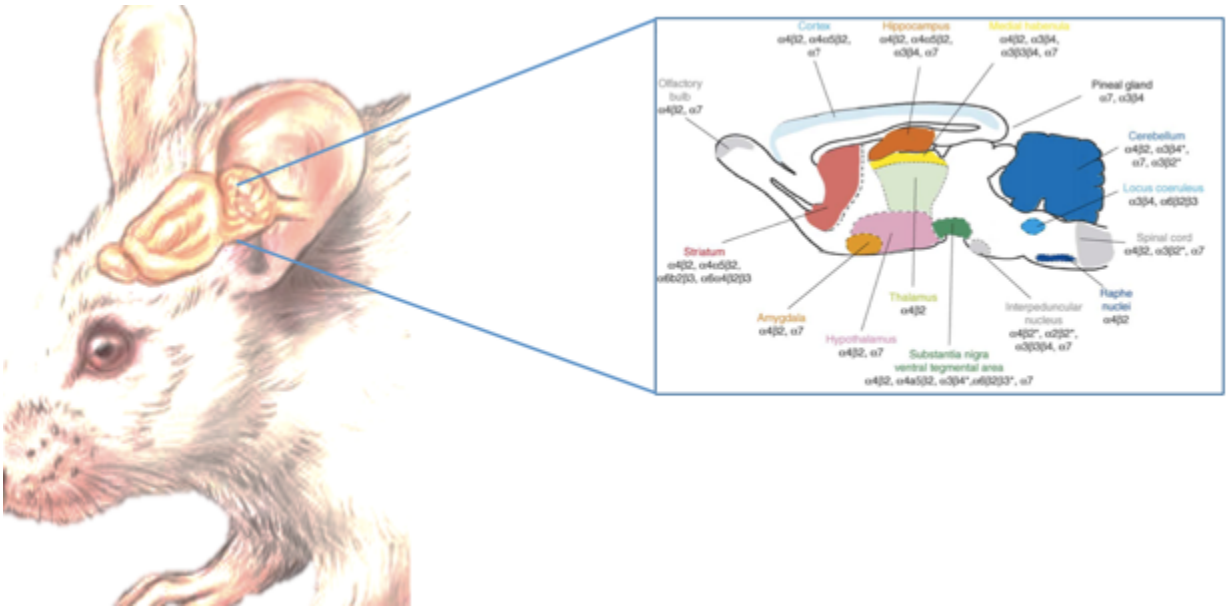


Figure 7. Regional distribution and subunit organization of the nicotinic acetylcholine receptors in rodents (Modified from Gotti C et al., 2006)

2.7 Regulation of native subtypes by chronic nicotine exposure

Nicotine induces psycho-stimulation, reward and reduces stress and anxiety. Nicotine, once in the bloodstream, rapidly crosses the blood brain barrier and accumulates in the brain where it exerts its pharmacological effects by binding the nAChRs (Changeux, 2010).

Chronic nicotine exposure induces neural adaptations that change cell physiology and behaviour and these effects are mainly due to the activation and/or desensitisation of nAChRs. Studies on animals and smoker brains, chronically exposed to nicotine, have shown an increase in the number of nAChRs (called up-regulation). The molecular mechanisms underlying the increased expression of cell surface nAChRs have been investigated *in vitro* and in *vivo.*

It has been found that in brains of mice treated chronically with nicotine there is an increase in ^{3}H-agonist labelled nAChRs, with no change in $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$ or $\beta2$ mRNA levels, indicating a post-transcriptional effect induced by nicotine. Autoradiography, immunoprecipitation and Western blotting experiments have shown that the $\alpha4\beta2$ is the most up-regulated subtype, but the presence of the accessory subunit $\alpha5$, in the $\alpha4\beta2\alpha5$ subtype, leads to the *in vivo* resistance to nicotine up-regulation (Mao et al., 2008).

The homomeric $\alpha7$ receptor is less up-regulated *in vivo* probably because this subtype needs very high concentration of nicotine that is not normally reached in the brain of smokers or animals treated with nicotine. *In vivo* chronic nicotine exposure decreases the number of $\alpha6*$ receptors expressed in midbrain dopaminergic neurons (Lai et al., 2005). The decrease of $\alpha6*$ is correlated with a decrease in $\beta3$-containing receptor, according to the fact that 70% of $\alpha6$*-receptors contain the $\beta3$subunit (Gotti et al., 2010).

In conclusion the *in vivo* effects of nicotine are very complex and depend on the nAChR subtype and the identity of the neuron expressing it. Moreover, the fact that several subtypes might co-exist in the same neuron suggests that the functional effect of nicotine depends on the neuronal circuit in which the nAChRs are expressed.

2.7.1 Molecular mechanisms for the up-regulation

Nicotine up-regulates nAChRs with post-transcriptional mechanisms: these processes are independent from the cell type and interfere with key steps of nAChRs (assembly, trafficking and cell surface expression).

Nicotine, by acting intracellular at the early stages of receptor biogenesis, increases the number of receptor subunits in the ER (Sallette et al., 2005) and induces a 5-fold increase

of the lifetime of AChRs on the cell membrane surface. Moreover Rezvani et al. (2007) have shown that nicotine reduce proteasomal degradation of the subunits and slowly stabilise the $\alpha4\beta2$ receptor in a high-affinity state for nicotine (Vallejo et al., 2005).

Nicotine also alter the subunit stoichiometry of nAChRs, in particular it shifts the $\alpha4\beta2$ receptor to the high affinity stoichiometry $(\alpha4)_2(\beta2)_3$ stoichiometry and these receptors are more efficiently transported to the plasma membrane

Chronic nicotine also affects the stoichiometry of the $\alpha3\beta4$ subtype towards the formation of the $(\alpha3)_2(\beta4)_3$ stoichiometry (Mazzo et al., 2013). Receptors with this stoichiometry are less prone to proteasomal degradation and more efficiently transported to the plasma membrane.

3. Nicotine effects on the CNS

The pharmacological effects of nicotine, the major reinforcing component of tobacco, on the CNS are due to its binding to presynaptic nAChRs expressed in different brain areas, where it stimulates the release of different neurotransmitters. Within a certain concentration range, nicotine binding activates nAChRs, but at the concentrations observed in the blood of regular smokers, it desensitises most heteromeric nAChRs and induces a long-term increase in receptor numbers known as up-regulation.

Recent studies suggest that the mesocorticolimbic system is responsible for the pleasant aspects of smoking that support the onset of nicotine dependence and is also responsible for the physical and physiological symptoms occurring during nicotine withdrawal (Livingstone and Wannacott, 2009; Picciotto and Kenny, 2013).

The mesocorticolimbic system is the central mediator of nicotine reward and reinforcement, and this neural pathway connects the neurons present in the ventral tegmental area (VTA) with two principal targets: the nucleus accumbens (NAc)) and the prefrontal cortex (PFC). Dopamine (DA) neurons that project to the NAc receive both excitatory afferents (glutamatergic and cholinergic), which mediate nicotine reward and inhibitory afferents (GABAergic), which mediate aversion (Lammel et al., 2011).

In the mesocorticolimbic pathway the reinforcing effects of nicotine are principally due to the activity of VTA DA neurons and nicotine exposure increases the release of dopamine (DA) in the NAc, which is critical for the nicotine-induced reward. In the VTA DA neurons are modulated by other neurotransmitters, whose release is modulated by the nAChRs expressed in cholinergic, glutamatergic and GABAergic terminals. Chronic nicotine

treatment activates the α7 receptors expressed on glutamatergic terminals, increases the release of glutamate that facilitates the burst firing of VTA DA neurons, increases the NMDA receptors activity and induces long-term potentiation (Zhao-Shea et al., 2011). In the mean time chronic nicotine induces the desensitisation of α4β2 receptors localised on GABAergic input, leading to a reduced GABA release and a lower DA neurons inhibition (Mansvelder et al., 2002). Finally nicotine directly activates the nAChRs present on DA terminals in the NAc and increases DA release.

4.The non-neuronal cholinergic system

The presence of ACh in primitive organisms, such as bacteria, protozoa and algae that do not have a real CNS and PNS, underlines the fact of considering ACh not only as a neurotransmitter. Free ACh is found in almost all mammalian non-neuronal cells and tissues where it acts in autocrine and paracrine manner with different biological activity depending on cell type (Grando, 2014).

Non-neuronal ACh is released from living cells and binds nAChRs expressed in non-neuronal cells such as epithelial cells (skin, digestive and respiratory tracts, kidneys, placenta) endothelial cells, immune cells, astrocytes, and mesenchymal cells (Wessler and Kirkpatrick, 2008).

Activation of the nAChRs can lead to cell proliferation or inhibition and this balance is crucial to maintain physiological functions. However, there are many pathophysiological diseases correlated with an aberrant expression of nAChRs in malignant cells stimulated by ACh and above all nicotine, which might contribute to health problems such as cancer.

4.1.Connection of nicotine to cancer

Most tumors are formed as a consequence of genetic alterations of cellular genes, which may be inherited or arise spontaneously as a result of DNA damage induced by environmental carcinogens or mutations arising from replication errors. These alterations confer a selective advantage to the cells that together with changes in the microenvironment promote tumor growth, progression and spreading.

The list of cancers connected with nicotine includes small-cell and non-small cell lung carcinomas, head and neck, gastric, pancreatic, gallbladder, liver, colon, breast, cervical and kidney cancers (Grando, 2014). Data present in literature show the mutagenic and tumour-promoting activity of nicotine: it can damage the DNA, disturb cell metabolic processes and facilitate growth and spreading of mutated cells. Nicotine also alters the

physiology of different organs such as the lung or mammary glands by inducing the release of different factors, which lead to tumour growth and metastasis (Schall and Chellappan, 2014).

Nicotine and its derivatives can both promote (nicotine itself) and initiate (NNN and NNK) smoking-induced carcinogenesis and their effects are mainly due to their binding to nAChRs.

4.1.2 Nicotinic acetylcholine receptors in cancer

nAChRs are expressed in the plasma membrane of many mammalian cells, including endothelial cells, gastrointestinal tissue, glia, immune cells keratinocytes, respiratory tract and also cancer cells. They regulate the release of growth, angiogenic and neurogenic factors that stimulate signal transduction in normal and malignant cells.

nAChRs are usually overexpressed in malignant cells where chronic exposure to nicotine or nicotine-derived nitrosamines (NNK and NNN) up-regulate cancer-stimulatory nAChRs (i.e. α7 nAChRs) and desensitise cancer-inhibitory nAChRs (i.e. $\alpha4\beta2$ nAChRs) (Schuller, 2009).

Both nAChR types are up-regulated when exposed to chronic nitrosamine or nicotine but the $\alpha4\beta2$ nAChRs desensitise very quickly at low agonist concentration, whereas the tumor-promoting α7 nAChRs remains active for longer time, might inducing cancer development and progression.

α7 receptor can activate the release of DA, which stimulates the proliferation of at least prostate and mammary cancers, regulates the release of glutamate, serotonin, noradrenaline and adrenaline as well as the neuropeptide mammalian bombesin, an autocrine growth factor involved in lung cancer (Olincy et al., 1999).

On the contrary the heteromeric $\alpha4\beta2$ nAChRs are the major inhibitors of malignancies: they induce the release of GABA, which acts as a tumour suppressor in lung, pancreas, breast and colon cancers and block the production of cyclic AMP that controls the release of endothelial and epidermal growth factors (Figure 8). The unbalance between the up-regulated α7 receptor and the desensitised $\alpha4\beta2$ receptor may be the driving force to the most common human cancers.

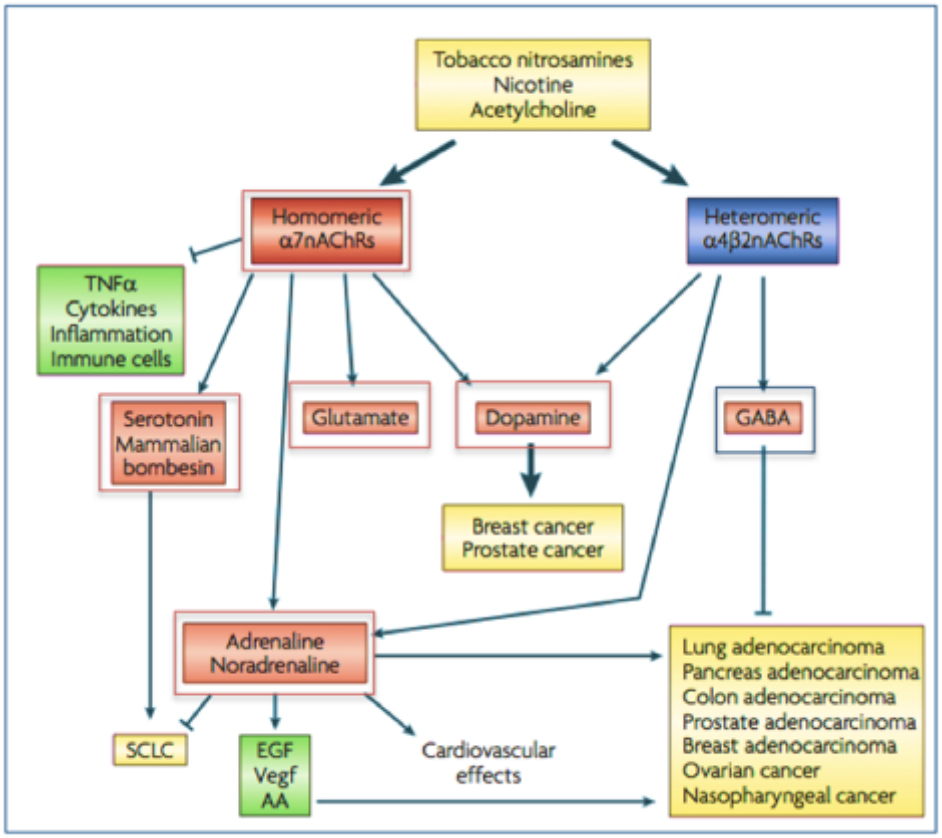


Figure 8. The α7 and α4β2 nAChRs opposite role in cancer cells. Their activation stimulates different neuro and not neuro -neurotrasmitters release involved in the balance between cancer development and inhibition (Modified from Schuller, 2009)

4.2. nAChR in lung cancer

It is well known that tobacco is one of the most harmful and preventable risk factor for cancer, particularly lung cancer. Indeed, it is responsible for the 80-90% of all lung cancer cases (Beasley et al., 2005).

4.2.1. Lung cancers

Lung cancer is one of the most common types of cancer, causing many people death every year worldwide. According to histological features, lung cancers are traditionally classified into two groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).

SCLC is the most aggressive tumour with a very high level of mortality. It originates from pulmonary neuroendocrine cells (PNEC) localised in the lungs, in the airways or grouped in small groups of neuroepithelial bodies. The PNEC cells synthesise neurotransmitters and neuropeptides, which show an autocrine and paracrine action as trophic factors or chemo- and mechano-receptors (Sunday et al., 1996).

NSCLC represents 80% of all lung cancers and is divided into three main subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Adenocarcinoma arises from epithelial cells of the small airways and alveolar type II cells, while squamous cell carcinoma develops from epithelial cells of the large lung airways (Improgo et al., 2011).

Tobacco use is a clear risk factor in the onset of cancer and in particular is associated with 90% of cases of SCLC and with 60% of NSCLC tumours (Egleton et al., 2008).

4.2.2. nAChR expression and function

Using RT-PCR, qRT-PCR, and in situ hybridization, nAChR subunit transcripts have been detected in the lung and airways of normal and malignant cells (Maus et al., 1998).

In SCLC cells there is an altered expression of the α3, α5, α7, β2 and β4 nAChRs subunits (Zhang et al., 2010), whereas the expression of nAChR in NSCLC cells are less clear and depends on the smoking history of the subject (Ambrosi and Becchetti, 2013).

The most convincing identification of nAChRs in lung cells has derived from pharmacological analysis using subunit-specific antagonists (Wu et al., 2011) and RNA interfering approaches.

Maouche et al. (2009) demonstrated that α7 nAChR, by restricting cell growth, promotes basal cells differentiation toward a mucociliary epithelium and α7 nAChRs are essential for the plasticity of the epithelium as α7 KO mice showed alteration in basal cell layer, hyperplasia and uncontrolled growth. This group also observed that the chronic inactivation of α7 nAChRs by the antagonist α–Bgtx prevents the *in vitro* differentiation of the normal human epithelium while in mice the absence of α7 nAChR induces modifications of the epithelium phenotype and an altered epithelial regeneration after wound healing.

On the contrary, in cancer cell lines it was found that α7-containing receptors, by activating different signalling pathways, mediate the proliferative effects of nicotine (Schaal and Chellappan, 2014). These results indicate that the regulation of cell proliferation by α7 nAChR is different in the normal airway epithelium and in primary cultures of normal lung cells from that in lung cancer tissue and cell lines.

Additionally it has been demonstrated that α7 nAChRs are expressed also on the mitochondrial membrane of non neuronal cells and nicotine, by permeating cells, may activate mitochondrial α7 nAChRs coupled to inhibition of the mitochondrial permeability transition pore opening, thus preventing cell apoptosis (Mucchietto et al., 2016).

Finally genome-wide association studies (GWAS) identified polymorphisms and a lung

cancer susceptibility locus on chromosome 15q24-25 which are correlated with increased risk of nicotine dependence and lung cancer development.

4.2.2.1 *CHRNA5, CHRNA3* and *CHRNB4* subunit genes and genetic variants

Genome-wide association studies have identified sequence variants in the region encoding nAChR α3, β4 and α5 subunits that increase risk of nicotine dependence, development of lung cancer and chronic obstructive pulmonary disease (Kuryatov et al., 2011) (Figure 9).

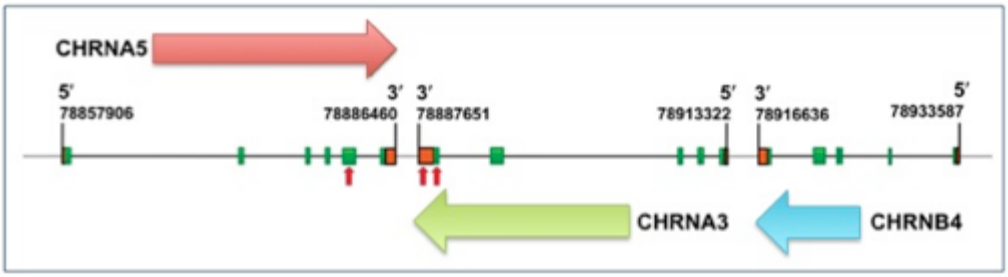


Figure 9. The human nAChR α3/α5/β4 gene clusters. Horizontal arrows indicate the transcription direction (Modified from Improgo et al., 2010)

Hung et al. (2008) have conducted a GWAS of DNA by analysing 317,139 single-nucleotide polymorphisms in 1,989 lung cancer cases and 2,625 controls from six different central European countries. They found a locus in the 15q25 chromosome region that is strongly associated with lung cancer. This region contains several genes including the genes coding for the α5–α3–β4 subunits that are predominantly expressed in neurons but also in alveolar epithelial cells, pulmonary neuroendocrine cells, and in lung cancer cell lines.

Two of the clustered nAChR genes coding for the α3–β4 subunits are significantly over-expressed in SCLC cells (Improgo et al., 2010) and silencing them decreases cell proliferation (Improgo et al., 2013). These findings and the recent demonstration that the surface expression of α3β4 receptors increases more than 5-fold with low concentration of nicotine (Mazzo et al., 2013), suggest that nicotine can increase the proliferative capacity of cancer cells expressing α3β4 receptors.

Genetic studies have shown that the SNP rs1051730 found in the non-coding region of the *CHRNA3* gene, and rs578776 found in *CHRNA3* gene un-translated region (Saccone et al., 2007) are correlated with nicotine dependence risk. These SNPs influence the onset of smoking, the daily intake in cigarettes and are associated with more severe nicotine dependence in adults (Weiss et al., 2008). Moreover, SNP rs1051730 is also associated

with increased risk for lung cancer and with a larger tumour size, especially in smokers (Shen et al., 2012).

Other studies have shown that the non-synonymous single nucleotide polymorphism (SNP) rs16969968 in the *CHRNA5* gene, leading to an aspartic acid to asparagine substitution (D398N) is also associated with lung cancer (Improgo et al., 2010). In the latter case, the disease appears not to be the consequence of smoking, but may be a direct consequence of altered functional properties of the variant receptor, as non-smokers bearing this polymorphism are also at increased risk of lung cancer.

Subjects who had one copy of the risk variant have a 1.3 fold increase in risk to develop nicotine dependence once exposed to smoking, and those with two copies have two-fold increase (Saccone et al., 2009). There was evidence for a recessive mode of inheritance for this SNP and the risk due to genotype was higher for males than females (Saccone et al., 2007).

The quantitative messenger RNA expression analyses investigated the association of 15q25 locus genes with lung cancer, demonstrating that *CHRNA5* mRNA is up-regulated 30-fold in lung adenocarcinoma compared to normal lung tissue (Falvella et al., 2009).

Falvella et al. (2010) found three variants in the 5' promoter region and three in the 3'-untraslated region of *CHRNA5*. The three promoter variants were associated with statistically significant *CHRNA5* expression, but those in the 3' region were not. These polymorphisms can modify the binding site for the transcription factors that alter *CHRNA5* expression levels and the risk of lung cancer. The same group (2013) have also found five mRNA isoforms that can encode for full-length or truncated proteins. All the isoforms are present both in normal and malignant lung tissue: in particular isoform 1 (corresponding to the full-length transcript) is more abundant in normal samples, while the others (corresponding to truncated proteins) in cancer tissues.

In the case of the *CHRNA5* gene, there are at least two distinct mechanisms underlying the risk of dependence and lung cancer: altered receptor function caused by the D398N amino acid variant in *CHRNA5* and variability in *CHRNA5* mRNA expression.

4.2.2.2 *CHRNA7* subunit gene and genetic variants

The α7–nAChR is highly and selectively expressed in the CNS where functions as ligand gated ion channel permeable to Na^+ and K^+ and Ca^{2+}. This receptor is characterised by a fast activation and desensitisation and is involved in the release of neurotransmitters.

α7–nAChR is expressed widely in both normal and malignant non-neuronal tissues and cells where it may act or not as an ion-channel (Chernyavsky et al., 2015). The ion

channel-independent events include activation of intracellular pathways through protein kinases, second messengers and transcription factors.

Different studies have shown the expression of α7 nAChRs, as mRNA and protein, in many different lung cancer cells obtained from human SCLC and NSCLC tumours.

One important characteristic of α7 is its ability to activate, after agonist (i.e. nicotine) binding, different downstream pathways, which in turn stimulate the proliferation and migration of NSCLC cells. An important variant of α7 gene is CHRFAM7A (see page 16).

4.2.2.3. *CHRNA9* subunit gene and genetic variants

The α9 nAChRs are homomeric receptors characterised by high affinity to the antagonist α–Bgtx and a very high Ca^{2+} permeability.

Lee et al. (2010) has recently shown that α9 nAChRs are ubiquitously expressed in many epithelial, lungs, and breast cancer cell lines, and most of the same cell lines also expressed α5 and α10-containing nAChRs. The α9 nAChRs are present in primary tumours and non-malignant breast tissues obtained from patients; however, breast cancer cells have increased α9 nAChR expression compared with the surrounding normal tissues. Silencing the α9 nAChR expression in breast cancer cells reduces proliferation and tumorigenic potential both *in vitro* and *in vivo* assays (Lee et al., 2010).

A very recent case control study of 340 non-small cell lung cancer patients and 435 controls has shown that an increased risk of developing lung cancer is also associated with two SNPs in the *CHRNA9* gene: rs56159866 and rs6819385 (Chikova et al., 2012).

Analysis of the mRNAs of human immortalised bronchial epithelial cell line (BEP2D) has shown the presence of four mRNA isoforms coding for the α9 protein: one coding for the full-length and three coding for truncated proteins. The full-length isoform may have a N442S aminoacid substitution, which induces, both in vitro and in vivo, proliferation and NNK-induced tumorigenesis (Chicova et al., 2011). On the contrary overexpression of a truncated isoform decreased proliferation and suppressed cell transformation.

4.3 nAChR-mediated nicotine effects

In 1989 Schuller reported the first evidence that nAChRs regulate cancer growth. Since then, various studies have described nAChRs as key molecules that act as central regulators of a complex network governing growth, angiogenesis, metastasis and apoptosis during carcinogenesis in response to the tumour microenvironment.

nAChRs stimulate in a cell type–specific manner intracellular signalling pathways and can proceed via ionic and non-ionic pathways activating different signalling cascades.

The major pathways activated in lung cancer cells are depicted in Figure 10.

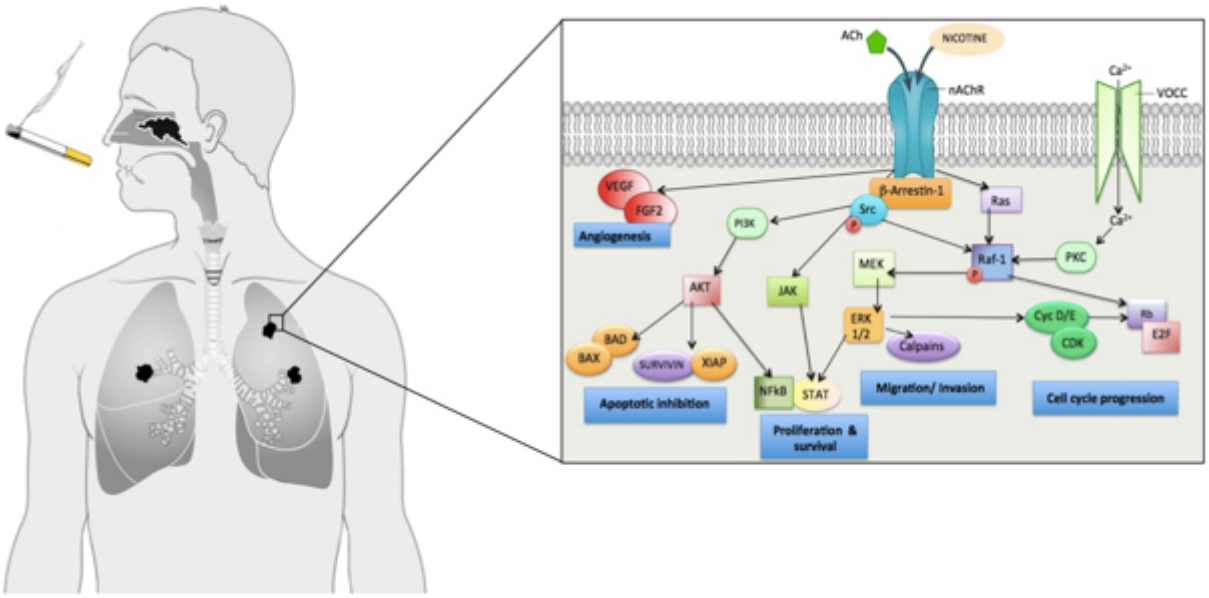


Figure10. Schematic representation of the nAChR-mediated lung cancer signalling pathways. Nicotine by binding nAChRs induces the entry of ions, which activate different intracellular cascade leading to cancer proliferation, survival, migration and angiogenesis (Modified from Mucchietto et al., 2016)

4.3.1. Proliferation

In SCLC and NSCLC lung cancers, nicotine induces cell proliferation through nAChR-associated downstream signal transduction pathways such as the MAP kinase (MAPK) cascade. Different studies showed that α7 is the most nAChR subtype involved in the stimulation of proliferation and is suppressed by the α–Bgtx and α–cobratoxin antagonists (Carlisle et al., 2007).

In SCLC nicotine induces, in a dose-dependent manner, MAPK kinase activation and this effect is blocked by the non specific antagonist mecamylamine (Cattaneo et al., 1997), whereas NNK induced-DNA synthesis and RAF-1 activation in SCLC cells or neuroendocrine cells is blocked by inhibitors of the protein kinase C (PKC) or by the α7 antagonist α-Bgtx (Jull et al., 2001).

Nicotine binding activates the extracellular-signal regulated kinase 2 isoform (ERK2) and the PKC. However, nicotine-induced PKC pathway activation is independent of the RAS-RAF-MEK-ERK pathway, and acts upstream of RAF-1 in the signalling sequence (Heusch et al., 1998).

Dasgupta et al. (2006) have demonstrated that mitogenic effects of nicotine in NSCLC are similar to that of growth factors but, unlike growth factor receptors, nAChRs do not have any intrinsic kinase activity. By binding to nAChRs, nicotine induces the recruitment of β-

arrestin-1 to the receptor and this actives the tyrosine-protein kinase Src (SRC). The same group have also shown that ablation of β-arrestin-1 decreases the nicotine-induced level of phosphorylated SRC kinase and ERK1/2 (Dasgupta et al., 2011). It appears that β-arrestin is a critical link between the mitogenic signals induced by nAChRs stimulation and the cell cycle machinery, and by activating β-arrestin–1 nicotine promotes tumour progression and metastasis.

It has been found that NSCLC release both excitatory (noradrenaline, NA) and inhibitory (GABA) neurotransmitters and their level change after nicotine stimulation: nicotine induces an increase in NA, which in turn stimulates P-CREB and P-ERK1/2 to support cell proliferation.

Nicotine exposure also increases the activation of the JAK/STAT pathway and its target genes and in NSCLC high concentration of nicotine or NNK increases the STAT3 phosphorylation.

In cells where the α7 nAChR has been silenced or in cells that do not express α7 (KO mice) nicotine does not activate the JAK/STAT pathway (Arredondo et al., 2006).

Studies in NNK-treated mice (West et al., 2003) showed that NNK activates the ERK/MAPK and PI3K/AKT cascades as well as STAT3 signalling.

In addition to proliferation, nicotine can also induce epithelial mesenchymal transition in NSCLC cell lines and tumours, leading to up-regulation of mesenchymal markers such as vimentin and fibronectin (Pillai et al., 2015).

4.3.2 Cell Cycle Regulation

Nicotine and nitrosamines also target different components of the cell cycle machinery.

Many studies have shown that the expression of cyclins and proteins involved in cell cycle checkpoints is influenced by nicotine (Baldi et al., 2011) and that nicotine and nitrosamine repress negative regulators of cell cycle progression such as cyclin-dependent kinase (CDK) inhibitors.

In normal human lung epithelial cells nicotine and NNK induce increase in proliferation by induction of cyclin D1 protein expression mediated by the nuclear factor κB (NFκB) (Ho et al., 2005). Similar results have been found in SCLC and NSCLC cells where nicotine activates cyclin D1 and NF-kB by activation of the PI3K/AKT intracellular pathway (Tsuratani et al., 2005).

In addition, nicotine and NNK enable cell-cycle progression via nAChR and β–AR-mediated induction of COX2 and prostaglandin E2 (PGE2), with an associated increase in cyclin D1 and decrease in CDK4/6 expression (Shin et al., 2008).

In NSCLC cell lines and primary lung lines it has been shown that nicotine activate the transcriptional repressor ID1 by acting on α7–nAChR (Pillai et al., 2011).

Moreover it has been defined that components of tobacco smoke inhibit in NSCLC also regulators of cell-cycle progression (Norton, 2000). These data suggest that tobacco smoke is able to modulate multiple components of the cell cycle, enabling cell cycle progression and spreading.

4.3.3. Antiapoptotic effects

In addition to the effect of nicotine on tumor growth, various epidemiological and clinical studies have linked the activation of nAChRs with the inhibition of apoptotic pathways and the possibility that nicotine may confer a survival advantage by promoting cell-cycle progression and by preventing apoptosis. Indeed, a large number of studies have shown that those patients who smoke have a poor prognosis with standard therapies.

The most important protein involved in the regulation of cell survival is the protein kinase AKT. This kinase can be activated by different factors such as growth factors as well as by nicotine in both normal and malignant cells. Nicotine effect on this pathway can be blocked by PI3K inhibitors or by α3/α4–specific nAChR antagonists but not by α7–specific antagonists (Tsurutani et al., 2005).

Nicotine exposure (1 μmol/L) also confers apoptosis resistance in presence of cysplatin and taxol, commonly used as chemotherapeutics, by upregulating XIAP and survivin two downstream proteins of AKT activation (Dasgupta et al., 2006).

AKT by phosphorylating XIAP inhibits its degradation by chemotherapeutic agents.

Apoptosis occurs as a result of the activation of an intrinsic or extrinsic pathway, and is largely regulated by the Bcl-2 family of apoptotic regulators. BAD and BAX are pro-apoptotic members of the Bcl2 family of proteins (Wei et al., 2001), expressed in human SCLC and NSLC cells, but their phosphorylation negatively regulates their pro-apoptotic activity (Jin et al, 2004). By activating the PI3K/AKT pathways, nicotine and NNK both increase BAD and BAX phosphorylation and cell survival.

4.3.4 Migration and Metastasis

One of the most harmful features of tumours is represented by their capacity to migrate and invade the surrounding tissue and metastasise. The movement of cancer cells into tissue surrounding the tumor and the vasculature is the first step in the spread of metastatic cancers. Cell migration and metastasis are fundamental processes during tumor cell invasion and these processes are the result of a complex interplay between numerous protein families critically regulated by different factors, which include growth

factors, kinases, phosphates and extracellular matrix (ECM) components. Nicotine increases EGF receptor expression in lung cells (Carlisle et al., 2007), which modulate different biological processes, including cell migration, by activating the signalling cascade of MAPK, PKC and PI3K/AKT.

Tumour invasiveness is determined by modification of cell adhesion, chemo-factors and activation of proteins with proteolytic activity (calpains) in order to migrate through he ECM. Calpains are a conserved family of cysteine proteinases that catalyse the controlled proteolysis of many specific substrates and have an altered expression during tumorigenesis. Xu et al. (2006) have demonstrated that nicotine induces the phosphorylation of both μ- and m-calpains via activation of protein kinase Cι, which supports the cleavage of various substrates in the ECM and facilitates metastasis and tumour progression. α–Bgtx potently blocks both nicotine-induced phosphorylation and activation of μ- and m-calpains together with the inhibition of wound healing, cell migration, and invasion. This indicates that nicotine-induced cell migration and invasion may occur through activation of the α7 nAChR signal transduction pathway involving α7 nAChR/c-Src/PKCι/calpains in lung cancer cells.

nAChR signalling modulates the motility of various epithelial cell types including bronchial cells. The proliferative effect of nicotine in these cells appears to be mediated by α7 nAChR, whereas cell migration is regulated by $(\alpha3\beta2)_2\alpha5$ receptors.

However the role of α5-containing receptor in cell migration is not clear and opposite conclusions derived from different studies. Krais et al. (2011) by silencing the α5 subunit in normal bronchial and in NSCLC has found a negative effect on nicotine signalling. In fact, upon nicotine stimulation the absence of α5-containing receptors leads to a higher migration and capacity to invade the surrounding tissue and these effects are blocked by α–Bgtx. On the contrary silencing the α5 subunit in the same adenocarcinoma cells, blocked nicotine-stimulated activation and suppressed cell migration and invasion (Sun et al., 2015).

Nicotine can also induce metastases by activating matrix metalloproteinases a family of proteins that remodel the ECM and facilitate cell invasion. These proteins are overexpressed in NSCLC cells and in biopsies of patients with NSCLC.

4.3.5 Angiogenesis

Angiogenesis, the development of new blood vessels from pre-existing vasculature is a complex, multi-steps process involving endothelial cells modifications and differentiation. In normal conditions there are factors, which contribute to start angiogenesis, as hypoxia

or inflammatory cytokines that in turn stimulate the release of pro-angiogenic factors (VEGF and FGF).

Many studies have demonstrated that nicotine increases angiogenesis in endothelial cell cultures as well as in tumour models. The endothelial cells express different nicotinic subunits and when nicotine binds the α7 nAChR induces endothelial tube formation and migration (Heeschen et al., 2002).

5. Oxidative stress and carcinogenesis

Deregulated cellular energetics is one of the cancer hallmarks. Mitochondrial dysfunction plays a critical role in cancer progression and targeting mitochondrial alterations and mitochondrial signalling might be an encouraging strategy for the development of selective anticancer therapy (Hsu et al., 2016).

Oxidative stress-induced tumour initiation or progression may result from the overproduction of reactive oxygen species (ROS) by different cell components. ROS are intracellular chemical species that contain oxygen, which react towards lipids, proteins and DNA. Production of ROS induces different biological outcomes depending on normal or malignant cells. In normal cells they increase lipid oxidation, alteration in the proliferation, genomic instability and at the end cell death, whereas in malignant cells elevated ROS levels can promote tumourigenesis (Wallace, 2005) (Figure 11).

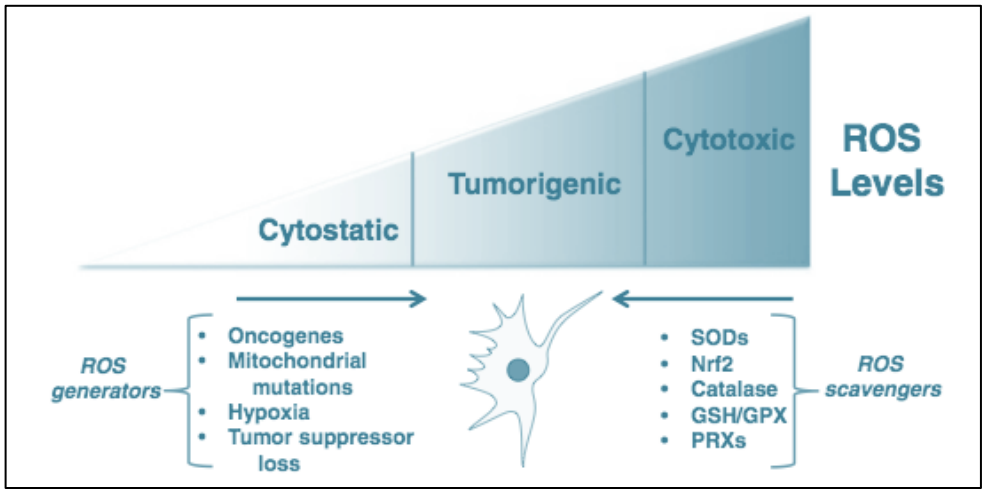


Figure 11. ROS balance in normal and malignant conditions (Modified from Sullivan and Chandel, 2014)

Particularly, in cancer cells ROS production shows two opposite effects: in a certain concentration range ROS can increase cellular proliferation, metabolic alterations, and angiogenesis leading to cancer progression and spreading while, at high concentrations,

can induce cell death signalling, senescence and cell cycle arrest. Various studies have shown that ROS elevation, either through ROS producers or antioxidant inhibitors, can selectively kill cancer cells and suppress tumour growth and progression in a variety of cancer cell lines (Glasauer and Chandel, 2014).

The two main sources of ROS are mitochondria and the family of NADPH oxidases (NOXs). NOXs are a family of enzymes that trigger the transfer of electrons from intracellular NADPH across the cell membrane to oxygen, producing superoxide and other ROS in the extracellular space. It has been shown that the over-expression of functional NOX proteins lead to tissue injury and DNA damage with excessive ROS production (Han et al., 2016). Recent studies suggest that NOXs activity is up regulated in lung cancer and ROS production by NOX species strongly influence both tumour growth and survival of lung cancer cells (Meitzler et al., 2014). In these cells NOX4 is the most up-regulated isoform and provides pro-oncogenic signals through stimulation of ROS production.

Moreover both oncogenes, such as RAS, MYC and AKT, as well as hypoxia lead to increase ROS production, in order to promote cancer growth.

These findings may lead to new more specific targets for cancer therapy in order to avoid the inefficacy of the main common therapies.

6. Common therapeutic strategies

Lung cancer is one of the most common cancers and depending on the type of cancer, SCLC or NSCLC, the treatment changes considerably.

For SCLC the common therapy is the chemotherapy based on cysplatin and dexorubucin. The chemotherapy is frequently associated with the radiotherapy to block the onset of relapses. Instead for NSCLC surgery is the therapy of choice, unless metastases are present, and in this case radiotherapy and chemotherapy follow the intervention.

In these years new molecularly targeted therapies are emerging to treat these cancers that, when compared with standard chemotherapy, have superior outcomes. Biological drugs as getfitinib (direct against the EGF receptor) and crizotinib (inhibitor of ALK tyrosine kinase receptor) are recommended as first-line treatment for patients with mutation in genes coding for these receptors, but unfortunately few people have these genetic mutations.

Vachhani and Chen (2016) have demonstrated that pembrolizumab, an humanised antibody, directed against the programmed cell death (PD-1) protein is efficacious in

some NSCLC patients and the US Food and Drug Administration has approved this treatment for patients with metastatic NSCLC.

Finally predictors of chemotherapy efficacy give a new challenge to novel technological advances encouraging perspectives for performing analytical validation of some promising candidates for developing novel types of predictive biomarkers such as DNA signatures or novel therapeutic targets (Olaussen and Postel-Vinay, 2016).

7. nAChRs as potential therapeutic targets

Nicotine and other tobacco compounds are able to promote lung cancer by activating multiple intracellular cascades mainly (but not always) as a result of nAChR-mediated effects (Mucchietto et al., 2016). Unfortunately the disadvantage of generalised nAChR antagonists is that they bind to all nAChR subtypes both in normal and malignant cells, and so they can alter the physiological role of nAChRs leading to negative side effects.

The most common antagonists that block the α7 nAChR include α–Bgtx, methyllylcaconitine (MLA), α–cobratoxin and α–conotoxin ImI. *In vitro* experiments have shown that these antagonists block the proliferation of SCLC (Jull et al., 2001) and NSCLC cells (Schuller, 2007). As both α–Bgtx and MLA also bind to α9-containing nAChRs with high affinity, it is possible that the *in vitro* blocking effects of the antagonists is due to the involvement of the α9 subtype. However the *in vivo* effect of these toxins is still controversial.

Other compounds emerging in the α7 drug development are the selective positive allosteric modulators such as the PNU-120596 that targets α7 receptors *in vivo*. PNU-120596 seems to be able to reverse or destabilise the desensitised states of α7 receptors and when given together with choline, an endogenous α7 agonist, can have cytotoxic effect on α7-expressing tumour cell line (Williams et al., 2011).

Another class of possible compounds is made by natural compounds (such as curcumin, resveratrol, lycopene and quercetin), which have been found to block different pathways altered in cancer growth (Feitelson et al., 2015). Epidemiological studies have shown that natural compounds can be active in chemoprevention and in cancer treatment for their lack of toxicity.

The issue to find new compounds able to bind and block the nAChRs responsible to lung cancer development and proliferation is a challenge of many scientific groups. In this field

the resveratrol- or stilbene-derivatives can provide new possible treatments for lung cancer cells.

7.1 Stylbene-derived drugs

Stilbenes are part of a vast group of natural defence polyphenols occurring in many plant species such as *Pinaceae families, Mirtaceae, Fagaceae, Liliaceae, Moraceae, Papilionaceae and Vitaceae*. The stilbene-compounds are widely distributed in nature and their biological functions have aroused great interest in the scientific world.

Indeed, the stilbenoids are small molecules with a large spectrum of properties (Jakubowska et al., 2014). *Trans*-resveratrol ((E)-3,5,4'-trihydroxystilbene) has an antioxidant activity and also blocks the proliferation on different types of cancer (i.e. lymphoid and myeloid cancers, breast, prostate, colon, pancreas cancers and others) (Aggarwal et al., 2004).

Another class of natural compounds are the phenols, which comprise combretastatin A4. This molecule is a potential new vascular disrupting agent and shows also a remarkable ability to inhibit gastric tumour metastasis and enhanced antitumor immune reactivity (Lin et al., 2007). Pterostilbene (3,5-dimethoxy-4'-hydroxy-(E)-stilbene), the analogue of resveratrol, exhibits anti-oxidant capacity and have concentration-dependent anticancer activity with inhibition of growth, adhesion and metastatic growth(Remsberg et al., 2008) (Figure 12).

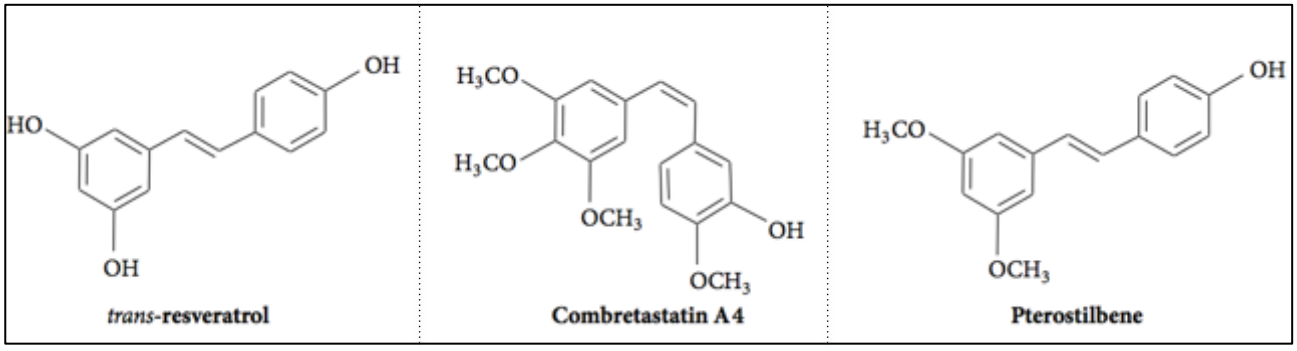


Figure 12. Chemical structure of natural compounds (Modified from Jakubowska et al., 2014)

In our laboratory different stylbene-derived drugs have been synthesised and analysed.

We have characterised the N,N,N-triethyl-2-(4-trans-stilbenoxy)-2-ethylammonium iodide, called MG624 or F1, firstly described by Cavallini et al. (1953) and subsequently the others N,N,N-trimethyl-1-(4-trans-stilbenoxy)-2-propylammonium iodide (F3) and N,N-diethyl-2-(4-trans-stilbenoxy)-2-ethylammonium iodide (F6) derivates (Figure 13).

Binding experiments have shown their high affinity for α7 nAChRs (nM concentration) and less for the heteromeric β2* or β4* (Gotti et al., 1998; Gotti et al., 2000).

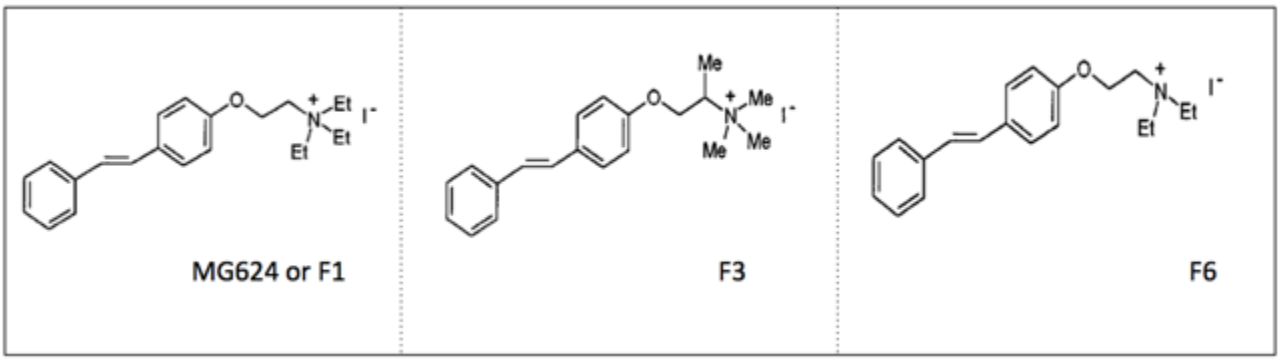


Figure 13. Chemical structures of the stylbene-derivates F1, F3 and F6.

Recently it has been shown that MG624 inhibits the nicotine-induced proliferation and angiogenesis of human lung microvascular endothelial cells (Brown et al., 2012). Furthermore, it suppressed the *in vivo* angiogenesis of NCI-H69 human SCLC tumours.
As tobacco components like nicotine promote the angiogenesis and metastasis of SCLC tumours in patients who are active smokers or exposed to second hand smoke the inhibition of α7-containing nAChRs by synthetic compounds such these, may be a useful means to attenuate nicotine-induced angiogenesis in SCLC.

Moreover, it has been recently reported that derivatives of resveratrol, mitochondria-targeted via a 4-triphenylphosphoniumbutyl (BTPI) group O-linked at either position -3 or 4, are cytotoxic in vitro and selectively induce necrotic death of fast-growing cancer cells when supplied in the low mM range (Sassi et al., 2014).

In conclusion nicotine contributes to cancer mainly by promoting growth and survival of mutated cell clones and protecting them from the chemo- and radiotherapy induced apoptosis. By doing this, nicotine increases the survival and expansion of cells with the genomic damage induced by carcinogens. On the other hand, inactivation of nAChRs can attenuate nicotine- or tobacco nitrosamine-induced cell proliferation, and suppress nicotine dependent chemoresistance. Therefore, nAChRs are viewed as a novel drug target for prevention and treatment of various forms of cancers.

AIM OF THE PROJECT

For decades, nAChRs have been considered a family of receptors expressed only in the central and peripheral nervous system (neuronal nAChRs) and at the neuromuscular junction (muscle nAChRs). However, recent findings have shown that they and their physiological agonist (ACh) are widely expressed in different mammalian cell types, including tumour cells. In non-neuronal tissues they regulate physiological functions such as cell proliferation and differentiation, cytoskeleton organisation, cell-cell contacts, cell locomotion and migration.

Lung cancer is one of the leading causes of cancer deaths worldwide. Smoking accounts for approximately 70% of non– small cell lung cancer (NSCLC) and 90% of small cell lung cancer (SCLC) cases, although there is a subset of patients who develop lung cancer without a history of smoking.

Tobacco smoke contains many classes of carcinogens and, although nicotine is unable to initiate tumorigenesis in humans and rodents, it promotes tumour growth and metastasis, and the secretion of growth factors, neurotransmitters and cytokines that alter the physiology of various organ systems.

Nicotine promotes tumour progression by binding and activating cell-surface nAChRs.

Furthermore, a genome-wide association study DNA from 1,989 lung cancer patients and 2,625 control subjects from six central European countries has found a locus in the 15q25 chromosome region that is strongly associated with lung cancer.

These findings highlight the importance of molecularly and biochemically investigating the mechanisms by which nAChRs act in lung cancer cells.

The aim of this thesis is to increase our knowledge of the pathophysiological role of nAChRs in lung cancer by: a) molecularly establishing which nicotinic receptor subtypes are expressed in lung cancer cell lines; b) investigating the molecular mechanisms by which nicotinic receptors regulate cell proliferation and intracellular signalling, and if this signalling are modulated by nicotine; and c) providing a framework for the development of new drugs that specifically target the receptors expressed in lung tumour cell lines.

MATERIALS AND METHODS

Compounds

Nicotine and α-bungarotoxin (α-Bgtx) were purchased from Tocris Bioscience (Bristol, UK). All other reagents (PMSF, proteases inhibitors, chemicals) were purchased from Sigma-Aldrich.

Drugs F1, F3, stylbene, RS12 and RS18 were synthesised from the group of Professor Marco Pallavicini (Department of Medicinal Chemistry PietroPratesi, Milano); NNN, NNK and ICH3 were synthesised from the group of Prof Marco De Amici from the same department; Toxins RgIA4 and ArIB [V11L; V16D] (called for convenience RG-I and AR) are a generous gift of Dr JM McIntosh (Department of Psychiatry, University of Utah, Salt Lake City, USA).

1. Antibody production and characterization

For the detection of nAChR subunits we used affinity-purified, subunit-specific, polyclonal antibodies (Abs), produced in rabbit against the human α7 and α5 subunits. For each of these subunits Abs against peptides derived from the C-terminal (COOH) or intra-cytoplasmic loop (Cyt) of human nAChR subunit sequences were produced as previously described (Gotti et al., 2006; Grady et al., 2009). The Abs directed to the human α5 subunit were made against the COOH peptide (PVHIGNANK) and the Cyt peptide (DRYFTQKEETESGSGPKSSRNTLEA). The Abs against the human α7 subunit were made against the C-terminal COOH (SAPNFVEAVSKDFA) and cytoplasmic loop peptide (ACSPTHDEHLLHGGQPPEGDPDL).

The specificity of the affinity-purified Abs was tested by immunoprecipitation studies using the hippocampus of α7 wildtype (WT) and know-out (KO) mice; the same Abs also were tested by means of Western blotting. In order to exclude any cross-reactivity between nAChR subunits, anti-α7 human subunit Abs were also tested by means of immunoprecipitation studies and Western blotting in HEK 293 cells transfected to express human α2β4-, α4β2-, α4β4, or α3β4-nAChR subtypes or in SH-SY5Y cells transfected to express human α7-nAChR. The α5 Abs was tested using HEK cells transfected with the α3β4 and α3β4α5 subunits.

2. Pharmacological and biochemical analyses

- **Purification of α-bungarotoxin-binding nAChR**

For our mice studies, hippocampus tissue from either WT or subunit-null mice (KO) was pooled in every experiment. The tissue was homogenised in 10 ml of 50 mM Na phosphate, pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA and 2 mM phenylmethylsufonylfluoride (PMSF) to covalently inactivate serine protease activity and the homogenates were diluted and centrifuged for 1.5 hour at 60,000g. The entire membrane homogenisation, dilution and centrifugation procedure was then repeated, and the resulting pellets were collected, rapidly rinsed with 50 mM Tris HCl, pH 7, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 2 mM PMSF. The washed pellets were then re-suspended in 2 ml of the same buffer, further supplemented with 10 μg/ml of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A and aprotinin. Triton X-100 at a final concentration of 2% was added to the washed membranes, which were extracted for 2 h at 4°C. The extracts were centrifuged for 1.5 hour at 60,000g, recovered and an aliquot of the supernatants was collected for protein measurement using the BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA), with bovine serum albumin used as standard. Extracts (2 ml) were incubated with 200 μl of Sepharose-α-Bgtx (concentration of coupled toxin 1 mg/ml of gel) and shaken overnight at 4 °C. The following day, the beads were centrifuged, the supernatant was recovered, and the resins were washed 4-6 times by re-suspension followed by centrifugation. After washing, the Sepharose-α-Bgtx beads with bound nAChR (purified α-Bgtx-binding receptors) were incubated with one-two volumes of Laemmli sample buffer (125 mM Tris phosphate, 4% SDS, 20% glycerol, 0,02% bromophenol blue and 10% 2-mercaptoethanol pH 6.8) and boiled for 2 min. The supernatant was then recovered by centrifugation.

In the case of α-Bgtx receptors purified from the A549, H1975 and SH-SY5Y human cells the same procedure as that used for mouse tissue was used starting from $100x10^6$ cells for each cell line.

- **Immunoprecipitation of nAChRs containing the α5 and/or α7 subunits**

The immunoprecipitation of receptors containing the α5 and α7 subunits was determined on two human lung cancer cell lines (H1975 and A549) and on a human neuroblastoma cell line (SH-SY5Y).

Affinity purified Abs against the α5 or α7 subunits were covalently immobilized on agarose-Protein A beads at a concentration of 4 mg/ml of wet resin. Immunoprecipitation was then performed by adding 20 µl of agarose-Protein A beads with bound, affinity-purified Abs or control IgG to the 2% TritonX-100 cells extracts. After overnight incubation, immunoprecipitates were recovered by centrifugation and washed three times with phosphate-buffered saline containing 0.1% Triton X-100 and then loaded on SDS gel.

- **Immunoblotting and densitometric quantification of western blot bands**

nAChR subunit contents of tissue immunoprecipitated extracts, affinity purified α-Bgtx receptors complexes or cell lysates stimulated by nicotine exposure were analysed by Western blotting. For the extracts were loaded 10 µg of proteins before the purification whereas for the α-Bgtx-purified receptors a constant volume (40 µl), that depending on the cell line, which represent 1/2 or 1/3 of the total recovered Laemmli sample buffer-eluted receptors, was loaded onto a 9% acrylamide (Biorad, Hercules, CA, USA) gel and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes with 0.45 mm diameter pores (Schleicher and Schuell, Dassel, Germany). The blots were blocked overnight in 4% non-fat milk in Tris-buffered saline (TBS), washed in a buffer containing 4% non fat-milk and 0.3% Tween 20 in TBS, incubated for two hours with the primary nAChRs antibody (1–2.5 mg/ml), and then incubated with the appropriate peroxidase conjugated secondary Abs (Sigma-Aldrich. St Louis, MO, USA). After 10 washes, peroxidase was detected using a chemiluminescent substrate (Pierce, Rockford, IL, USA).

In the case of Abs directed against ERK, P-ERK, AKT and P-AKT proteins, we used cell-signalling Abs. Blots after overnight in 4% non-fat milk in Tris-buffered saline were incubated with the primary antibody (used 1:1000 as the datasheet; p44/42 MAPK (ERK1/2) and phospho-p44/42 MAPK (Thr202/Tyr 204) were purchased from Cell Signalling Technology; AKT BD Transduction Laboratories, AKT [pS473] MAB from Life Technologies).

They were then incubated for 1h with the appropriate secondary antibody (anti-rabbit Ly-Cor IRDye800RD; anti-mouse Ly-Cor IRDye680RD). After another series of washes, the membranes were dried overnight in the dark at RT. The IR signal was measured using an Odyssey CLx -Infrared Imaging System. The signal intensity of the Western blot bands was

quantified using iStudio software. The optical density ratio was calculated by taking the optical density of the control saline as 100%.

The data are expressed as mean values ± S.E.M. of at least three separate experiments using each antibody.

- **Binding Studies and determination of the Ki values of oxystylbene compounds**

The binding of [^{125}I]-α–Bgtx to 2% Triton X-100 extracts of mouse tissues was determined by collection onto DEAE-Sepharose™ Fast Flow (GE Healthcare, Uppsala, Sweden). Triton extracts (250 µl) from each experimental group were incubated overnight with a saturating concentration (5 nM) of [^{125}I]-α–Bgtx at 20°C in the presence of 2 mg/ml bovine serum albumin. Specific radioligand binding was defined as total binding minus the non-specific binding determined in the presence of 1 µM un-labeled α–Bgtx. Non-specific binding averaged around the 30-40% of total binding.

Binding to α7-nAChR could also be measured in an immunoprecipitation assay format: receptor extracts were labeled with [^{125}I]-α-Bgtx (5 nM in the presence or absence of 1 µM unlabeled α–Bgtx to define total and non-specific binding). The labelled extract could then be bound to protein A beads via anti-α7 subunit Abs (Moretti et al., 2014). Similar amounts of specific binding were recorded in either assay format, and non-specific binding was between 10-15% of total binding.

The oxystylbene compounds were tested using SH-SY5Y cells transfected with the human α7cDNA. The inhibition of [^{125}I]-α-Bgtx binding was measured by pre-incubating membranes containing transfected human α7 receptors with increasing concentrations (10 pM - 10 mM) of the drug for 30 min at RT followed by overnight incubation with a final concentration of 2 nM [^{125}I]- α–Bgtx at RT. After the incubation the membranes were separated by filtration on GFC filters hydrated with PEI, and the radioactivity is counted in the gamma counter.

The prism software, version 6 was used to calculate the K_i values of all tested compounds obtained from at least three independent saturation and competition binding experiments at RT.

3. *In vitro* cell culture experiments

- **Cell culture**

Human non-small cell lung adenocarcinoma cell lines (NSCLC) A549 and H1975 were obtained from the American type culture collection (ATCC). The neuroblastoma cell line SH-SY5Y were obtained from European collection of authenticated cell culture (ECACC) and Hepg2 (ATCC).

NSCLC cells and the neuroblastoma were grown in RPMI medium (Lanza) supplemented with 10% fetal bovine serum (FBS), 1% of penicillin-streptomycin and 1% of L-glutamine, HepG2 in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin and 1% of L-glutamine. The cells were maintained in an environment of 37 °C containing 5 % CO_2.

Microscopy for cells:

EVOS XL core

Lplan PH2 40X/0,65

Lplan PH2 20X/0.40

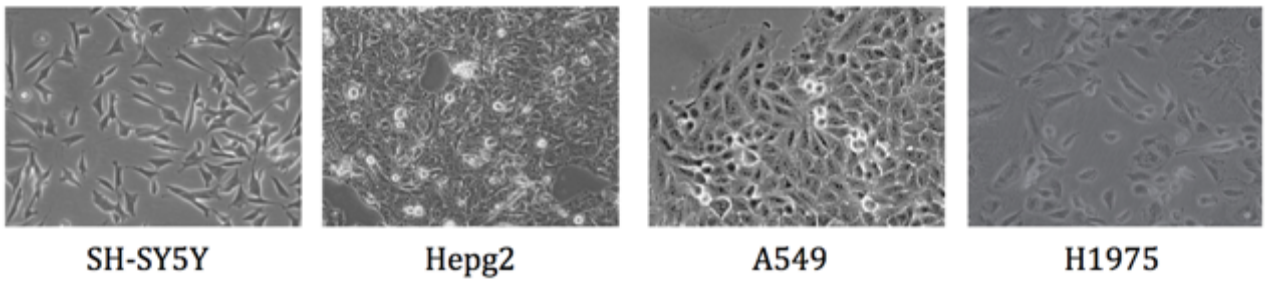


Figure 1. Morphological features of the cell lines

Each cell line was treated with different protocol in order to optimise the proper condition for the experiments.

- **Total RNA Extraction and Reverse Transcription**

Total RNA was extracted using the RNeasy Mini kit and accompanying QIAshredder (Qiagen), according to the manufacturer's instructions. Briefly, a maximum of 9x106 cells was collected by centrifugation and the cells lysed with 600 µl of buffer RLT, previously added with β-mercaptoethanol (10 µl/ml RLT buffer). The lysate was homogenized by means of QIAshredder column centrifuged for 2 minutes at maximum speed.

To avoid DNA contamination, samples were on-column incubated with DNAse I for 15 minutes and RNA eluted with 50 µl of RNase-free water. The amount of eluted total RNA

was determined by spectrophotometer at 260 nm and its purity was evaluated using the 260/280 ratio; 1 μg per sample was reverse transcribed using the GoScriptTM Reverse Transcriptase (Promega), according to information provided by the company.
This part of the work was done in collaboration with Dr. Roberta Benfante (Institute of Neuroscience, CNR, Milano).

- **Quantitative Real-Time PCR**

Gene expression analyses were performed by quantitative Real-Time PCR assay using the ABI Prism Thermocycler QuantStudio 5. The target sequences were amplified from 50 ng of cDNA in the presence of TaqMan® Gene expression master mix (Life Technologies, Inc.).
The TaqMan® primer and probe assays used were human CHRNA2 (ID #Hs00181237_m1), CHRNA3 (ID #Hs01088199_m1), CHRNA4 (ID #Hs00181247_m1), CHRNA5 (ID #Hs00181248_m1), CHRNA6 (ID #Hs00610233_m1), CHRNA7 (ID #Hs01063373_m1), CHRFAM7A (ID #Hs04189909_m1), CHRNA9 (ID #Hs00214034_m1), CHRNA10 (ID #Hs00220710_m1), CHRNB2 (ID #Hs00181267_m1), CHRNB3 (ID #Hs00181269_m1), CHRNB4 (ID #Hs00609520_m1). GADPH (ID #Hs99999905_m1) was used as endogenous control. The $2-\Delta CT$ or $2-\Delta\Delta CT$ method was used to calculate the results, as described in the figure legends, thus allowing the normalization of each sample to the endogenous control, and comparison with the calibrator for each experiment (set to a value of 1).

- **ERK and AKT pathways activation**

To evaluate the activation of ERK and AKT signalling pathways, the NSCLC cell lines were seeded in 24-well plates ($80X10^4$ cells/well) and starved overnight before to be treated with different concentration of nicotine in presence or absence of the antagonists or of the compound to be tested.
The signal detection was obtained by Western blotting analyses with Abs directed against ERK, P-ERK, AKT and P-AKT.

- **siRNA transfection**

Sequences of small interfering RNA (siRNA), obtained from Dharmacon (GE Healthcare), were used to knockdown the endogenous nAChR α7, α5 and α9 subunits.
For each subunit (α7, α5, α9) four different siRNAs were analysed and a non-targeting scrambled siRNA sequence was used as a control in the transfection experiments.

A549 cells were plated to 70-80% confluence in 6-well plate ($4X10^5$ cells/well) and transfected in Opti-MEM with 75 pmoli of siRNA (scrambled or subunit specific siRNA) (GE Healthcare, Buckinghamshire, UK) using Lipofectamine 3000 reagent (Invitrogen) in RPMI minus penicillin/streptomycin. Five hours after transfection, cells were trypsinised, counted and plated in 24-well plate (10-100 X 10^3 cells/well) and the medium was replaced by complete medium. The cells were collected at different times (48-72 hours) in order to isolate their mRNA and determine the mRNA and protein levels of the subunits.

The siRNAs that were more active in decreasing the expression of mRNA and subunit proteins were used for the cell proliferation and signalling experiments. To study the effect of nicotine, siRNA transfected A549 were rendered quiescent for 72 hours using serum-free RPMI. Subsequently, they were treated with nicotine (100 nM) for 48 hours and then were counted to assess their proliferative ratio.

To evaluate the activation of ERK and AKT signalling pathways, A549 cells, transfected with siRNAs, were starved for 24 hours and then treated with nicotine (100 nM) for different times (from five minutes to one hour). The cells were then examined by means of Western blotting.

- **Proliferation assay**

The different cell lines were seeded in 24-well plates and rendered quiescent by 72 hours of serum deprivation. After this time, cells were treated with nicotine or the other drugs for 48 hours. The capacity of antagonists to block the effect of nicotine was assessed by pre-incubating the cells for 30 minutes with specific antagonists at the appropriate concentration before adding nicotine.

At the end of the treatment, the cells were washed once with PBS 1X, trypsinised, suspended in 500 μl of medium, and manually counted using a hemocytometer (Burker camera) at the optical microscope.

- **Viability assay**

Cell viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). The assay is based on the reduction of MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] by viable cells to generate a colour formazan product that is soluble in cell culture media. This conversion is carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells. The formazan dye produced is quantified by measuring the absorbance at 490 and is directly proportional to the number of living cultured cells.

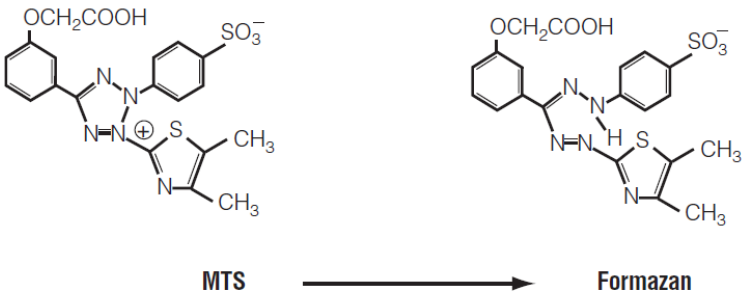


Figure 2. Structure of MTS tetrazolium converted to its soluble product as a result of cell metabolism

Cells were plated in 24-well plates at different densities depending on the metabolic activity of each cell line (i.e. 8X10^3 A549cells/well, 15X10^3 SHSY5Y and H1975 cells/well) and were treated with nicotine, nicotinic antagonists or other compounds synthesised by the groups of Prof. Pallavicini and Prof De Amici (University of Milan) for 48 or 72 hours in complete medium (RPMI or DMEM).

At the end of the treatment, the cells were washed and 50 µl/well MTS solution was added to each well. The plates were incubated for two hours and absorbance was read using spectrophotometer at a wavelength of 490 nm.

Optical density (OD) of each plate was measured in triplicate, and normalised by taking the OD of untreated cells as 100%.

- **Wound-healing assay**

Wound-healing assay was used to assess cell migration.

Cells were grown to a confluence of 90% and the monolayer was wounded by scratching the surface of the 24-well plate as uniformly as possible using a P200 pipette tip. The wells were rinsed twice with phosphate buffered saline (PBS) and fresh medium containing 100 nM nicotine, with or without nicotinic antagonist, was added. The antagonists were added 30 minutes before nicotine. Cells were incubated at 37°C for different times.

The initial wounding and movement of the cells in the scratched area were photographed using an Olympus CKX41 inverted microscope equipped with a digital imaging system (time 0) and subsequently until the wound closure. The healing wound width was measured at time 0, and after 24 and 48 hours. All of the values were normalised to the control cells.

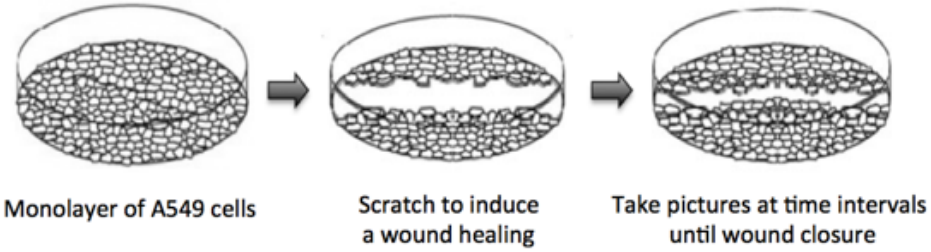


Figure 3. Scheme of the Wound healing protocol

- **ROS production**

ROS generation in A549 cells was assessed by using 2',7'-diclhorofluorescein di-acetate (DCFH-DA), a fluorogenic dye that measures intracellular hydroxyl, peroxyl and other ROS activity. DCFH-DA is deacetylated in cells by esterases, and becomes highly fluorescent when oxidised by ROS.

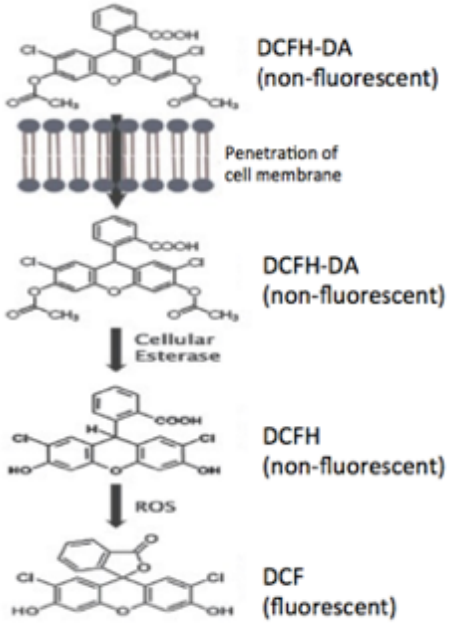


Figure 4. DCFH-DA conversion in cells upon ROS stimulation

Briefly, A549 cells line were seeded in a black 96-well plate (1,2X10^4 cells/well), washed with HBSS buffer, and incubated with 20 mM DCFH-DA for 45 min at 37°C. After incubation, DCFH-DA was removed and the cells were treated with different concentrations of oxystylbene compounds and derivatives for 24 hours. ROS levels were measured during the treatment period at emission wavelengths of 485-535 nm. The results were normalised using the protein value of each sample.

4. Electrophysiological studies

- ***Xenopus* oocytes and RNA injection**

These experiments were done in collaboration with Dr Michel Mcintosh Utah.

Xenopus oocytes were purchased from Ecocyte Bioscience US (Austin, TX) and incubated upon arrival at 13°C. The tips of pulled glass micropipettes were broken to achieve an outer diameter of ~40 µm (resistance of 2-6 MΩ), and pipettes were used to inject 20-60 nl containing 10 ng of cRNA/oocyte. To improve functional expression of α7*-nAChR, Ric-3 mRNA was also co-injected (Halevi et al., 2002). A ratio of 1:50 Ric-3: α7 subunit mRNA by mass was determined to be optimally effective in pilot experiments (data not shown).

- **Two-electrode voltage-clamp recording of α7- and α9 α10-nAChR function**

Two-electrode voltage-clamp recordings were made at room temperature (20˚C) in oocyte saline (OR2) solution (containing 82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1.8 mM CaCl2·2H2O, and 1 mM MgCl2·6H2O, pH 7.4). Seven to fourteen days after injection, *Xenopus* oocytes expressing concatenated α7 or α9- α10 -nAChRs were voltage clamped at −70 mV with an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA, USA). Recordings were sampled at 10 kHz (low-pass Bessel filter: 40Hz; high-pass filter: DC), and the resulting traces were saved to disk (Molecular Devices Clampex v10.2). Data from oocytes with leak currents (I_{leak}) > 50 nA were excluded from recordings.

- **Nicotinic receptor pharmacology**

Fresh stock drug solutions of ACh, methyllycaconitine (MLA) or α-Bungarotoxin (α-Bgtx), F1 or F3, were made daily and diluted as required. Agonists and antagonists were applied using a sixteen channel, gravity-fed, perfusion system with automated valve control (AutoMate Scientific, Inc.; Berkeley, CA, USA). All solutions were supplemented with atropine sulphate (1.5 μM) to ensure that muscarinic Ach receptor responses were blocked and thus not recorded. Oocytes expressing α7 or α9- α10 -nAChRs were perfused with ACh for 5 seconds with 60second washout times between each subsequent application. Oocytes were preincubated with F1 or F3 for 2 minutes prior to activation with ACh (100 uM; 5 seconds). All of the data of the F1 and F3 inhibition of Ach currents were normalised to those obtained upon superfusion of the same oocytes with 100 μM ACh.

5. Statistical analyses

All the experiments were performed in triplicate or quadruplicate, and the results are expressed as mean values ± SE.

The data from the binding, cell proliferation and western blotting studies were expressed as mean values ± SEM and analysed by means of Student *t* test for comparisons of two groups or one-way ANOVA followed by a Bonferroni *post hoc* test (paramatric data) or by Kruskal–Wallis test followed by Dunn's *post hoc* test (non parametric data). The accepted level of significance was $p < 0.05$. All of the statistical analyses were made using Prism software, version 5 (GraphPad).

RESULTS

1. Molecular studies

• Expression of nAChRs in NSCLC cell lines

We used RT-PCR analysis to compare the expression of the α2-α10 and β2-β4 nAChR subunits and CHRFAM7A in A549 and H1975 NSCLC cell lines with that of the same subunits in the SH-SY5Y neuroblastoma cell line (Figure **1**). The two lung cancer cell lines had a high level of transcripts for the α5 subunit and the CHRFAM7A protein and low level of α9 and α3 subunits whereas the other subunits were only found in one or the other NSCLC cell line.

The SH-SY5Y neuroblastoma cell line had higher levels of almost all of the subunits, particularly α3, α7, β2 and β4.

In order to confirm the expression of nAChR subunits at protein level, we performed Western blotting analyses and immunoprecipitation studies.

• Antibody specificity

The specificity of the antibodies (Abs) has been checked in preliminary experiments. Figure **2Aa** shows the specificity of anti-α7 Abs tested on α7 receptors obtained from hippocampus of wild type (WT) (left) and α7 knockout (KO) (right) mice. The same Abs where also tested on human α7 receptors purified using α-Bgtx covalently bound to Sepharose 4B (Figure **2Ab**) in the absence (left) or presence (right) of an excess of 2 μM cold α-Bgtx. The α7 Abs recognised the presence of the α7 subunit in WT mice (Figure **2Aa** left) and SH-SY5Y cells (Figure **2Ab** left), but not in α7 KO mice (Figure **2Aa** right), or when the binding to the resin was competed by cold α-Bgtx (Figure **2Ab** right).

As detailed in the methods we have raised two Abs against the human α7 subunit, one against a peptide located at the terminus (COOH Abs) and one against a peptide located in the intracellular M3-M4 loop (Cyt Abs). These α7 Abs were tested in SH-SY5Y and HEK cells transfected with the human α7 cDNA. As shown in the Figure **2Ac** the Abs directed against the COOH and Cyt α7 peptides recognised a peptide of identical molecular weight in transfected HEK and SH-SY5Y cells and this signal was very low in un-transfected SH-SY5Y cells. No signal was found in the un-transfected HEK cells.

We also tested the specificity of an anti human α5 Abs using HEK cells stably transfected with the α3β4 and α3β4α5 subtypes. As shown in Figure **2B**, the anti-α5 Abs only recognised a band in the α3β4α5 subtype.

- **Western blotting and Immunoprecipitation analyses**

nAChR were originally described in neuronal tissues but it has been recently reported that other cell types, including non-small cell lung cancer (NSCLC) cells, express nAChR subunits.

After having checked the specificity of the Abs, we used them to characterise the nicotinic subunits expressed in the NSCLC (A549 and H1975) and SH-SHSY neuroblastoma cell lines.

We first performed immunoprecipitation experiments on 2% Triton extracts obtained from the A549, H1975 and SH-SY5Y cells, using anti-α5 Abs (Figure **3Aa**), anti-α7 Abs (Figure **3Ab**), and control IgG bound to protein A (Figure **3Ac**). Western blotting analysis with anti-α5 Abs showed that the anti-α5 Abs recognised a 50 kDa peptide in all the cell lines (Figure **3A**). The peptide recognised by α5 Abs was absent when the extracts were immunoprecipitated by anti-α7 (Figure **3Ab**) or control IgG (Figure **3Ac**).

As the level of endogenous α7-containing receptors is very low in the SH-SY5Y cells, we purified the receptors by means of affinity purification using the α-Bgtx coupled to Sepharose 4B. As shown in Figure **3B**, after affinity purification the Western blotting with the α7 Abs demonstrated that α-Bgtx receptors were highly enrichment in α7-containing receptor.

When measured by RT-PCR α7 mRNA expression was lower in A549 cells than in SH-SY5Y cells, and so we analysed the expression of the α7 protein only after affinity purifying the α7-containing α-Bgtx receptors on a Sepharose-aBgtx column.

Figure **3B** shows a diagram of the purification of α-Bgtx receptors from A549, H1975 and SH-SY5Y cells, and the Western blotting analyses of the α–Bgtx-purified receptors probed with anti-α7 Abs. The α7 Abs recognised a specific band in the receptors purified from A549 cells but not in those purified from H1975 cells, which is in line with RT -PCR findings.

As the α5 protein is expressed at high level in all the three cell lines we also probed whether the α5 subunit could be associated with the purified α-Bgtx receptors. Figure **3B** (left) shows the probing of the α-Bgtx receptors with anti-α5 Abs. No specific

labelling was determined in the α-Bgtx receptors whereas the labelling of the α5 subunit was determined in the positive control (HEK α3β4α5 subtype) run in the same gel.
These data suggest that the α5 subunit is expressed in the tumour cell lines A549 and H1975, whereas the α7 subunit is only expressed in A549 cells. Moreover we determined that the α5 subunit is not associated with α7 receptors in the A549 cells.

2. Nicotine-induced effects

- **Nicotine increases cell viability and proliferation in NSCLC**

In order to study the role of nicotinic receptors in the proliferation of NSCLC cells we treated the A549 and H1975 cell lines with increasing nicotine concentrations (10 nM-100 mM). As shown in Figures **4A** and **4B**, 48 hours exposure to nicotine increased A549 cell viability (measured by means of an MTS assay) and proliferation (measured by counting cells), but not the viability or proliferation of H1975 cells. The nicotine concentrations that increased cell proliferation were comparable with those found in the serum of habitual tobacco smokers (50 -1000 nM). As nicotine 100 nM gave a significant result and falls in the range of the blood nicotine levels of habitual smokers, we used this dose for most of the follow-up experiments.

- **Nicotine induces A549 cell migration**

We evaluated the effects of nicotine on A549 cell migration using a wound-healing assay. The level of wound healing was measured by the average decrease in distance between the edges of the wounds at different time points in the presence or absence of nicotine stimulation (100 nM). The results indicate that, after treatment with nicotine for 24 and 48 hours, the cells migrated more quickly to close the wounds than control cells (not treated) (Figure **4C**).

- **Nicotine stimulates activation of ERK and AKT intracellular pathways**

Previous work by other groups has shown that nicotine increases the cell proliferation of a number of tumour cells by activating the ERK pathway, or inhibits cell apoptosis by activating the AKT pathway.
We used Western blotting analyses to investigate the possible nicotine-induced activation of ERK and AKT pathways in A549 and H1975 lung cancer cells. Nicotine (100 nM) by increasing the P-AKT and P-ERK levels activated both pathways in A549 cells, but not in H1975 cells (Figure **5A-B**).

This activation was time-dependent and nicotine-stimulated phosphorylation peaked five and ten minutes after nicotine stimulation and decreased permanently after 30 minutes. Nicotine did not affect the protein levels of total AKT or ERK. The lack of effect of nicotine on H1975 cells suggests that nicotine-mediated effects are due to specific nAChR subtypes, which are little or not expressed in H1975 cell line.

3. Pharmacological studies

Previous studies have shown that α7–, α5– and α9– containing nAChRs are involved in tumour growth and promote resistance to apoptosis, cancer cell proliferation, migration and intracellular activation when stimulated by nicotine. As we have found that NSCLC cells express α5, α7 and α9 mRNAs, we evaluated the involvement of receptors containing these subunits in the proliferative effect of nicotine by studying the effects of subtype-specific antagonists (pharmacological studies) and by using interfering RNAs (siRNAs) against the *CHRNA5, CHRNA7* and *CHRNA9* genes to knock down the expression of the subunits (silencing experiments). We also analysed the effects of nAChR antagonists and siRNAs on the intracellular pathways activated by nicotine.

- **α–bungarotoxin (α-Bgtx)**

α-Bgtx is a selective nAChR antagonist of homomeric and/or heteromeric receptors containing α7 or α9 subunits (Gotti et al., 2006; Olsen et al., 2008) and was used to study the nicotine–induced proliferation of A549 and H1975 cells. Both cell lines were pre-treated with 1 μM α-Bgtx for 30 minutes, and then with nicotine100 nM. The effect of α-Bgtx on nicotine-induced proliferation was evaluated after 48 hours.

We found that α-bgtx abrogated the nicotine–induced proliferation of A549 cells as measured by means of an MTS assay and counting assay (Figure **6A**).

We also evaluated the effect of α-Bgtx on the nicotine-induced activation of P-ERK and P-AKT, and found that in the presence of the antagonist, nicotine did not activate either in A549 cells (Figure **6B**). The presence of α-Bgtx had any effect on H1975 cell line (Figure **6C**).

- **Methyllycaconitine (MLA)**

MLA is a selective antagonist of homomeric or heteromeric nAChRs containing the α7 or α9 subunits (Gotti et al., 2006), and we tested its effect on the nicotine-induced proliferation of A549 and H1975 cells.

As in the case of α-Bgtx, 30 minutes of pre-treatment with 500 nM MLA followed by 100

nM nicotine for 48 hours blocked nicotine-induced proliferation and cell viability as measured by MTS viability assay and counting assay (Figure **7A**).

Wound healing assay showed that nicotine-treated A549 cells migrated more quickly than control cells and this effect was slowed in the presence of MLA (Figure **7B**).

MLA (500 nM) also blocked the nicotine-induced increase in P-ERK and P-AKT levels in A549 cells (Figure **7C**) but had no effect on H1975 cells (Figure **7D**).

α-Bgtx and MLA toxins act on α7 and α9-containing recepors and both toxins blocked the effects of nicotine on A549 cells, to discriminate the subtypes involved the same proliferation and signalling experiments were carried out using a toxin selective for the α7-containing subtype (AR) and a toxin selective for the α9-containing subtype (RG-I).

- **Effects of subtype-specific toxins**

The inhibitory activity of AR (Whiteaker et al., 2007; Innocent et al., 2008) was tested by pre-incubating A549 cells with AR 1 μM and then treating the cells for 48 hours with 100 nM nicotine. As shown in Figure **8A** this toxin alone did not affect the proliferation of untreated cells but blocked nicotine induced proliferation.

We also analysed the effect of AR 1 μM in nicotine-induced AKT and ERK signalling and found that it significantly decreased the nicotine-induced activation of P-AKT (Figure **8B**) after 10 minutes of nicotine stimulation but had no significant effect on the nicotine-induced modulation of the ERK pathway. This suggests that the inhibition of α7 nAChR has a greater effect on the anti-apoptotic pathway induced by P-AKT than on the proliferative pathway induced by P-ERK and that nicotine exposure induces cell proliferation and signalling activation through the α7-containing nAChRs in the NSCLC A549 cell line.

The α9 nAChR specific toxin RG-I 1 μM completely abolished proliferation of A549 cells induced by 48 hours treatment with nicotine (100 nM) (Figure **9A**) and when administrated alone the toxin had no effect on basal cell proliferation.

When tested for the activation of intracellular pathways we found that RG-I toxin blocked the nicotine-activated downstream cascades of P-AKT and P-ERK (Figure **9B**).

- **Nicotine-derived nitrosamines and an α7-selective agonist (ICH3) induce A549 cell proliferation**

It has been previously shown that two nicotine-derived nitrosamines (NNK and NNN) can induce carcinogenesis and tumour progression by binding nAChRs. For this reason,

we tested their activity on the A549 cell line using the MTS assay. The cells were treated separately with NNK and NNN at different concentrations (10 nM-100 μM) for 48 hours. Our results show that NNK and NNN stimulate A549 cell viability only at a very low dose (10 nM) and not at higher doses. This effect may be explained by the fact that the higher concentration may lead to nAChR desensitisation (Figure **10A-B**).

We also investigated the role of a selective α7 agonist called ICH3 previously identified by our group (Dallanoce et al., 2011b). This agonist increased cell viability 1-1.5-fold in a dose-dependent manner at doses ranging from 100 nM to 10 μM (Figure **10C**).

3.1 Silencing gene studies

Small (or short) interfering RNA (siRNA) is the most commonly used RNA interference (RNAi) tool for inducing short-silencing of protein coding genes.

As α7- and α9-containing nAChRs are associated with various types of cancer and the α5 subunit is associated with a high risk of nicotine dependence and lung tumour onset, we investigated whether knocking-down the nAChR containing these subunits affected their nicotine-induced, pro-tumorigenic effects in the A549 NSCLC cell line.

- **The effect of siRNAs on α7-containing nAChR**

In preliminary experiments we screened among four different siRNAs (Dharmacon) using RT-PCR and Western blotting analysis.

In order to evaluate the ability to knock down the gene of interest, A549 cells were transfected for 48 hours with scrambled siRNA (used as negative control) or four siRNAs targeting specific CHRNA7 sequences (Figure **11A**).

RT-PCR analysis shows that, in comparison with control untreated cells, scrambled siRNA had no significative effect on the α7 mRNA level but three α7 siRNAs knocked down more than 50% of CHRNA7 gene transcript: siRNA II (48 %), siRNA III (58%) and siRNA IV (60%).

As said above we could not detect the endogenous α7 protein using our α7-specific Abs and so, in order to investigate the effect of the different siRNAs on α7 expression, we co-transfected α7 cDNA into A549 cells for 48 hours. We measured the level of silencing by means of Western blotting with the anti-α7 Abs. Under these conditions, we found that the siRNAs that were most potent in silencing the transfected α7 protein were siRNA II and siRNA IV (Figure **11B**).

We next analysed the effect of scrambled siRNA, siRNAs II or IV on the nicotine-induced

stimulation of cell proliferation. To this end the cells were first transfected with scrambled or siRNAs II or IV, starved, by means of FBS deprivation, and then treated with 100 nM nicotine for 48 hours. As shown in Figure **11C**, transfection with the scrambled siRNA did not affect basal proliferation or the pro-proliferative effect of 100 nM nicotine and transfection with siRNAs II and IV did not change basal proliferation but abolished nicotine-induced proliferation (**Figure 11C**).

We also examined whether knocking down the α7 subunit affects the nicotine-activation of the intracellular ERK and AKT pathways. A549 cells were transfected with scrambled, siRNA II or siRNA IV for 48 hours and then stimulated with 100 nM nicotine for different times, from five minutes to one hour, and analysed by means of Western blots. Figures **12A and B** showed that knocking down α7 protein with siRNA II and siRNA IV blocked the nicotine activation of P-AKT at all times, and treatment with siRNA IV and siRNAII also decreased the P-ERK activation induced by nicotine stimulation (**Figure 12B**) although it was only significant with siRNA IV. These data suggest that α7-containing receptors are mainly involved in AKT pathway activation and are maybe not so essential for mediating the effect of nicotine on the ERK pathway.

- **The effect of siRNAs on α5-containing nAChRs**

It is well known that α5–nAChR subunit is involved in lung cancer susceptibility and nicotine addiction and genetic studies demonstrated that specific polymorphism in this gene can lead to a higher risk of developing lung cancer (Amos et al., 2008).

We therefore assessed whether nAChRs containing the α5 subunit are involved in the effects on A549 cells induced by nicotine.

We first used RT-PCR to analyse the activity of four siRNAs directed against α5 subunit transcript and found that all four decreased the expression of the CHRNA5 gene by 65% or more (Figure **13A**). As the most effective were siRNA II and siRNA IV we tested them for their ability to decrease the expression of the α5 subunit. The level of endogenous α5 protein in A549 cells is too low to be correctly detected by our α5 subunit specific Abs, and so we co-transfected the cells with α5 cDNA and the siRNAs. Western blot analysis showed that in comparison with control α5 transfected cells, scrambled siRNA had no effect but both siRNA II and siRNA IV reduced the α5 protein expression (**Figure 13B**).

We then transfected A549 cells with scrambled siRNA, siRNA II or IV, starved them by means of FBS deprivation, and then treated them with 100 nM nicotine for 48 hours. As shown in the Figure **13C,** transfection with scrambled siRNA or siRNA IV did not affect

the basal proliferation of the A549 cells or the pro-proliferative effect of 100 nM nicotine, but siRNA IV abolished the nicotine-induced proliferation (Figure **13C**) while siRNA II did not abrogated the nicotine-induced proliferation (data not shown). Knocking down the α5 protein in A549 cells with siRNA IV also completely blocked nicotine-induced P-AKT and P-ERK activation (Figure **13D**).

These results indicate that α5–containing nAChRs are also involved in nicotine-mediated effects on cell proliferation.

- **The effect of siRNAs on α9-containing nAChR**

α9–containing nAChRs play an important role in the development of smoking-induced breast cancer (Lee et al., 2010), and human variants of this receptor can dramatically affect cell proliferation and neoplastic transformation in human bronchial cells (Chicova and Grando, 2011). For these reasons, we analysed the possible role of these receptors in nicotine-induced effects on A549 cells.

Our preliminary RT-PCR studies showed that the α9 subunit is expressed in A549 cells and so we analysed the ability of four α9-directed siRNAs to knowdown the α9 subunit. Using the same protocol as that used to study the effect of α5 and α7 siRNAs, we found that all four siRNAs down regulated the α9 mRNA but the most potent were siRNAs II, III and IV (Figure **14A**). Unfortunately, we could not use Western blotting to assess the effect of α9 siRNAs on the overexpressed α9 subunit because no α9 subunit specific Abs are available.

We used siRNA II and siRNA IV to determine whether α9-containing receptors were involved in the nicotine-induced proliferation and intracellular signalling pathways of A549 cells (Figure **14B**).

Figure 14C shows that siRNAs II and IV both inhibited nicotine-induced cell growth, and that siRNA II reduced the nicotine-induced activation of P-AKT after 10 and 30 minutes (but not at the other times) without inducing any change in the ERK pathway. siRNA IV showed a tendency to block the activation of P-AKT, but it had no effect on P-ERK.

4. Oxystilbene derivatives

In collaboration with the group of Pallavicini (University of Milan), our group have previously synthesised and characterised 4-oxystilbene derivatives as selective α7 antagonists (Gotti et al., 1998; Gotti et al., 2000) and found that two of these compounds, F1 (previously called MG624) and F3, bind with high affinity to the human α7 subtype

(Ki values of respectively 161 and 100 nM). At that time these compounds were not characterised for their effects on α9-containing receptors, but now, in collaboration with Dr. McIntosh, we have done this. Electrophysiological experiments were used to test the effects of F1 and F3 on oocytes expressing the α9-α10 receptor subtype. As shown in Figure **15**, both compounds reduced the ACh activation of the α9-10 subtype in a dose–dependent manner with IC_{50} values of 10.1 nM (C.I. 5.6-18 nM) in the case of F1 and 4.8 nM (C.I. 3.1-7.3 nM) in the case of F3. These values are very similar to the values determined in the human α7 subtype expressed in oocyte, tested in parallel, and were F1 IC_{50} value was 33.5 nM (C.I. 21-53 nM) and 16.8 nM for F3 (C.I. 13.8 -19.8 nM) (**Figure 15**).

- **Inhibition of A549 cell viability, proliferation and migration by 4-oxystilbene derivatives**

We then characterised the activity of F1 and F3 by treating A549 cells with increasing concentrations of each compound (10 nM- 100 μM) for 72 hours and using an MTS assay. Both compounds blocked the proliferation and viability of A549 cells, with an IC_{50} of 4.3 μM (F1) and 11 μM (F3), but were much less active on SH-SY5Y and in HepG2 cells (Figure **16A**).

As F1 was more potent than F3 in A549 cells, we co-incubated nicotine (100 nM) with three different doses of F1 (1 μM, 5 and 10 μM) for 48 hours, and then manually counted the cells. Our results showed that the lowest concentration of F1 (1 μM) was not toxic but blocked nicotine-induced proliferation, while the higher concentrations (5 and 10 μM) administrated alone decreased the viability of control cells. For this reason the following experiments were conducted using F1 at the concentration of 1 μM (Figure **16B**).

We next investigated the anti-migratory activity of F1 in the A549 cell line using the wound-healing assay. The cells treated with nicotine (100 nM) quickly closed the scratch (as previously shown), whereas those treated with nicotine and F1 had increased the time to wound closure. This suggests that F1 inhibits the A549 cell migration induced by nicotine (Figure **16C**).

We analysed the effect of F1 on intracellular ERK and AKT signalling by treating the A549 cells with nicotine (100 nM) for different times (from five minutes to one hour) alone or in combination with F1 (1 μM). Western blotting analyses showed that F1

significantly abrogated the nicotine-induced activation of AKT and ERK after five and ten minutes of nicotine stimulation (Figure **17A**).

- **F1 activates ROS production**

It is becoming gradually clear that ROS play an important role in the biology of tumourigenesis (Sullivan and Chandel, 2014). As cancer cells have increased ROS levels, they may be selectively sensitive to the damaging effects of further increasing ROS.

We examined the possible role of F1 in mediating ROS production.

A549 cells were treated with increasing concentrations of F1 (10 nM-10 μM) for 24 hours. ROS production was measured using the DCF-DA probe, and the results showed that F1 increases ROS production at concentrations of 50, 100, 250 and 500 nM (Figure **17B**).

However, we still do not know whether this mechanism is mediated by nAChRs or is an intrinsic feature of the drug, passing through the cell membrane.

4.1 New oxystilbene derivatives

It has recently been reported that derivatives of resveratrol, mitochondria-targeted via a 4-triphenylphosphoniumbutyl iodide (BTPI) group O-linked at position 3- or 4′-, are cytotoxic *in vitro* and selectively induce mainly the necrotic death of fast-growing tumour cells when administrated in the low μM range. It has also been shown that "capping" the resveratrol's free hydroxyls with acetyl or methyl groups increases the cytotoxicity of such derivatives and their effectiveness as respiratory chain inhibitors, and promotes ROS generation (Sassi et al., 2014).

As our 4-oxystylbene derivatives F1 and F3 share the stylbene scaffold and a *para* positioned ω-onium alkyloxy group with the BTPI derivatives of 3,5-dimethyl resveratrol, we decided to synthesise, a the 3,5-dimethyl resveratrol-analogue of F1 (RS12), and a derivative (RS18) that was further modified by replacing trialkylammonium with a triphenylphosphonium head (Figure **18A**).

We determined the α7affinity and the effect on cell viability of the new compounds RS12 and RS18 and found that the former has similar affinity (Ki 186 nM) but lower potency (IC_{50} of 12 μM) to those of F1, whereas the latter has lower affinity than F1 (Ki 1.52 μM) but slightly higher potency (IC_{50} 2.5 μM). The effects of these compounds are cell specific because they had no effect on HepG2 or SH-SH5Y cells (Figure **18B**).

FIGURES

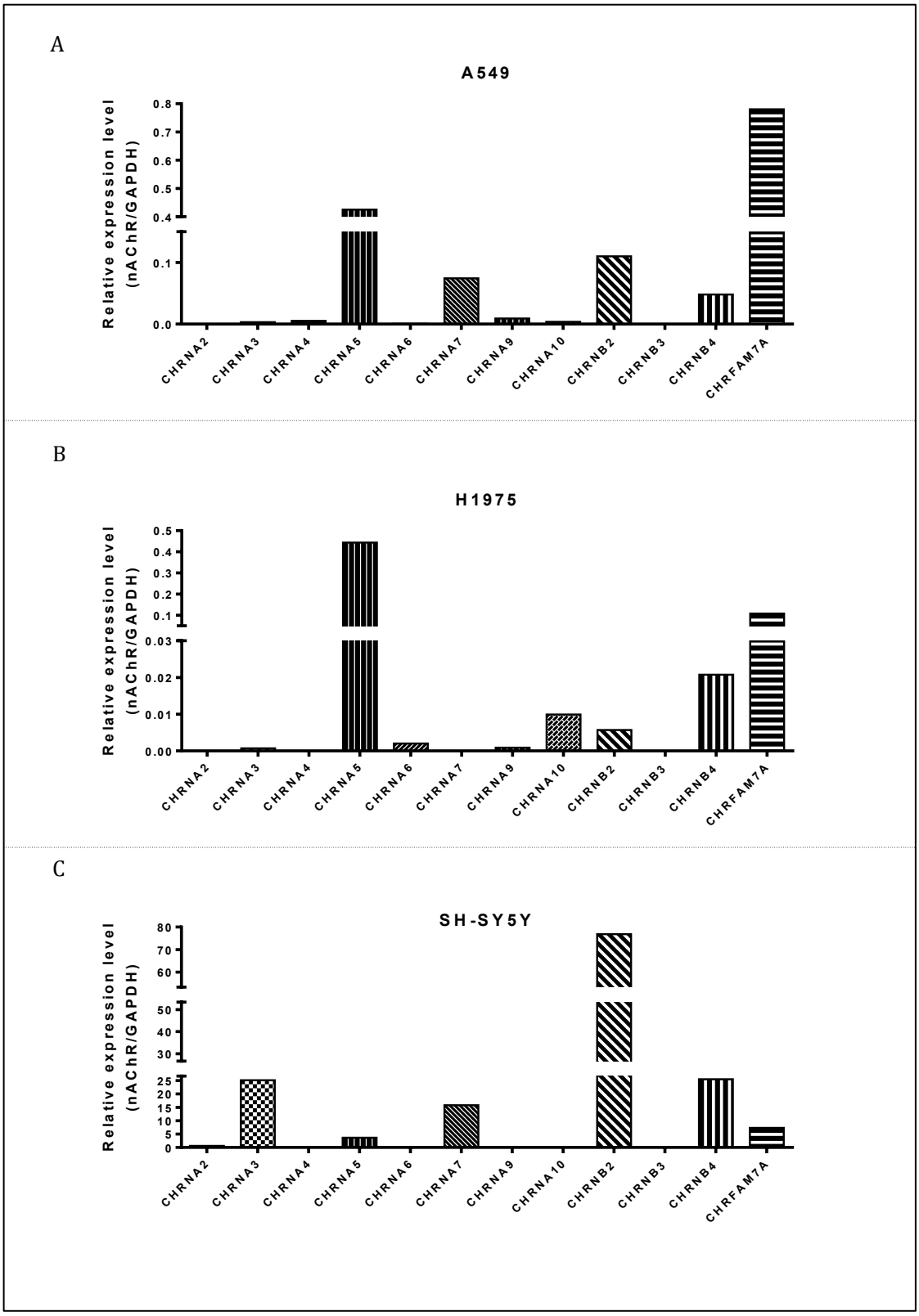


Figure 1: mRNA expression level of different nAChRs subunits in non-small cell lung cancer cell lines A549 (A) and H1975 (B)

qRT-PCR analysis of the human adenocarcinoma cell lines A549 (A) and H1975 (B) and the neuroblastoma cell line SH-SY5Y (C) in the presence of specific TaqMan® primers and probe assay for nAChR subunits. Data are shown as relative expression normalised to endogenous GAPDH expression, according to the 2^-ΔCt method.

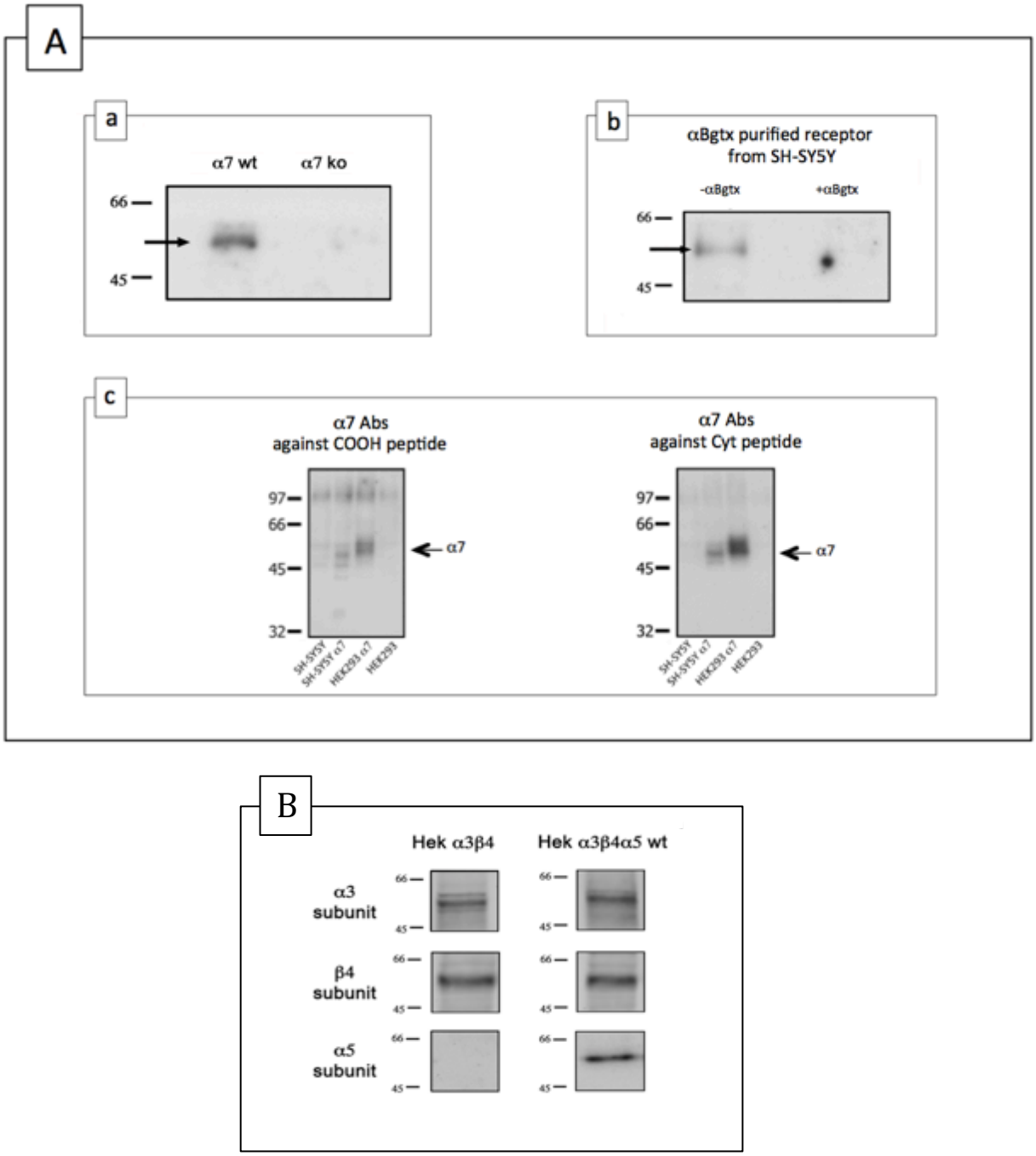


Figure 2: Analysis of antibody specificity

A) Characterisation of α7 Abs tested on hippocampus membrane of wildtype (WT) and α7 knockout (KO) mice (a), and α–Bgtx receptors purified from SH-SY5Y cells (b). Anti- α7 Abs directed against the α7 COOH peptide and the Cyt peptide were tested on untransfected and α7 transfected SH-SY5Y and HEK cells (c).

B) Anti-α3, α5 and b4 Abs tested on HEK cells transfected with the a3b4 and a3b4a5 subtypes.

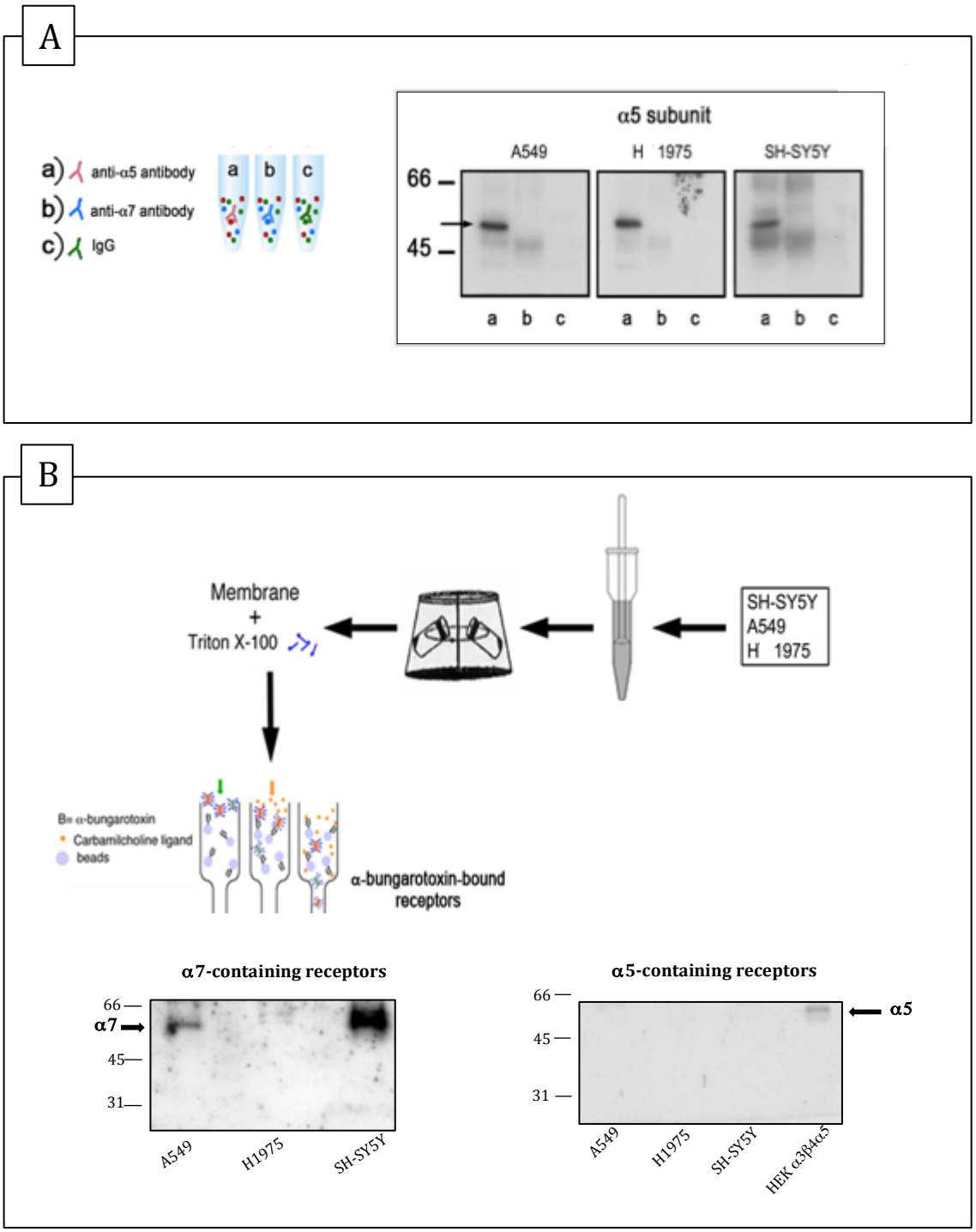


Figure 3: Analysis of native nAChR receptors expressed in NSCLC and neuroblastoma cells

A) Western blotting analysis of A549, H1975 and SH-SY5Y cell extracts immunoprecipitated with anti-α5 (lane a), anti-α7 (lane b) and control IgG (lane c) and probed with anti-α5 Abs.

B) Diagram of the purification experiment and Western blot analysis of the α–Bgtx affinity receptors purified from A549, H1975 and SH-SY5Y cells and Western blotting analysis of the purified α-Bgtx receptors probed with anti α7 (left) or anti α5 (right) Abs.

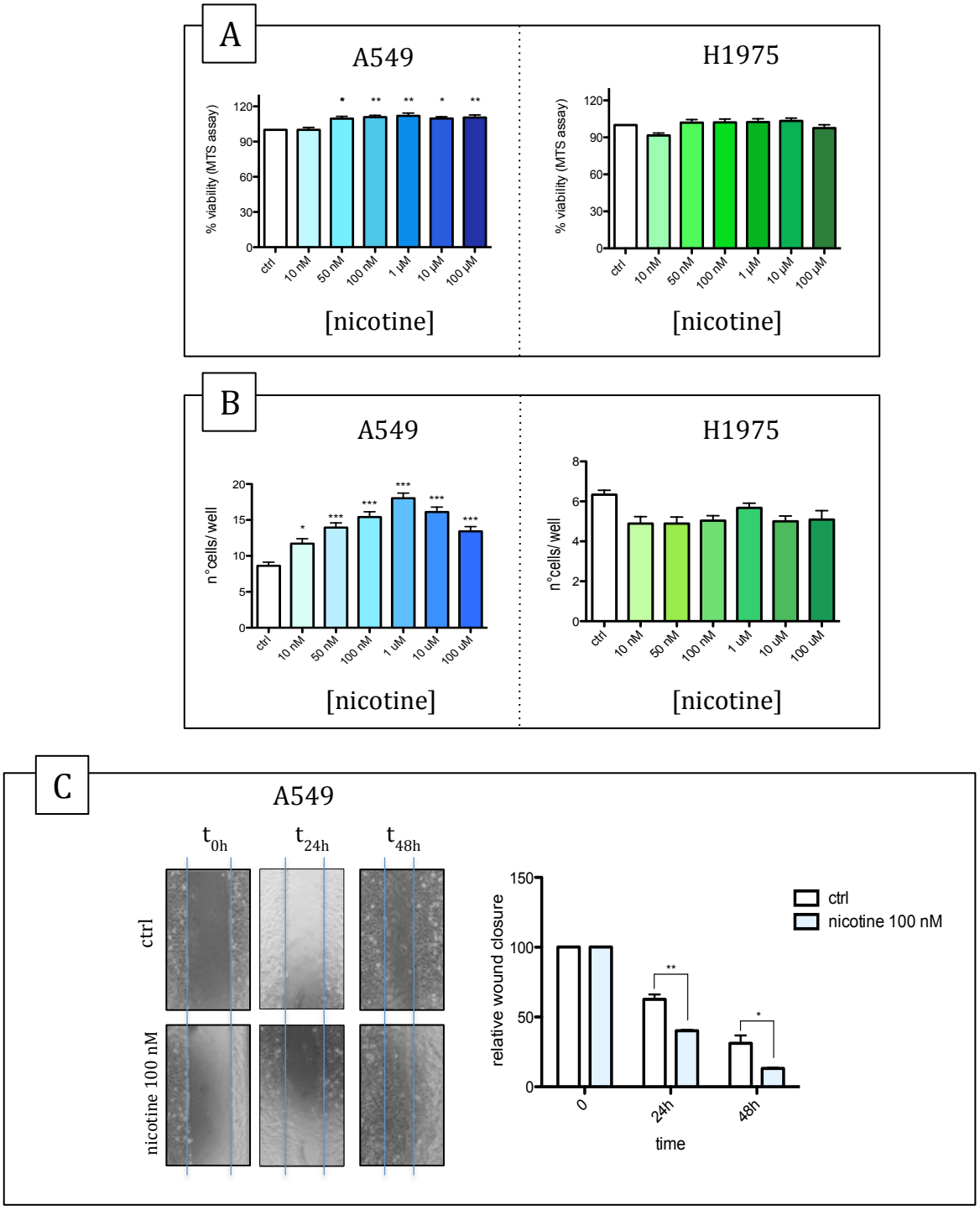


Figure 4: Effects of chronic nicotine exposure on cell viability, proliferation and migration in NSCLC A549 and H1975 cells

Chronic (72 hours) exposure to the indicated concentrations of nicotine increased the viability (A left) and number (B left) of A549 cells, but not H1975 cells (A and B right). Results are the average of three experiments performed in triplicate.

The analysis was made using one-way ANOVA followed by post hoc Bonferroni test for cell count or Dunn test for cell viability (*P< 0.05, ** P< 0.01 and ***P< 0.001).

C) Nicotine-induced migration was evaluated using the wound-healing assay. At time 0 wells containing A549 cells were scratched and not exposed (ctrl) or exposed to 100 nM nicotine. The cells were imaged after 24 and 48 hours, and the wound width of nine random views of the nicotine-treated cells was measured and compared with that of the untreated cells.

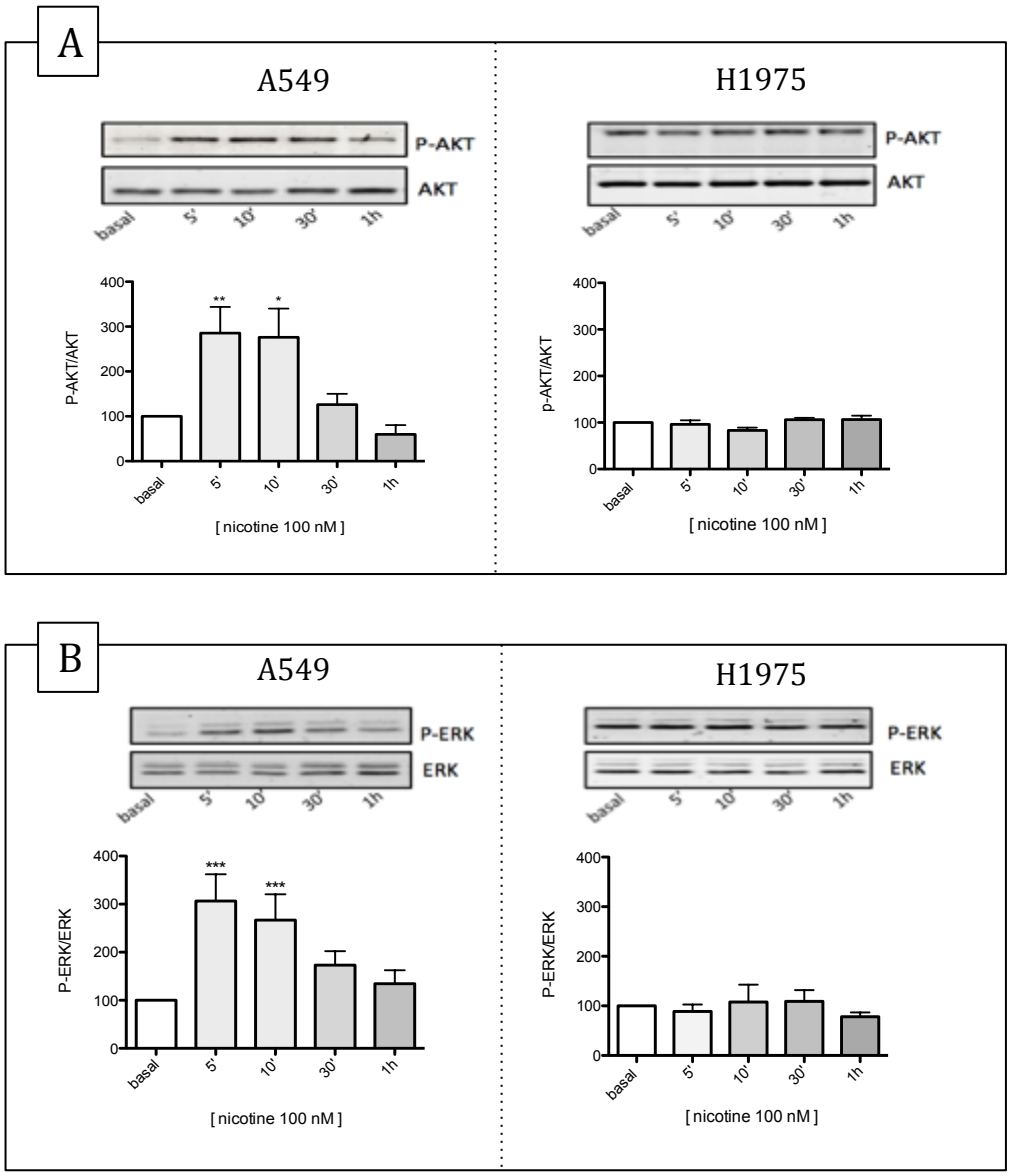


Figure 5: Analysis of nicotine-induced AKT and ERK pathway activation in A549 and H1975 NSCLC cells

A549 (A and B left) and H1975 (A and B right) cells were treated with 100 nM nicotine for the indicated times and the incubation was blocked by adding sample buffer. Proteins were separated on 9% acrylamide SDS gels, transferred to nitrocellulose and probed with antibodies directed against AKT and P-AKT, ERK and P-ERK as described in the Methods.

The Western blot analysis is expressed as the P-AKT /AKT and P-ERK/ ERK ratio at the indicated time of nicotine stimulation (0, 5, 10, 30 and 60 minutes). The graphs show the mean values ± SEM obtained in at least three different experiments performed in duplicate or triplicate. A representative blot at the corresponding times is shown above the graph. The Western blotting data were statistically analyzed using one-way ANOVA followed by Dunn's test (*P< 0.05, ** P< 0.01 and ***P< 0.001, versus untreated cells).

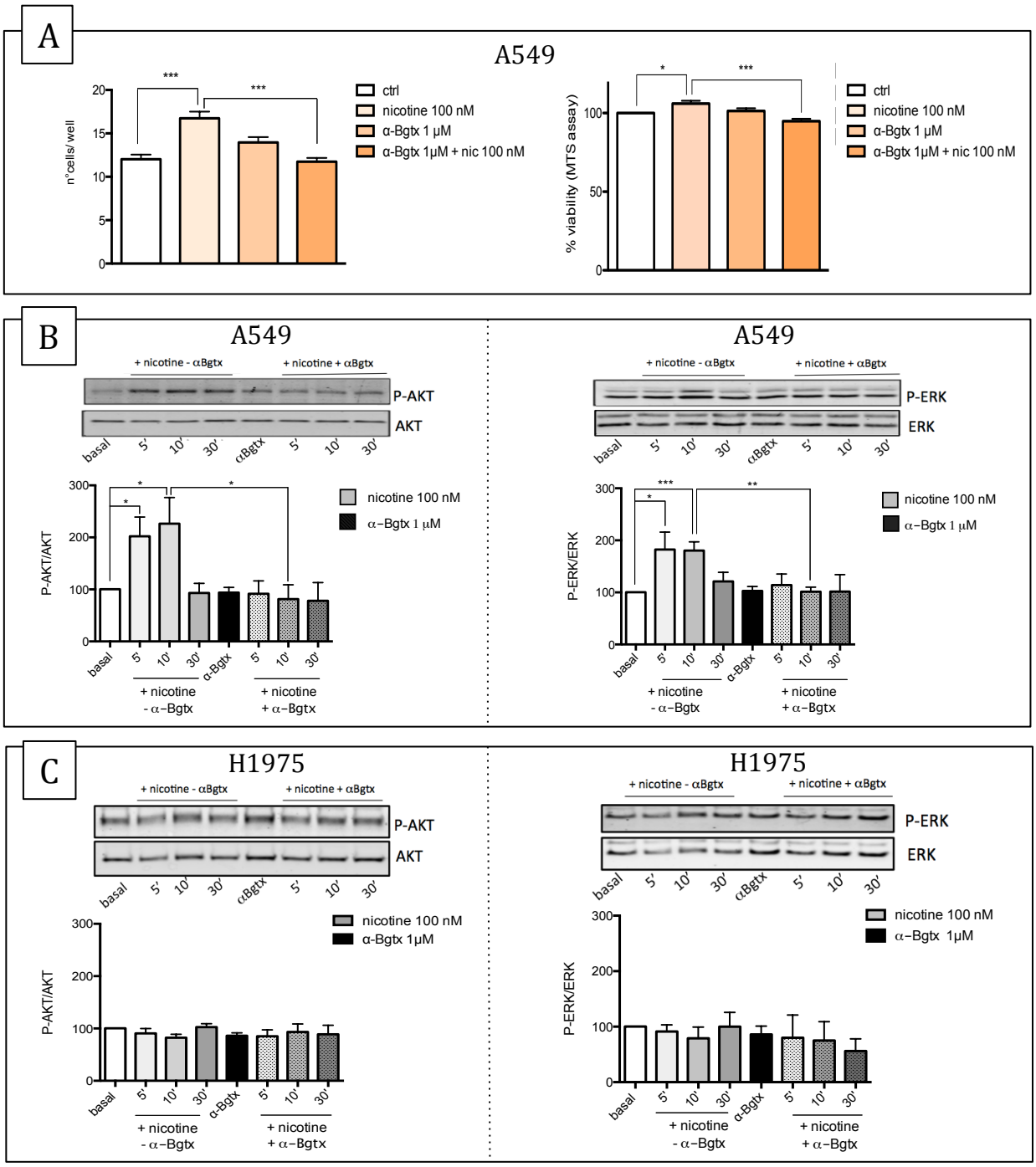


Figure 6: α–Bungarotoxin inhibits nicotine-induced A549 cell proliferation and signalling activation

A) Effect of 100 nM nicotine treatment for 48 hours in presence or absence of 1uM α-Bgtx on A549 cell number (left) and viability (right). Graphs show the mean values ± SEM obtained by analysis of three different experiments performed in triplicate. The analysis was made using one-way ANOVA followed by post hoc Bonferroni test for cell count or Dunn test for cell viability (*P< 0.05, ** P< 0.01 and ***P< 0.001).

B-C) Western blot analysis of AKT and ERK pathway activation in A549 and H1975 cells exposed to nicotine in the presence or absence of 1 μM α–Bgtx. Cells were pre-incubated or not for 30 minutes with 1 μM α–Bgtx and then treated with 100 nM nicotine for the indicated times. The graphs show the mean values ± SEM obtained by analysing at least three different experiments performed in duplicate or triplicate. A representative blot at the corresponding times is shown above each graph.

Western blotting data were statistically analysed using one-way ANOVA followed by Dunn's test (*P< 0.05, ** P< 0.01 and ***P< 0.001).

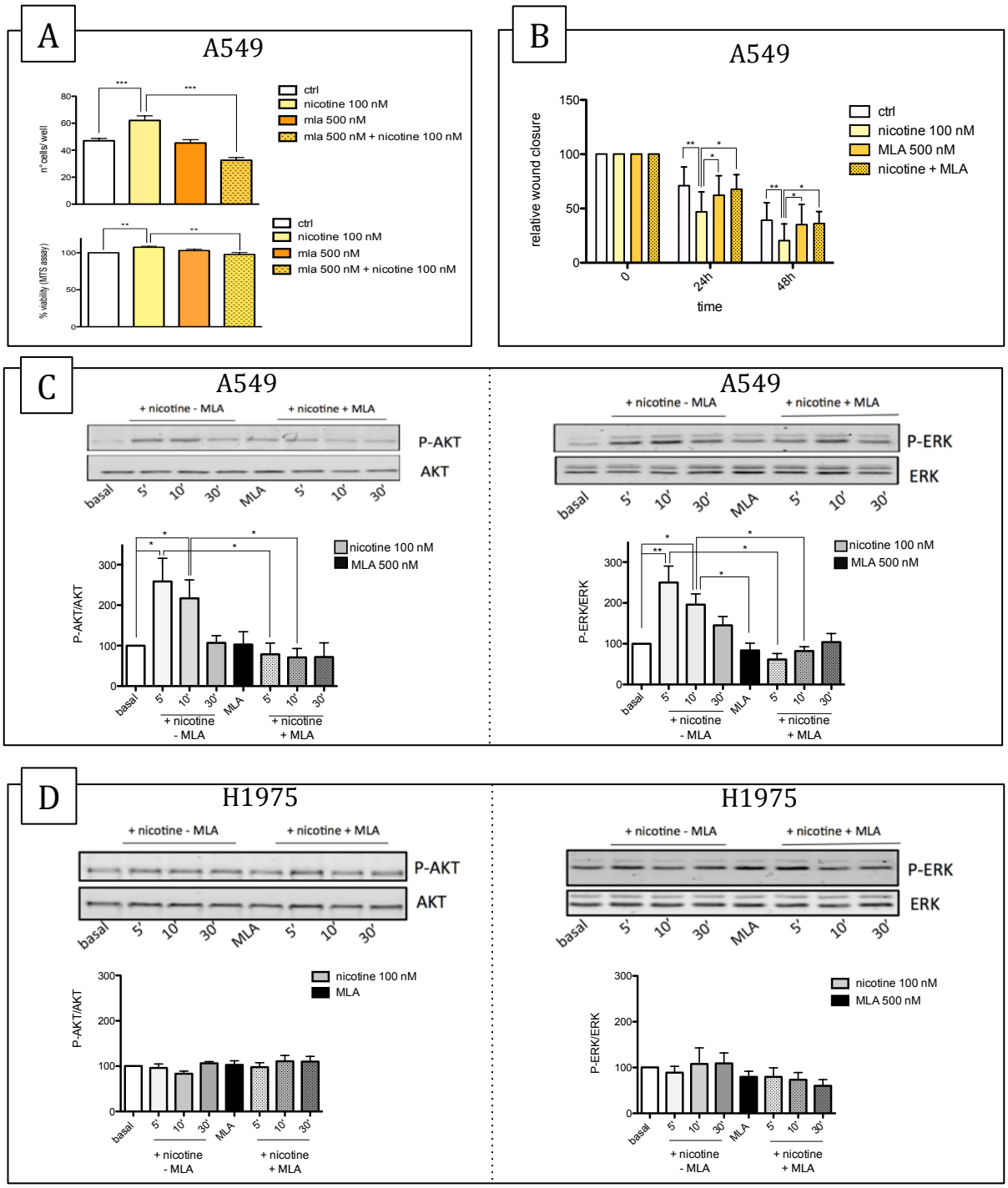


Figure 7: Methyllycaconitine inhibits nicotine-induced A549 cell proliferation and migration

A) The effect of treatment with 100 nM nicotine for 48 hours in the presence or absence of 500 nM MLA on A549 cell number (left) and viability (right) is shown. The analysis was made using one-way ANOVA followed by post hoc Bonferroni test for cell count or Dunn test for cell viability (*P< 0.05, ** P< 0.01 and ***P< 0.001).

B) Nicotine-induced migration was evaluated using wound-healing assay. Wells containing A549 cells were scratched at time 0 and not exposed (ctrl) or exposed to 100 nM nicotine in the presence or absence of 500 nM MLA.The graph shows the results obtained by imaging the cells 24 and 48 hours after the scratch and measuring the wound width in nine random views compared to untreated cells.

C-D) Western blot analysis of ERK and AKT pathway activation in A549 and H1975 cells by 100 nM nicotine in the presence or absence of 500 nM MLA for the indicated times. Cells were then lysed and loaded onto 9% acrylamide SDS gels and probed with antibodies against AKT, P-AKT, ERK and P-ERK.

The graphs show the mean values ± SEM obtained by analysing at least three different experiments performed in duplicate or triplicate. A representative blot at the corresponding times is shown above each graph. The Western blotting data were statistically analysed using one-way ANOVA followed by Dunn's test (*P< 0.05, ** P< 0.01 and ***P< 0.001).

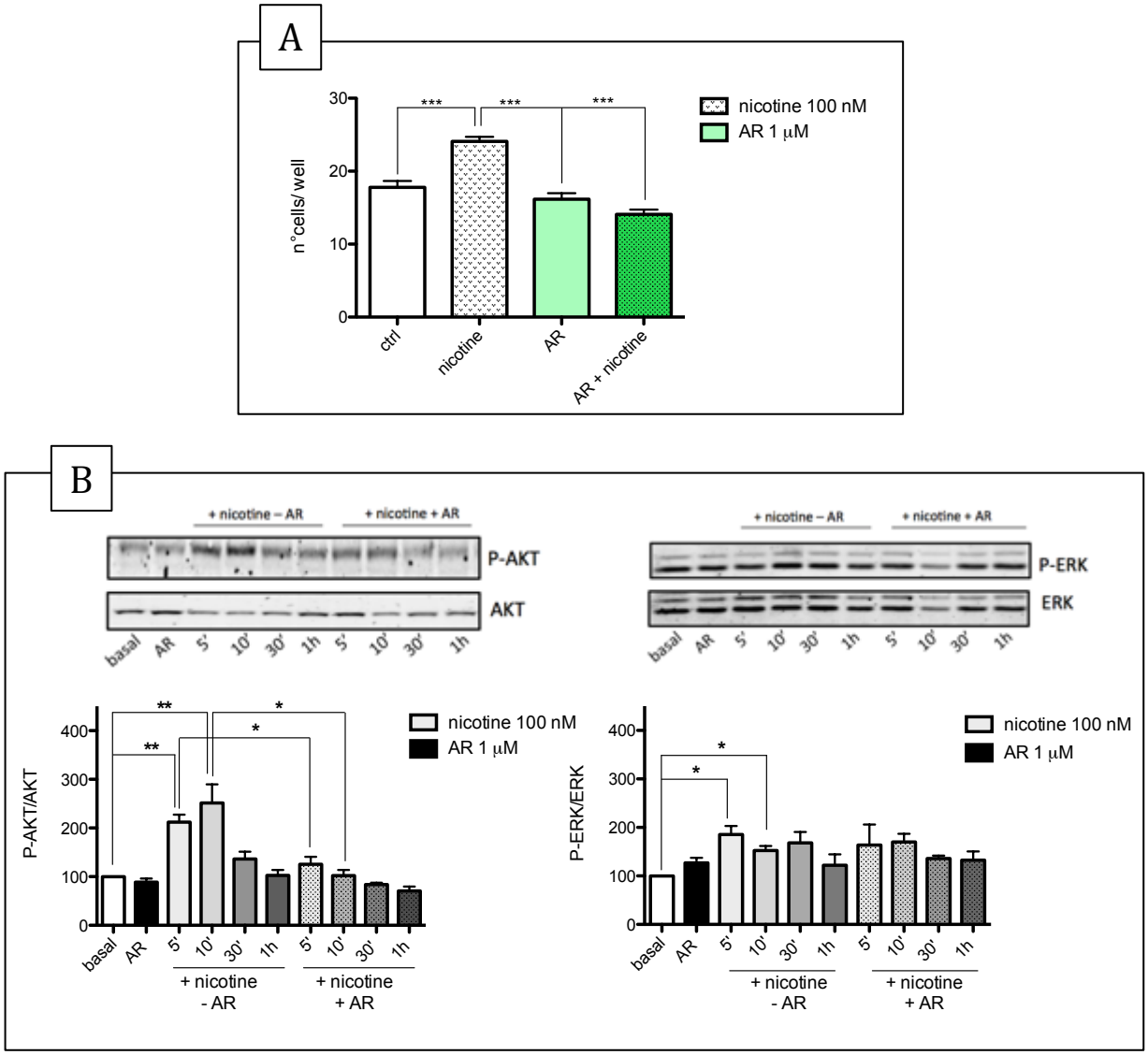


Figure 8: The α7 subtype-selective toxin AR blocks the proliferative effect of nicotine

A) The effect of treatment with 100 nM nicotine for 48 hours in the presence or absence of 1 μM of toxin AR on the number of A549 cells is shown. The results are the average of three experiments performed in triplicate, and are expressed as the number of proliferative cells in each well. The statistical analysis was made using one-way ANOVA followed by a post hoc Bonferroni test (*P< 0.05, ** P< 0.01 and ***P< 0.001)

B) Western blot analysis ERK and AKT pathway activation in A549 cells in the presence or absence of 1 μM toxin AR. The method is the same as that described in part C-D of Figure 6 except for the fact that 1 μM toxin AR was used. The Western blotting data were statistically analysed using one-way ANOVA followed by Dunn's test (*P< 0.05, ** P< 0.01 and ***P< 0.001).

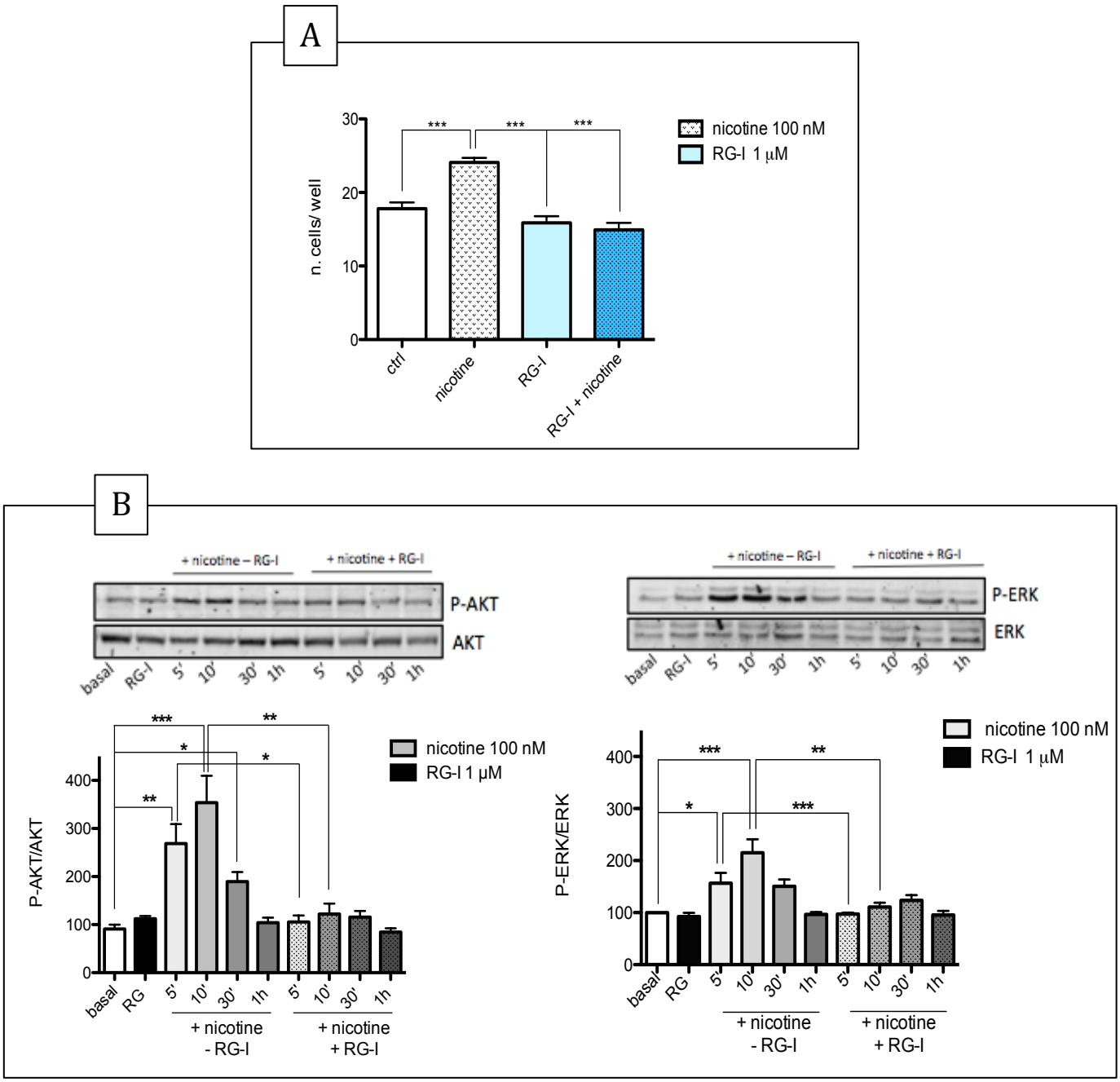


Figure 9: The α9 subtype-selective toxin RG-I blocks the proliferative effect of nicotine

A) The effect of treatment with nicotine (100 nM) for 48 hours in the presence or absence of 1 μM RG-I on the number of A549 cells is shown. The results are the average of three experiments performed in triplicate, and are expressed as the number of proliferative cells in each well. The statistical analysis was made using one-way ANOVA followed by a post hoc Bonferroni test (*P< 0.05, ** P< 0.01 and ***P< 0.001)

B) Western blot analysis ERK and AKT pathway activation in A549 cells in the presence or absence of 1 μM RG-I. The method is the same as that described in part C-D of Figure 6 except for the fact that 1 μM RG-I was used.

The Western blotting data were statistically analysed using one-way ANOVA followed by Dunn's test (*P< 0.05, ** P< 0.01 and ***P< 0.001).

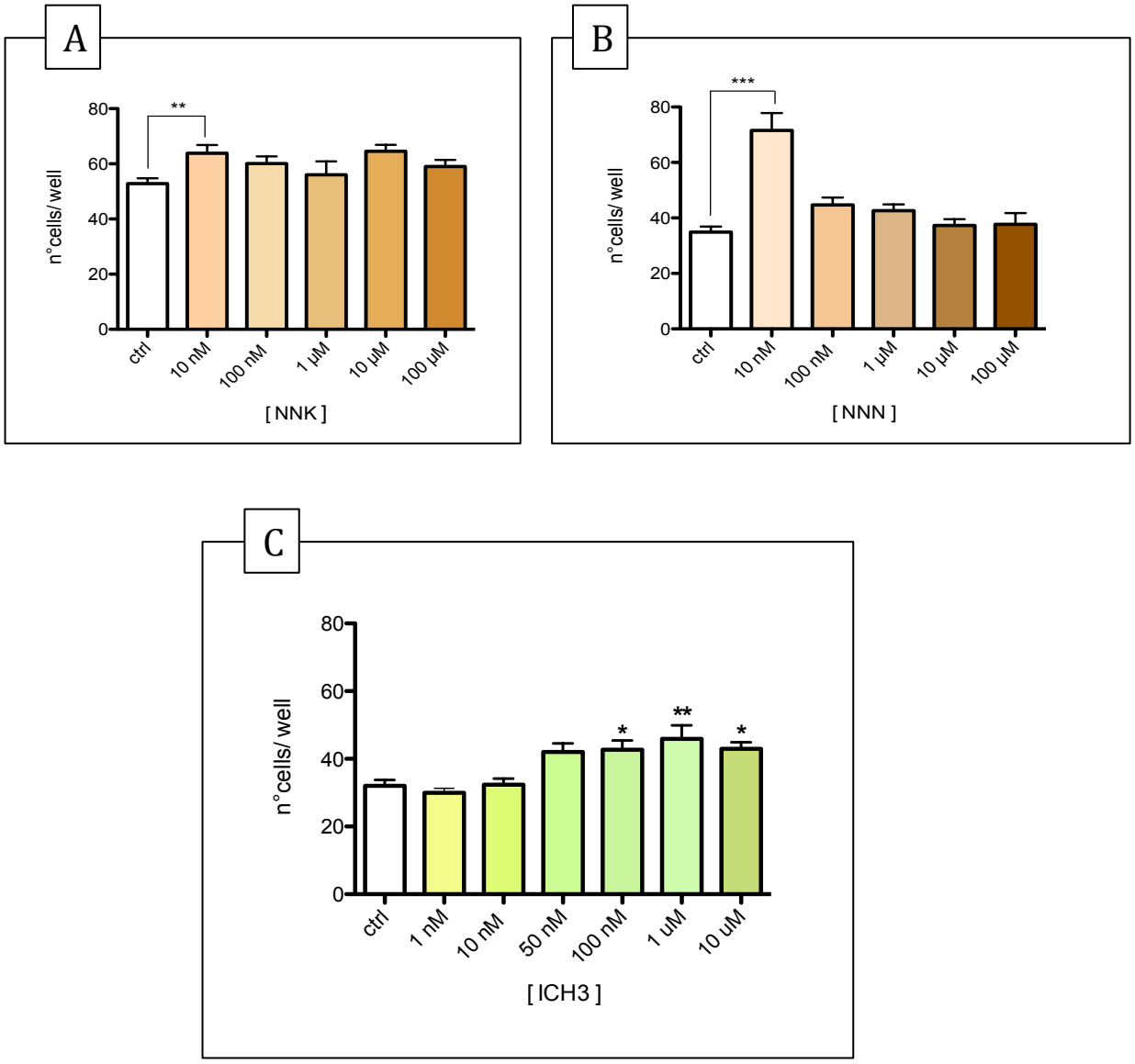


Figure 10: Chronic treatment with nicotine-derived nitrosamines or the α7 agonist (ICH3) increases the number of A549 cells

A549 cells were chronically treated (48 hours) with increasing concentrations of nitrosamines NNN (A) or NNK (B) or (C) the α7 agonist (ICH3). The two nicotine- derived nitrosamines increased cell proliferation at low doses (10 nM), whereas ICH3 increased the number of A549 cells in a dose-dependent manner.

The results are the average of three experiments in triplicate, and are expressed as the number of viable cells in each well. The statistical analysis was performed using one-way ANOVA followed by post hoc Bonferroni test (*P< 0.05, ** P< 0.01 and ***P< 0.001versus untreated cells).

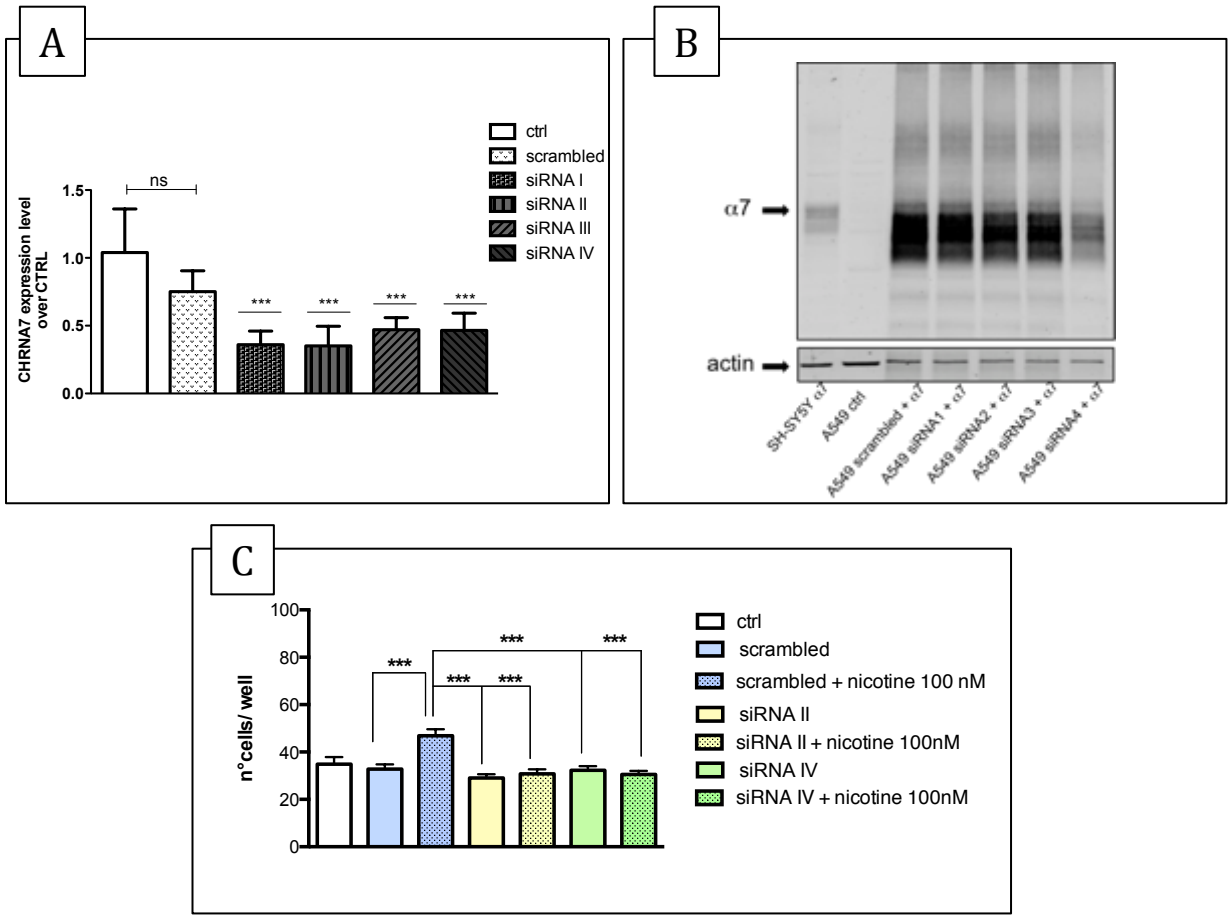


Figure 11: siRNA knockdown of the α7 subunit in A549 cells

A) RT-PCR analysis of α7 subunit mRNA level in A549 cells transfected for 48 hours with 75 pmoli of the indicated α7 siRNAs. The values are expressed taking the level of a7 subunit mRNA present in cells trasfected with scrambled siRNA as 100%.

B) Western blot analysis of A549 cells transfected with α7 cDNA and or not the α7 siRNA II and siRNA IV.

C) Effect of knocking down the α7 subunit on nicotine-induced cell proliferation.

A549 cells were transfected with 75 pmoli of siRNA II or siRNA IV, treated for 48 hours with100 nM nicotine, and then counted. The results shown in the graph are the average of three experiments performed in triplicate, and are expressed as the number of proliferative cells in each well. The statistical analysis was made using one-way ANOVA followed by a post hoc Bonferroni test (*P< 0.05, ** P< 0.01 and ***P< 0.001). Both siRNA II and IV reduced the nicotine-induced proliferation of A549 cells.

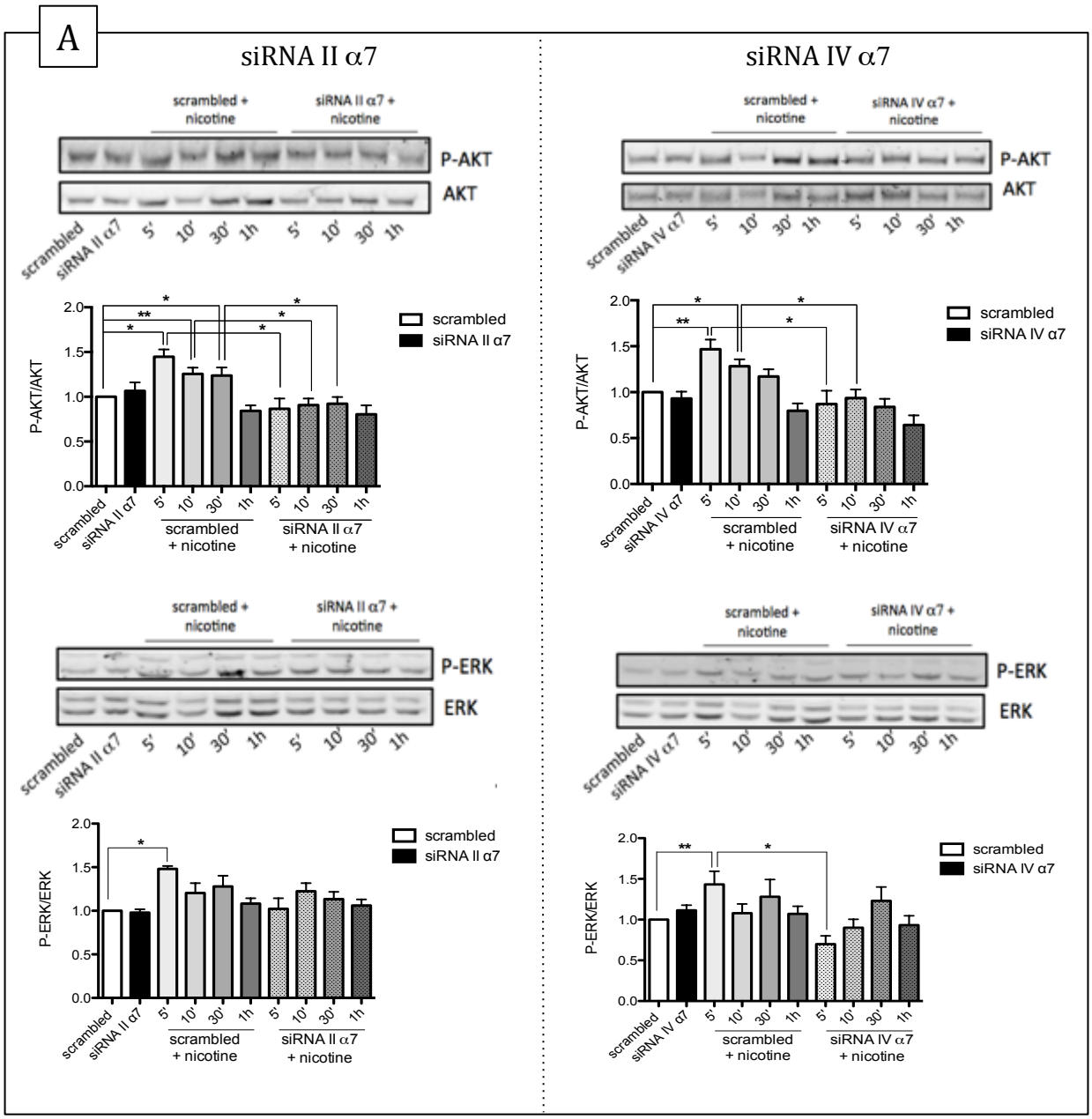


Figure 12: α7 siRNA reduces the nicotine-induced activation of P-ERK and P-AKT in A549 cells

A549 cells were transfected with scrambled siRNA or α7 siRNAs II and IV, starved one day, and then stimulated with nicotine 100 nM for the indicated times and then lysed. Proteins were separated on 9% acrylamide SDS gels, electro-transferred to nitrocellulose and then probed with the antibodies directed against AKT, P-AKT, ERK and P-ERK. The bar graphs show the quantifications as mean values ± S.E.M of at least three independent experiments.

The Western blotting data were statistically analysed using one-way ANOVA followed by Dunn's test (*P< 0.05, ** P< 0.01 and ***P< 0.001).

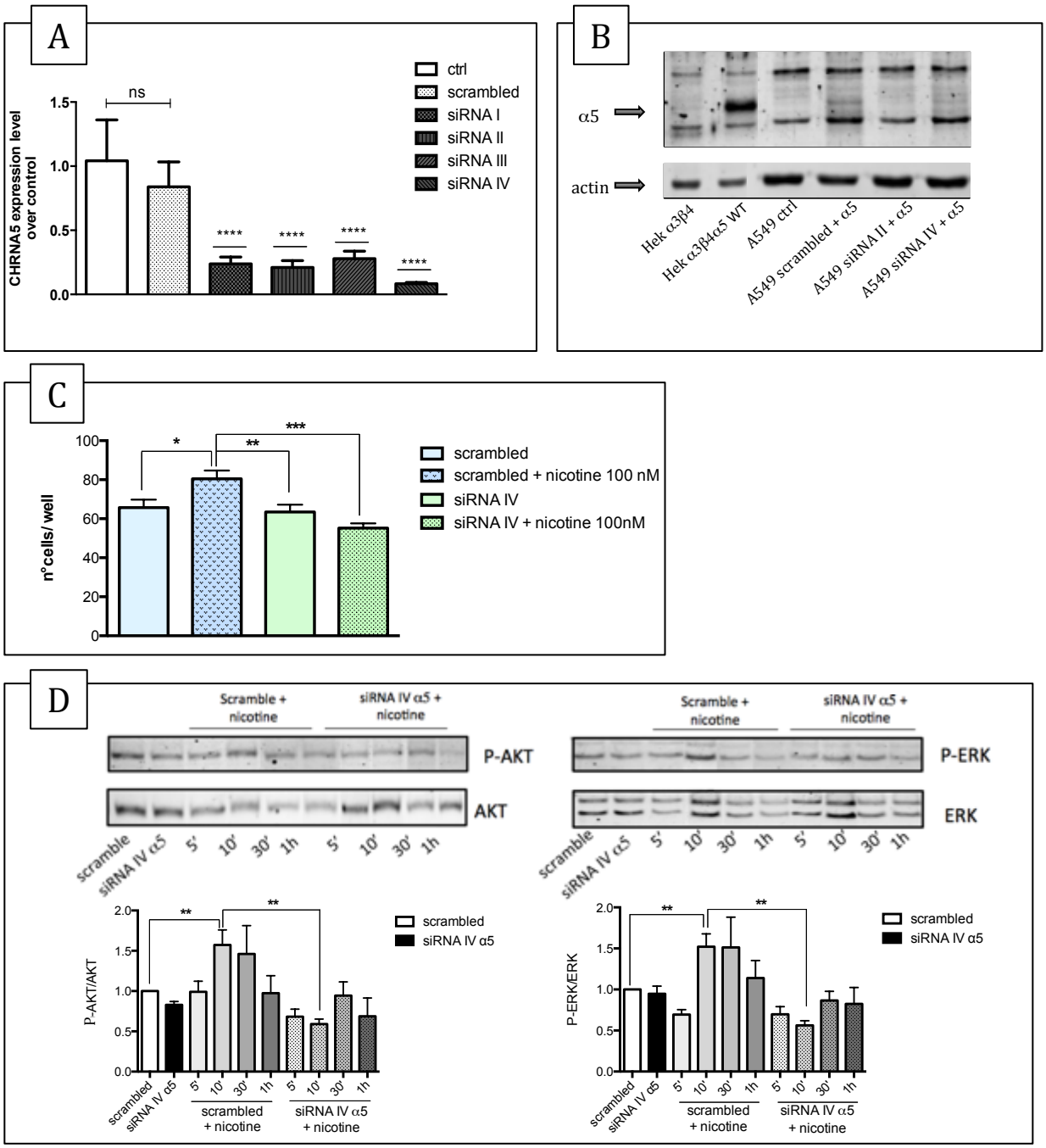


Figure 13: siRNA knockdown of the α5 subunit reduces nicotine-induced proliferation and activation of P-ERK and P-AKT in A549 cells

A) RT-PCR analysis of α5 subunit mRNA level in A549 cells transfected for 48 hours with 75 pmoli of the indicated siRNAs. The values are expressed taking the scrambled siRNA as 100%.

B) Western blot analysis of A549 cells transfected with α5 cDNA with or without α5 siRNA II and siRNA IV.

C) Effect of siRNA IV on nicotine-induced cell proliferation. A549 cells were transfected with siRNA IV, treated for 48 hours with 100 nM nicotine, and then counted.

D) Western blot analysis of P-ERK and P-AKT pathway activation in A549 cells in the presence or absence of 100 nM nicotine and α5 siRNA IV.

Nicotine-induced proliferation as ERK and AKT pathway activation in the presence of scrambled siRNA or α5 siRNAs were measured in at least three independent experiments performed in triplicate. The statistical analysis was made using one-way ANOVA followed by Bonferroni's post hoc test (*P< 0.05, ** P< 0.01 and ***P< 0.001 versus untreated cells) or Dunn test.

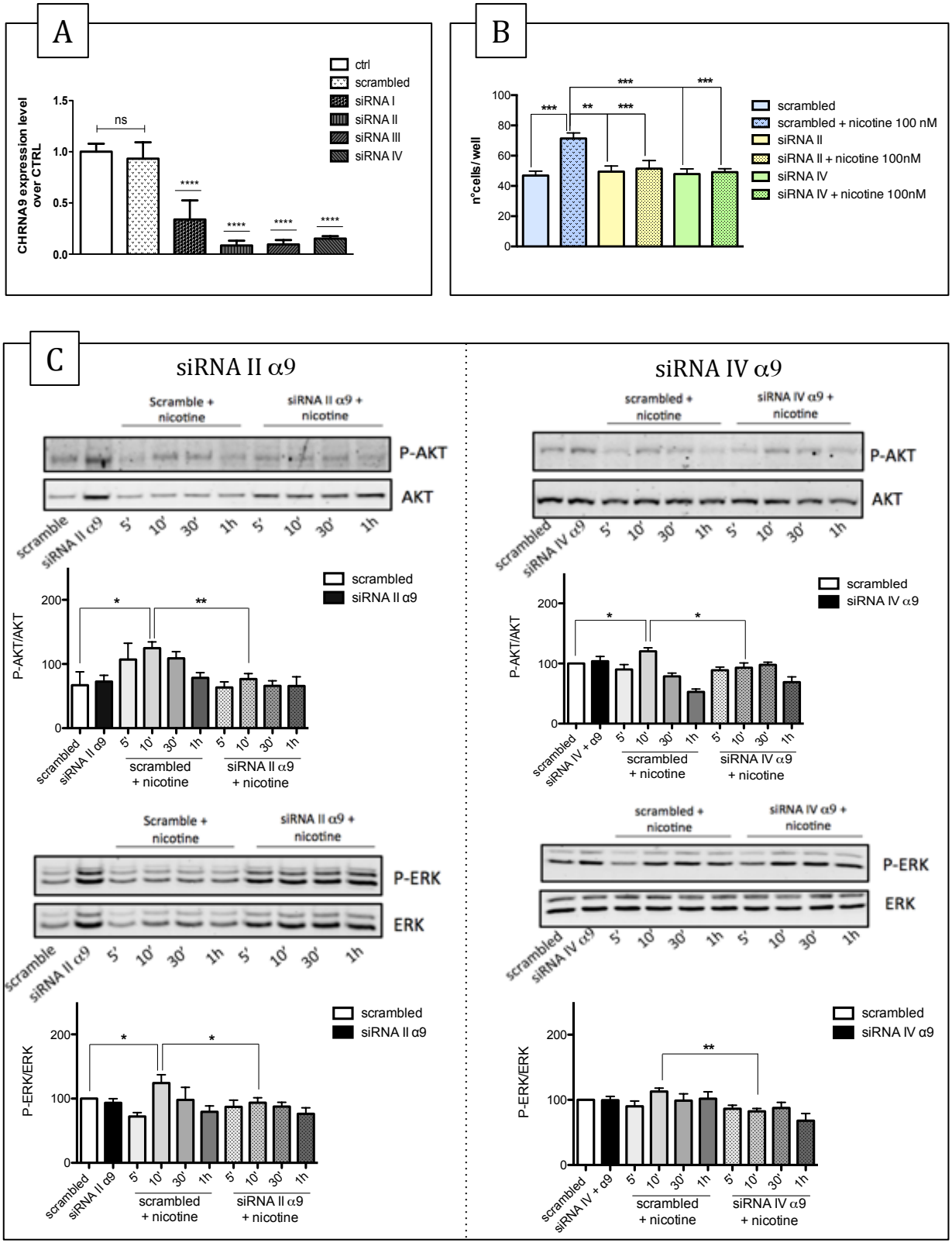


Figure 14: siRNA knockdown of the α9 subunit reduces nicotine-induced proliferation and activation of P-ERK and P-AKT in A549 cells

A) RT-PCR analysis of α9 subunit mRNA level in A549 cells transfected for 48 hours with the indicated α9 siRNAs .The values are expressed taking the level of α9 subunit mRNA present in the cells transfected with scrambled siRNA as 100%.

B) Effect of knocking down the α9 subunit on nicotine-induced cell proliferation. A549 cells transfected with siRNA II or siRNA IV were treated for 48 hours with100 nM nicotine, and then counted.

C) Western blot analysis of ERK and AKT pathway activation in A549 cells in the presence or absence of 100 nM nicotine with or without the α9 siRNA II or siRNA IV.

The statistical analysis of nicotine-induced proliferation and ERK and AKT pathway activation in the presence of scrambled siRNA or α9 siRNAs were made in at least three independent experiments performed in triplicate using one-way ANOVA followed by Dunn test (*P< 0.05, ** P< 0.01 and ***P< 0.001, respectively versus untreated cells) or Dunn test.

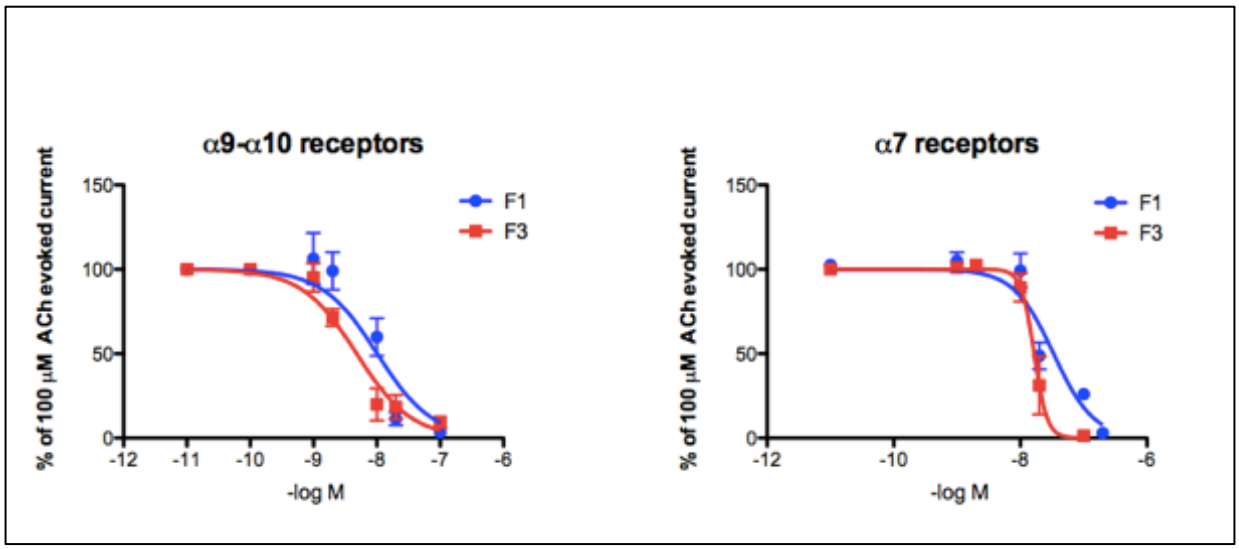


Figure 15. Effects of F1 and F3 on oocyte-xpressed α9-α10 and α7-containing nicotinic receptors

Oocytes were injected with mRNA encoding the human α9-α10 (left) or α7 (right) subunits. Maximal function (I_{max}) was measured in each group by means of perfusion with the full agonist ACh (100 μM).

Concentration–effect curves of F1 and F3 inhibition of 100 μM ACh-induced ion currents. Within each group, all responses were normalized to an initial control stimulation with 100 μM ACh. The data points represent mean values ± SEM. Drug potency and efficacy parameters were calculated using non-linear least-squares curve fitting to the Hill equation (see Methods).

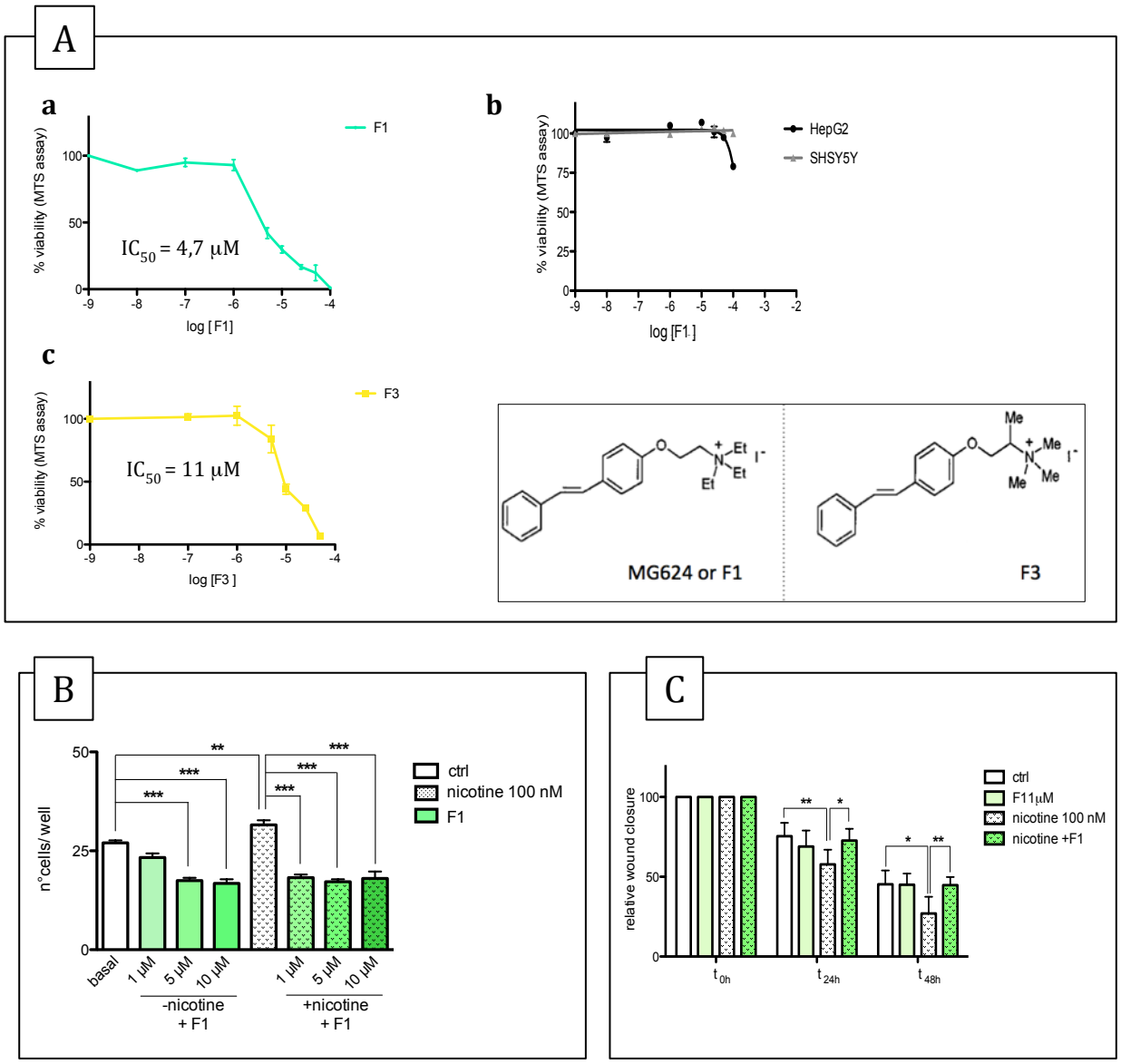


Figure 16: F1 decreases A549 line cell viability, proliferation and migration

A) A549 cells viability was tested using the MTS assay. Cells were plated and incubated with F1 (a) or F3 (c) for 72 hours at increasing concentration. F1 was tested also in SH-SY5Y and HepG2 cells (b).

The data points are the mean values ± SEM of three experiments in which each drug concentration was tested in triplicate.

B) F1 (1 µM) abolished the chronic (72 hour) nicotine-induced increase in the number of A549 cells.

The analysis was made using one-way ANOVA followed Dunn test for cell viability or by post hoc Bonferroni test for cell count (*P< 0.05, ** P< 0.01 and ***P< 0.001).

C) F1 (1 µM) decreased nicotine-induced migration by wound-healing assay. At time 0, wells containing A549 cells were scratched and not exposed (ctrl) or exposed to 100 nM nicotine in the presence or absence of 1 µM F1. The cells were imaged after 24 and 48 hours, and the wound width of nine random views of the nicotine-treated cells was measured and compared with that of the untreated cells.

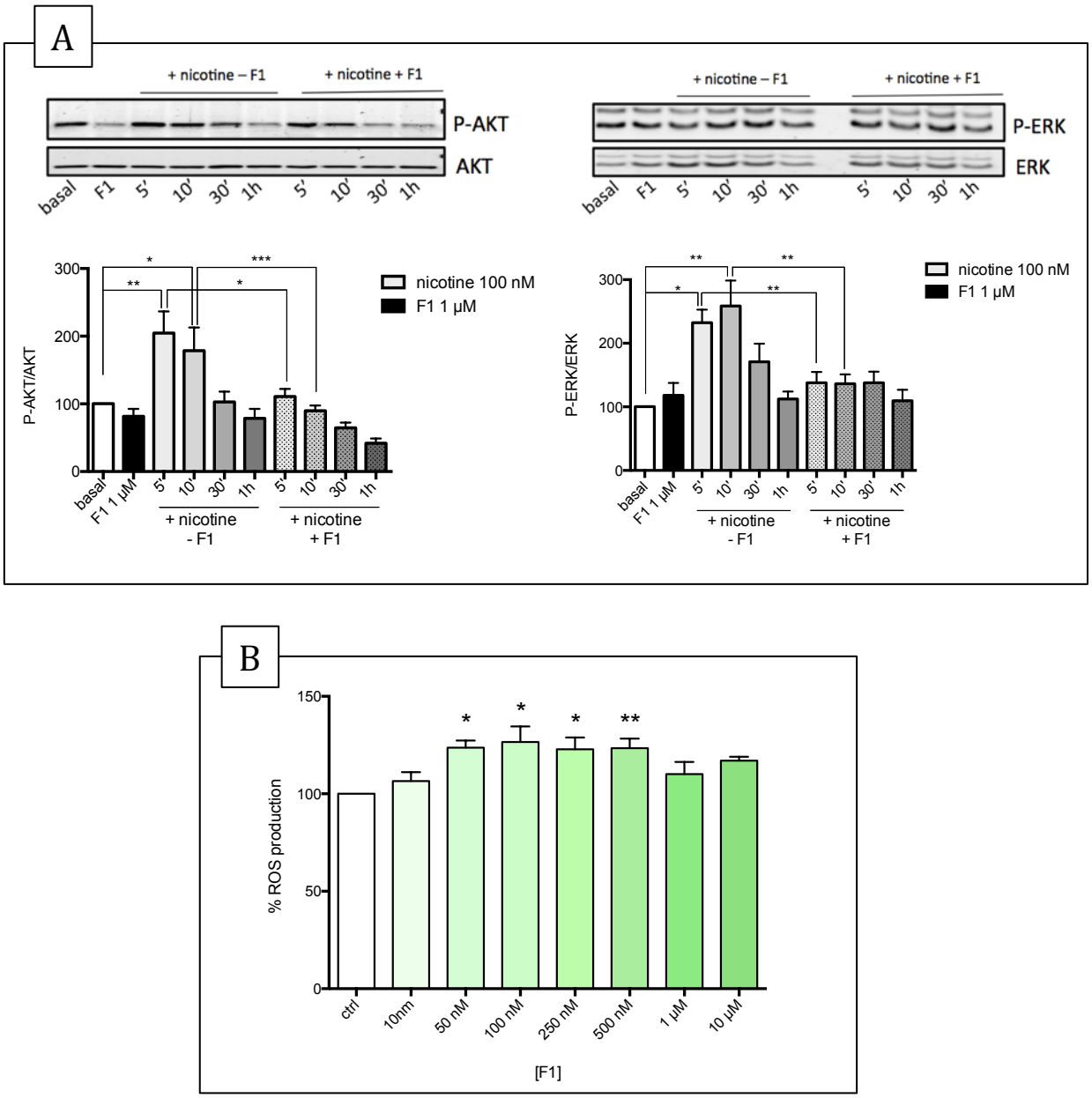
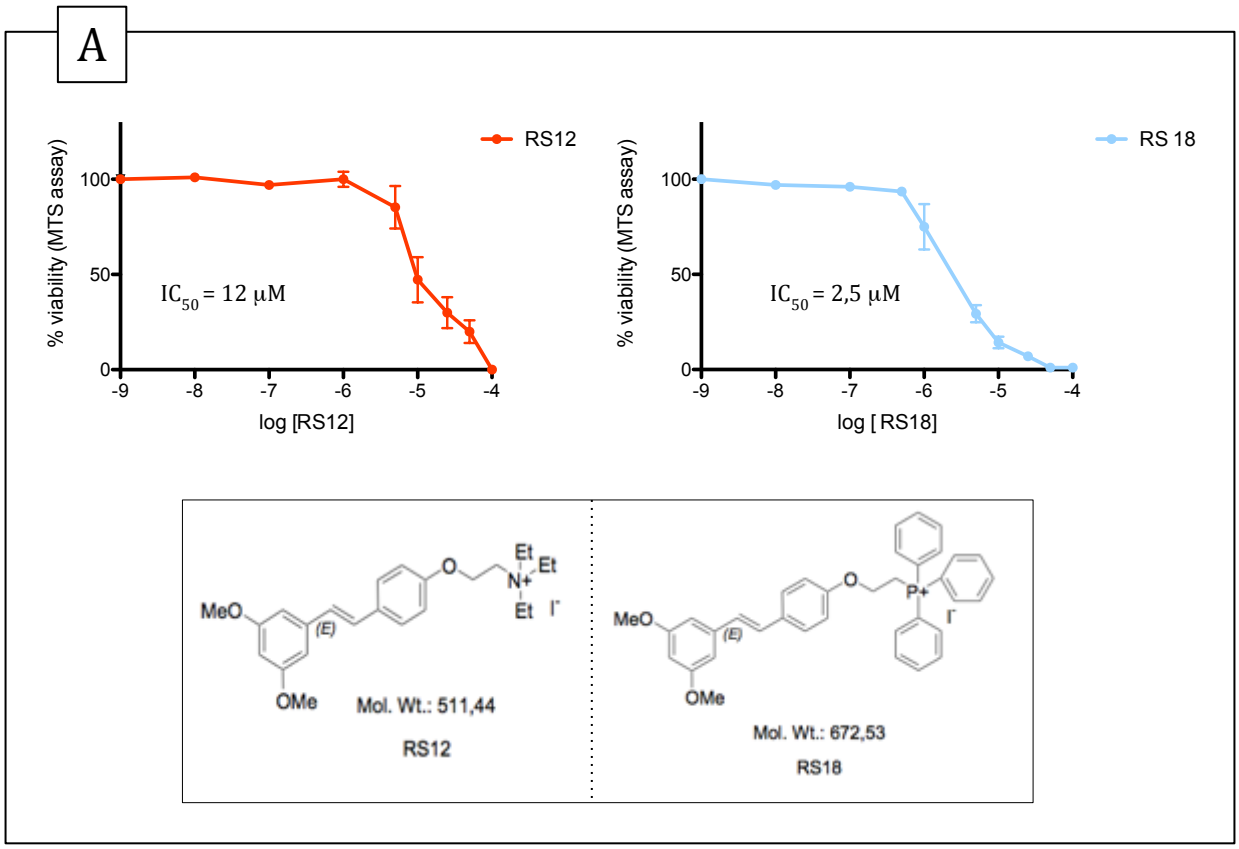


Figure 17: F1 blocks the nicotine-induced activation of P-ERK and P-AKT and increases ROS production

A) Western blot analysis of ERK and AKT pathway activation upon nicotine stimulation in A549 cells with or without F1 (1 μM).

B) The A549 cells were seeded in black 96-well plates, pre-treated with the indicated concentrations of F1 for 24 hours, and then incubated with 25 μM DCFH-DA for 30 minutes at 37°C. Fluorescence was measured at 485 nm_{ex}/530 nm_{em} using a microplate reader. The results are expressed as the increase in fluorescence units/μg of cell proteins taking as the production of ROS in untreated cells as 100%.



B

	α7 affinity (Ki, nM)	A549 IC_{50} µM	SH-SY5Y IC_{50} µM	HepG2 IC_{50} µM
F1	161 (86-303)	4.1 (3.6-5.3)	>50	>50
F3	100 (60-250)	9.7 (6.3-15)	>50	>50
RS12	186 (54-325)	4.5 (1.8-10.1)	>50	>50
RS18	1520 (383-6075)	1.9 (1.4-2.7)	>50	>50

Figure 18. Characterisation of new oxystilbene derivatives

The new componds RS12 and RS18 were tested for their α7 affinity (B) and effect on A549 cell viability by MTS assay (A).

B) Ki affinities were determined from inhibition of ^{125}I α-Bgtx binding by inhibition curves whereas the IC_{50}values were determined from dose-response curves with the MTS assay.

DISCUSSION

Many literature data indicate that in lung tumours nAChRs are central regulatory elements, which mediate nicotine and nicotine derivates cellular responses (Schall and Chellapan, 2014; Grando, 2014).
Delineating the biological and pharmacological effects of nicotine on lung cancer cells at receptor subtype level represents a big challenge for cancer prevention and treatment.
As in the case of the nAChRs express in the CNS, the function, ligand-binding affinity and pharmacological profile of lung tumoural nAChRs depend on their subunit composition. For this reason we used RT-PCR to analyse the nAChR subunit gene expression levels in order to make a quantitative comparison between the A549 and H1975 NSCLC cell lines.

The H1975 cell line was established in July 1988, while the A549 cell line was developed in 1972 by D.J. Giard, et al. through cultures of explants of lung carcinoma tissue of a 58-year-old Caucasian male. In nature A549 cells are responsible for the diffusion of substances, such as water and electrolytes, across the alveoli of lungs. They grow adherently, and form confluent monolayers *in vit*ro.
Many scientific papers have reported that this cell line expresses the mRNAs for different nAChR subunits, but the native subtypes are not yet well identified. It has also been reported that nicotinic drugs can influence the proliferation of these cells and that A549 luciferase-labelled cells, commercially available, can be used for *in vivo* bioluminescence imaging to study the effects of nicotinic toxins or compounds in NOD/SCID mice xenografted with A549 cells. The properties of A549 cell line to be very manageable, resistant and little subject to contamination or cell death make them the most suitable scientific model for our *in vitro* experimental analyses.

We demonstrated that both cell lines showed a high level of transcripts for the α5 subunit and the α7 dup protein; the other subunits were only found in one or the other cell line. In particular H1975 cells did not express α7 mRNA and the expression of α9 mRNA was very low.
Treatment of A549 and H1975 cells with nicotine concentrations in the range of those detected in serum of smokers (10^{-8} – 10^{-7} M) induced proliferation in A549 but not in H1975 cells. Nicotine induced a dose-dependent increase in S-phase entry of A549 cells that was greatest at a nicotine concentration of 100 nM - 1μM thus suggesting the

involvement of cell-specific nAChRs in nicotine-mediated effects expressed in this cell line. We found that the proliferation of A549 cells was also induced by a low concentration (10 nM) of the nitrosamines NNN and NNK, the most harmful tobacco components, because they directly bind the DNA, form DNA adducts and cause mutations.

In order to identify the subtypes involved in this proliferative effect we used two nAChR antagonists (α–Bgtx and MLA) that act on α7 and α9 receptors, and found that both blocked the nicotine-induced proliferation in A549 cells. In order to discriminate the activity of the two subtypes, we performed separate proliferation assays in the presence of α7 (AR) and α9 (RG-I) selective toxins, and found that both blocked nicotine–induced proliferation. The involvement of the α7 subtype in nicotine-induced proliferation was also confirmed by the fact that a very low concentration of the subtype selective α7 agonist ICH3 also increased cell proliferation.

It has been widely reported that cigarette smoking not only induces cancer cell proliferation but might also promote their metastatic spread (Murin et al., 2004). Xu et al. (2006) have demonstrated that invasion and metastasis are parts of a sophisticated process that involves alterations in cell adhesion molecules and changes in cadherin composition.

We found that A549 cells increased their migratory activity upon nicotine stimulation, and that MLA blocked this increase. Our findings are in agreement with those of other studies showing the importance of the α7-receptor in nicotine-induced cell proliferation and migration.

However the role of α7 receptors in A549 cell proliferation is debated because one study, originally found that the proliferation of transplanted A549 cells in nude mice can be inhibited by α-cobratoxin, an α7 receptor antagonist (Paleari et al., 2009), but the authors subsequently retracted some of their findings and later studies of *in vivo* tumour growth in orthotopic mice treated with the same dose of a-cobratoxin did not find any significant reduction in tumour growth or survival (Alama et al., 2011). However, our data indicating that *in vitro* MLA abrogated the pro-invasive effects induced by nicotine suggests that blocking nAChRss containing the α9 and/or α7 subunit can reduce specific lung cancer invasiveness.

Although nAChRs are ion channels mediating the influx of Na^+ and Ca^{2+} and the efflux of K^+, their activation by a ligand, such as nicotine, may elicit both ionic and non-ionic signalling events regulating the phosphorylation and dephosphorylation of target proteins. We therefore analysed which intracellular pathways are involved in the nicotine-induced proliferation and migration of A549 cells.

Nicotine stimulation of nAChRs triggered several protein kinase-signalling cascades, thus simultaneously altering gene expression and inducing cellular changes. We confirmed that nicotine increased P-ERK/ERK and P-AKT/AKT ratios after very short exposure (five –ten minutes), and that the α7/α9 toxins (MLA and α–Bgtx) as well as the α9-selective toxin (RG-I), and α7-selective toxin (AR) abrogated these intracellular signalling activations.

We chose these pathways because many studies have shown that the ERK pathway modulates cell proliferation and survival. It is thought that ERK activation triggers cell cycle progression and inhibits cell death by inactivating pro-apoptotic factors and/or overexpressing and activating anti-apoptotic factors such as Bcl2 (Balmanno and Cook, 2009) and AKT, is a critical pathway for survival signals in cancer cells (Sheng et al., 2009). The overexpression of serine 473-phosphorylated AKT has been observed in a wide range of human cancers, including colorectal and lung cancer (Roy et al., 2002). Moreover, AKT activation seems to be correlated with advanced disease and/or a poor prognosis (Bellacosa et al., 2005).

The use of nAChR-subunit antibodies, has shown that nAChR subunits are also expressed on the mitochondrial outer membrane of lung cells (mt-nAChRs) (Kalashnyk et al., 2012; Gergalova et al., 2011) and that these receptors non-covalently connect to voltage-dependent anion channels and control cytcrome C release by inhibiting the mitochondrial permeability transition pore opening (Saxena et al., 2011; Yu et al., 2011). Although the specificity of many antibodies against nAChRs has been questioned and the presence of mt-nAChR is still an open question, the fact that nicotine-induced cell proliferation and intracellular signalling in A549 cells was blocked by peptide toxins, that are not cell permeable and can only act at the plasma membrane, indicate that the effects of nicotine are due to the activation of nAChRs in the plasma membrane.

We also verified whether the nAChR subunit genes *CHRNA5 CHRNA7* and *CHRNA9*, were involved in nicotine-induced A549 cell growth and proliferation by silencing the α5, α7

and α9 subunits. We found that subunit-specific siRNAs abrogated nicotine induced cell proliferation and the activation of ERK and AKT intracellular signalling pathways. The involvement of α7 in ERK and AKT activation has already been demonstrated in other cancer cell lines, but we demonstrated that also α9-containing receptors play important role in the nicotine-induced activation of P-AKT and P-ERK. This is significant because α9-containing receptors have so far been primarily associated with breast cancer (Lee CH et al., 2010). Various groups have shown that the specific inhibition of α9 nAChRs concomitantly inhibit breast cancer cell growth, soft-agar colony formation, and tumour growth in SCID mice (Chen et al., 2011), whereas our pharmacological and silencing studies demonstrate that they are involved in the development of lung cancer, suggesting a potential therapeutic target in NSCLCs, expressing this subunit.

RT-PCR analysis showed that the α5 subunit mRNA is highly expressed in A549 cells, and that silencing this accessory subunit decreases nicotine-induced cell proliferation and abrogates nicotine-induced P-AKT activation. Our results are in agreement with those reported by Ma X. et al. (2014). He found that silencing α5-containing nAChRs in A549 cells significantly inhibited nicotine-induced cell proliferation and attenuated nicotine-induced up-regulation of HIF-1α and VEGF. However these results are different from those reported by Krais et al. (2011), who demonstrated that silencing the CHRNA5 gene or inhibiting α5-containing receptors nAChRs in non-transformed bronchial cells and lung cancer cell lines increased cell motility and invasiveness, thus indicating that these receptors are negative regulators of nicotine-induced cell migration.

These different results may be explained by the fact that the silencing effect was quantitatively different in the different studies and/or that different sub-clones of A549 cells were used.

α5 is an accessory subunit and can only exert its effect when it is associated with other nicotinic subunits, and so we tried to identify the possible subtype(s) containing this subunit. Previous studies in normal bronchial cells have shown that (α3β2)2α5 receptors are expressed during wound healing in normal mucosa and that this expression is particularly high at the edges of wounds (Tournier et al., 2006). We used subunit-specific anti-α5 Abs to immunoprecipitate α5 subunits from A549 cell extracts and probed them with Abs directed against other nAChR subunits. The fact that there was no anti- α3, anti-β2 and anti- β4 subunit antibody labeling excluded the presence of the α3α5β2 or α3α5β4 subtypes in A549 or H1975 cells.

It has been reported that a possible α7α5 subtype is present in cancer cells. In order to verify this we purified the nAChRs from A549 cells using α–Bgtx covalently bound to Sepharose 4B. Western blot analysis of the affinity purified α–Bgtx receptors shows the presence of the α7 subunit but not the α5 subunit (**Figure 3B**). Although other findings suggest that α5-containing nAChRs contributes to cancer cell growth and progression, further studies are needed to identify the subunit composition of the subtype that mediates this effect in A549 cells.

Stimulated by recent data (Brown et al., 2012) showing that an old molecule MG624 (also called F1) previously characterized by our group, potently suppresses the proliferation of primary human micro vascular endothelial cells of the lung (HMEC-Ls) and suppresses the *in vivo* angiogenesis of NCi-H69 SCLC tumours in the chorioallantonic membrane and nude mice model, we decided to characterise its anti-proliferative effects on A549 cells.

We have previously reported that F1 is highly selective on α7 nAChRs expressed in chicken, rat, monkey and human brain membranes (Maggi et al., 1999; Tanibuchi et al., 2010) and, in collaboration with Dr McIntosh (USA) we have recently found that it also binds the oocyte-expressed α9–α10 subtype and blocks its Ach-induced current at very low concentration (nM range).

We found that F1 binds both α7- and α9- containing nAChRs, potently abrogates the viability and proliferation of A549 cell line after nicotine stimulation, but has no effect on control cell lines (HepG2 and SH-SY5Y). As nicotine stimulates NSCLC cell invasion and the epithelial to mesenchymal transition (EMT) preferentially via α7 nAChRs (Zhang et al., 2016), we assessed whether F1 abrogates nicotine-induced cell migration in the A549 cell line and found that a low concentration inhibits cell invasion and decreases nicotine-induced activation of the P-ERK and P-AKT pathways.

In addition F1 display a stylbene structure very similar to that of resveratrol and ptero-stylbene, so we investigated whether F1 exerts some of its effects by simply passing the membrane and/o increasing ROS production.

ROS play contradictory roles in tumorigenesis: they play a causal role in tumor development and progression by inducing DNA mutations, genomic instability, and aberrant pro-tumorigenic signalling, while very high levels might induce cell death in cancer cells. There is published evidence of a correlation between increased oxidative stress and apoptosis, and Luo et al. (2010) have shown that the isoflavanone, cajanol induces apoptosis via a ROS-mediated mitochondria-dependent pathway. We therefore

tested F1 and found that it stimulates ROS production at relatively low concentrations (100-250 nM). Given this finding it is important to clarify the molecular mechanisms by which F1 and its derivatives can increase cancer cell apoptosis.

Starting from the F1 structure we have synthesised and pharmacologically characterised the new compounds RS12 and RS18. Both compounds seem to have a cytotoxic effect, but more studies are necessary to define their possible cytotoxic effect on A549 cells.

CONCLUSIONS

The findings of this study show that nAChRs are selectively expressed in different NSCLC cell lines. We present substantial evidence of the expression of three major nAChR subtypes containing the α5, α7 and α9 subunits in NSCLC. The activation of α7- and α9-containing nAChRs stimulates nicotine-induced cancer cell growth and migration, and inhibition of nAChR subunit levels can significantly attenuate nicotine cell proliferation, invasion and the activation of two important intracellular signalling pathways.
However, there is no currently a non-peptidic specific antagonist that could definitely target these nAChR subtypes *in vivo*. The development of nAChR-specific antagonists for clinical purposes is crucial and will help to increase our understanding of the carcinogenic role of nAChRs.

Our findings suggest that stylbene-derived compounds may provide a molecular means of investigating lung cancer cell growth inhibition and death and become a lead structure for the development of new therapeutic agents for human lung cancer.
We believe that this study is important because concerns about the safety of nicotine-containing products make it urgent to discover the molecular mechanisms underlying the effects of nicotine on tissues subject to the development of tobacco-related malignancies.

BIBLIOGRAPHY

- Aggarwal BB, Bhardwaj A et al. (2004) Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res 24: 2783-840
- Alama A, Bruzzo C et al. (2011) Inhibition of the nicotinic acetylcholine receptors by cobra venom α-neurotoxins: is there a perspective in lung cancer treatment? PLoS One 6(6): e20695
- Albuquerque EX, Pereira EF et al. (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev 89(1): 73-120
- Ambrosi A, Becchetti A (2013) Targeting neuronal nicotinic receptos in cancer: new ligands and potential side-effects. Recent Patents on Anti-Cancer Drug Discovery 8: 38-52
- Amos CI, Wu X et al. (2008) Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. Nat Genet 40(5): 616-22
- Araud T, Graw S et al. (2011) The chimeric gene CHRFAM7A, a partial duplication of the CHRNA7 gene, is a dominant negative regulator of α7*nAChR function. Biochem Pharmacol 82(8): 904-14
- Arredondo J, Chernyavsky AI et al. (2006) Receptor-mediated tobacco toxicity: cooperation of the Ras/Raf-1/MEK1/ERK and JAK-2/STAT-3 pathways downstream of alpha7 nicotinic receptor in oral keratinocytes. FASEB J (12): 2093-101
- Baldi A, De Luca A et al. (2011) Tumor suppressors and cell-cycle proteins in lung cancer. Patholog Res Int 2011: 605042
- Balmanno K, Cook SJ (2009) Tumour cell survival signalling by the ERK1/2 pathway. Cell Death Differ 16(3): 368-77
- Beasley MB, Brambilla E et al. (2005) The 2004 World Health Organization classification of lung tumors. Semin Roentgenol 40(2): 90-7
- Bellacosa A, Kumar CC et al. (2005) Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv Cancer Res 94: 29-86
- Benowitz NL (1999) Nicotine addiction. Prim Care 26(3): 611-31
- Bertrand D, Gopalakrishnan M (2007) Allosteric modulation of nicotinic acetylcholine receptors. Biochem Pharmacol 74(8): 1155-63
- Bierut L (2009) Convergence of genetic findings for nicotine dependence and smoking related diseases with chromosome 15q24-25. Cell press 31 (1): 46-51
- Brody AL (2006) Functional brain imaging of tobacco use and dependence. J Psychiatr Res 40(5): 404-18

- Brown KC, Lau JK et al. (2012) MG624, an α7-nAChR antagonist, inhibits angiogenesis via the Egr-1/FGF2 pathway. Angiogenesis 15(1): 99-114
- Campbell NR, Fernandes CC et al. (2010) Endogenous singaling through alpha7-containing nicotinic receptors promotes maturation and integration of adult born neurons in hippocampus. J Neurosci. 30(26): 8734-44
- Carlisle DL, Liu X et al. (2007) Nicotine activates cell-signaling pathways through muscle-type and neuronal nicotinic acetylcholine receptors in non-small cell lung cancer cells. Pulm Pharmacol Ther 20(6): 629-41
- Cattaneo MG, D'atri F et al. (1997) Mechanisms of mitogen-activated protein kinase activation by nicotine in small-cell lung carcinoma cells. Biochem J 328 (Pt 2):499-503
- Changeux, JP, Edelstein, SJ (1998) Allosteric receptors after 30 years. Neuron 21(5): 959-980
- Changeux, JP, Edelstein, SJ (2001) Allosteric mechanisms in normal and pathological nicotinic acetylcholine receptors. Curr Opin Neurobiol 11(3): 369-377
- Changeux JP (2010) Nicotine addiction and nicotinic receptors: lessons from genetically modified mice. Nat Rev Neurosci 11: 389-401
- Chen CS, Lee CH, et al. (2011) Nicotine-induced human breast cancer cell proliferation attenuated by garcinol through down-regulation of the nicotinic receptor and cyclin D3 proteins. Breast Cancer Res Treat 125(1): 73-87
- Chernyavsky A.I., Shchepotin IB et al. (2015) Mechanisms of tumor-promoting activities of nicotine in lung cancer: synergistic effects of cell membrane and mitochondrial nicotinic acethylcoline receptors. BMC Cancer 15(152): 1-12
- Chikova A, Bernard HU et al. (2012) New associations of the genetic polymorphisms in nicotinic receptor genes with the risk of lung cancer. Life Sci 91(21-22): 1103-8
- Colombo SF, Mazzo F et al. (2013) Biogenesis, trafficking and up-regulation of nicotinic ACh receptors. Biochem Pharmacol 86(8): 1063-73
- Dallanoce C, Magrone P et al. (2011b) Design, synthesis, and pharmacological characterization of novel spirocyclic quinuclidinyl-Delta2-isoxazoline derivatives as potent and selective agonists of alpha7 nicotinic acetylcholine receptors. ChemMedChem 6: 889-903
- Dasgupta P, Rastogi S et al. (2006) Nicotine induces cell proliferation by β-arrestin-mediated activation of Src and Rb-Raf-1 pathways. The Journal of clinical investigation 116(8): 2208-2217
- Dasgupta P, Rizwani W et al. (2011) ARRB1-mediated regulation of E2F target genes in nicotine-induced growth of lung tumors. J Natl Cancer Inst 103(4): 317-33

- Dawkins L, Turner J, Hasna S, Soar K (2012) The electronic-cigarette: effects on desire to smoke, withdrawal symptoms and cognition. Addict Behav 37(8): 970-3
- Dawkins L, Turner J et al. (2013) 'Vaping' profiles and preferences: an online survey of electronic cigarette users. Addiction 108(6): 1115-25
- de Lucas-Cerrillo AM, Maldifassi MC, et al. (2011) Function of partially duplicated human α7 nicotinic receptor subunit CHRFAM7A gene: potential implications for the cholinergic anti-inflammatory response. J Biol Chem 286(1): 594-606
- Egleton RD, Brown KC et al. (2008) Nicotinic acetylcholine receptors in cancer: multiple roles in proliferation and inhibition of apoptosis. Trends in Pharmacological Science 29(3): 151-158
- Falvella FS, Galvan A et al. (2009) Transcription deregulation at the 15q25 locus in association with lung adenocarcinoma risk. Clin Cancer Res 15(5): 1837-42
- Falvella FS, Galvan A et al. (2010) Promoter polymorphisms and transcript levels of nicotinic receptor CHRNA5. J Natl Cancer Inst 102(17): 1366-70
- Falvella FS, Alberio T, et al. (2013) Multiple isoforms and differential allelic expression of CHRNA5 in lung tissue and lung adenocarcinoma. Carcinogenesis 34(6): 1281-1285
- Feitelson MA, Arzumanyan A (2015) Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. Seminar Cancer Biol 35 Suppl: S25-54
- Fowles J, Dybing E. (2003) Application of toxicological risk assesment principles to the chemical constituents of cigarette smoke. Tob Control 12(4): 424-30
- Gault J, Robinson M et al. (1998) Genomic organization and partial duplication of the human α7 neuronal nicotinic acetylcholine receptor gene. Genomics 52: 173–85
- Gergalova GL, Skok MV (2011) Nicotine effects on mitochondria membrane potential: participation of nicotinic acetylcholine receptors. Ukr Biokhim Zh 83: 13–21
- Giard DJ Aaronson SA (1973) In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 51(5): 1417-23
- Glasauer A, Chandel NS (2014) Targeting antioxidants for cancer therapy. Biochem Pharmacol 92(1): 90-101
- Gotti C, Fornasari D et al. (1997) Human neuronal nicotinic receptors. Prog Neurobiol 53(2): 199-237
- Gotti C, Balestra B et al. (1998) 4-oxystilbene compounds are selective ligands for neuronal nicotinic alphaBungarotoxin receptors. British Journal of Pharmacology 124(6): 1197-1206

- Gotti C, Carbonnelle E et al. (2000) Drug selective for nicotinic receptor subtypes: a real possibility or a dream? Behavioural Brain Research 113: 183-192
- Gotti C, Clementi F (2004) Neuronal nicotinic receptors: from structure to pathology. Progress in Neurobiology 74: 363–396
- Gotti C, Riganti L et al. (2006a) Brain Neuronal Nicotinic Receptors as New Targets for Drug Discovery. Current Pharm Design 12(4): 407-28
- Gotti C, Zoli M et al. (2006) Brain nicotinic acetylcholine receptors: native subtype and their relevance. Trends Pharmacol Sci 27(9): 482-491
- Gotti C, Clementi F et al. (2009) Structural and functional diversity of native brain neuronal nicotinic receptors. Biochemical Pharmacology 78: 703–711
- Gotti C, Guiducci S et al. (2010) Nicotinic acetylcholine receptors in the mesolimbic pathway: primary role of ventral tegmental area alpha6beta2* receptors in mediating systemic nicotine effects on dopamine release, locomotion, and reinforcement. J Neurosci 30: 5311–25
- Grando SA (2014) Connections of nicotine to cancer. Nature Reviews Cancer 14: 419-429
- Han M, Zhang T et al. (2016) Association between NADPH oxidase (NOX) and lung cancer: a systematic review and meta-analysis. J Thorac Dis. 8(7): 1704-11
- Han ZY, Le Novère N et al. (2000) Localization of nAChR subunit mRNAs in the brain of Macaca mulatta. Eur J Neurosci 12(10): 3664-74
- Hatsukami DK, Grillo M et al. (1997) Safety of cotinine in humans: physiologic, subjective, and cognitive effects. Pharmacology, Biochemistry, and Behavior 57(4): 643–50
- Hatsukami DK, Stead LF et al. (2008) Tobacco addiction. Lancet 371(9629): 2027-38
- Heeschen C, Weis M et al. (2002) A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. J Clin Invest 110(4): 527-36
- Heusch WL and Maneckjee R (1998) Signalling pathways involved in nicotine regulation of apoptosis of human lung cancer cells. Carcinogenesis 19(4): 551-6
- HHS (2004) 2004 Surgeon General's Report-The Health Consequences of Smoking. US Department of Health and Human Services, Public Health Service, Office of the Surgeon General
- Ho YS, Chen CH et al. (2005) Tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces cell proliferation in normal human bronchial epithelial cells through NFkappaB activation and cyclin D1 up-regulation. Toxicol Appl Pharmacol 205(2): 133-48

- Hogg RC, Raggenbass M et al. (2003) Nicotinic acetylcholine receptors: from structure to brain function. Rev Physiol Biochem. Pharmacol 147: 1-46
- Hsu CC, Tseng LM et al. (2016) Role of mitochondrial dysfunction in cancer progression. Exp Biol Med (Maywood) 241(12): 1281-95
- Hughes JR (2006) Clinical significance of tobacco withdrawal. Nicotine Tob Res 8(2): 153-6
- Hukkanen J, Jacob P 3rd et al. (2005) Metabolism and disposition kinetics of nicotine. Pharmacol Rev 57(1): 79-115
- Hung RJ, McKay JD et al. (2008) A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. Nature 452(7187): 633-7
- Hurst R, Rollema H et al. (2013) Nicotinic acetylcholine receptors: from basic science to therapeutics. Pharmacology Therapy 137: 22-54
- Improgo MRD, Scofield MD et al. (2010) From smoking to lung cancer: the CHRNA5/A3/B4 connection. Oncogene 29: 4874-4884
- Improgo MR, Tapper AR et al. (2011) Nicotinic acetylcholine receptor-mediated mechanisms in lung cancer. Biochem Pharmacol 82(8): 1015-21
- Improgo MR, Soll LG et al. (2013) Nicotinic acetylcholine receptor mediated lung cancer growth. Frontiers in physiology 4(251): 1-6
- Innocent N, Livingstone PD et al. (2008) Alpha-conotoxin Arenatus IB[V11L,V16D] [corrected] is a potent and selective antagonist at rat and human native alpha7 nicotinic acetylcholine receptors. J Pharmacol Exp Ther 327(2): 529-37
- Jakubowska J, Mikuła-Pietrasik J et al. (2014) Cytotoxicity studies of novel combretastatin and pterostilbene derivatives. Biomed Res Int 2014: 320895
- Jin Z, Gao F et al. (2004) Nicotine induces multi-site phosphorylation of Bad in association with suppression of apoptosis. J Biol Chem 279(22): 23837-44
- Jones IW and Wonnacott S (2004) Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. J Neuroscience 24 (50): 11244-11252
- Jull Ba, Plummer HK et al. (2001) Nicotinic receptor-mediated activation by the tobacco-specific nitrosamine NNK of a Raf-1/MAP kinase pathway, resulting in phosphorylation of c-myc in human small cell lung carcinoma cells and pulmonary neuroendocrine cells. J Cancer Res Clin Oncol 127(12): 707-17
- Kalashnyk OM, Gergalova GL et al. (2012) Intracellular localization of nicotinic acetylcholine receptors in human cell lines. Life Sci 91: 1033–7

- Kalappa BI, Sun F et al. (2013) A positive allosteric modulator of α7 nAChRs augments neuroprotective effects of endogenous nicotinic agonists in cerebral ischaemia. Br J Pharmacol 169(8): 1862-78
- Karlin A (2002) Emerging structure of the nicotinic acetylcholine receptors. Nat Rev Neurosci 3(2): 102-14
- Keenan RM, Hatsukami DK et al. (1994) Pharmacodynamic effects of cotinine in abstinent cigarette smokers. Clinical Pharmacology and Therapeutics 55(5): 581–90
- Keller SH, Lindstrom J et al. (2001) Adjacent basic amino acid residues recognized by the COP I complex and ubiquitination govern endoplasmic reticulum to cell surface trafficking of the nicotinic acetylcholine receptor alpha-Subunit. J Biol Chem 276(21): 18384-91
- Krais AM, Hautefeuille AH et al. (2011) CHRNA5 as negative regulator of nicotine signaling in normal and cancer bronchial cells: effects on motility, migration and p63 expression. Carcinogenesis 32(9): 1388-95
- Krashia P, Moroni M et al. (2010) Human alpha3beta4 neuronal nicotinic receptors show different stoichiometry if they are expressed in Xenopus oocytes or mammalian HEK293 cells. PLoS One 5: e13611
- Kuryatov A, Onksen J et al. (2008) Roles of accessory subunits in alpha4beta2(*) nicotinic receptors. Mol Pharmacol 74(1): 132-43
- Kuryatov A, Berrettini W et al. (2010) Acetylcholine receptor (AChR) α5 subunit variant associated with risk for nicotine dependence and lung cancer reduces $(\alpha4\beta2)_2\alpha5$ AChR function. Molecular Pharmacology 79(1): 119-125
- Lai A, Parameswaran N et al. (2005) Long-term nicotine treatment decreases striatal alpha 6* nicotinic acetyl- choline receptor sites and function in mice. Mol Pharmacol 67: 1639–47
- Lammel S, Ion DI et al. (2011) Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. Neuron 70: 855–862
- Lee CH, Huang CS et al. (2010) Overexpression and activation of the a9-nicotinic receptor during tumorigenesis in human breast epithelial cells. J Natl Cancer Inst 102 (17): 1322-1335
- Lin HL, Chiou SH et al. (2007) Combretastatin A4-induced differential cytotoxicity and reduced metastatic ability by inhibition of AKT function in human gastric cancer cells. J Pharmacol Exp Ther 323(1): 365-73
- Lindstrom J (1997) Nicotinic acetylcholine receptors in health and disease. Mol Neurobiol 15(2): 193-222

- Lindstrom J (2000) The Structures of Neuronal Nicotinic Receptors. Handbook of Experimental Pharmacology: 144: 101-162
- Livingstone PD, Wonnacott S (2009) Nicotinic acetylcholine receptors and the ascending dopamine pathways. Biochem. Pharmacol 78(7): 744-55
- Liu Z, Zhang J et al. (2007) Role of endogenous nicotinic signaling in guiding neuronal development. Biochemical pharmacology 74(8): 1112-9
- Luo M, Liu X et al. (2010) Cajanol, a novel anticancer agent from Pigeonpea [Cajanus cajan (L.) Millsp.] roots, induces apoptosis in human breast cancer cells through a ROS-mediated mitochondrial pathway. Chem Biol Interact 188(1): 151-60
- Ma X, Jia Y, et al. (2014) α5 Nicotinic acetylcholine receptor mediates nicotine-induced HIF-1α and VEGF expression in non-small cell lung cancer. Toxicol Appl Pharmacol 278(2): 172-9
- Maggi L, Palma E et al. (1999) Selective effects of a 4-oxystilbene derivative on wild and mutant neuronal chick alpha7 nicotinic receptor Br J Pharmacol 126(1): 285-95
- Mansvelder HD, Keath JR et al. (2002) Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. Neuron 33: 905–919
- Mao D, Perry DC et al. (2008) The alpha4beta2alpha5 nicotinic cholinergic receptor in rat brain is resistant to up-regulation by nicotine in vivo. J Neurochem 104: 446–56
- Maouche K, Polette M et al. (2009) {alpha}7 nicotinic acetylcholine receptor regulates airway epithelium differentiation by controlling basal cell proliferation Am J Pathol 175(5): 1868-82
- Maus AD, Pereira EF et al. (1998) Human and rodent bronchial epithelial cells express functional nicotinic acetylcholine receptors. Mol Pharmacol 54: 779–88
- Mazzo F, Pistillo F et al. (2013) Nicotine-modulated subunit stoichiometry affects stability and trafficking of α3β4 nicotinic receptor. J Neurosci 33(30): 12316-28
- Meitzler JL, Antony S et al. (2014) NADPH oxidases: a perspective on reactive oxygen species production in tumor biology. Antioxid Redox Signal 20: 2873-89
- Millar NS, Harkness PC (2008) Assembly and trafficking of nicotinic acetylcholine receptors. Mol Membr Biol 25(4): 279-92
- Millar NS, Gotti C (2009) Diversity of vertebrate nicotinic acetylcholine receptors. Neuropharmacology 56(1): 237-46
- Moretti M, Zoli M et al. (2014) The novel α7β2-nicotinic acetylcholine receptor subtype is expressed in mouse and human basal forebrain: biochemical and pharmacological characterization. Mol Pharmacol 306-17

- Moroni M, Zwart R et al. (2006) alpha4beta2 nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry and sensitivity to long-term exposure to nicotine. Mol Pharmacol 70: 755-68
- Mucchietto V, Crespi A et al. (2016) Neuronal acetylcholine nicotinic receptors as new targets for lung cancer treatment. Current Pharmaceutical Design 22: 2160-2169
- Murin S, Pinkerton KE, et al. (2004) The effect of cigarette smoke exposure on pulmonary metastatic disease in a murine model of metastatic breast cancer. Chest 125(4): 1467-71
- Norton JD (2000) ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J Cell Sci 113 (Pt 22): 3897-905
- Olaussen K. A., Postel-Vinay S (2016) Predictors of chemotherapy efficacy in non-small-cell lung cancer: a challenging landscape. Annals of Oncology 27: 2004–2016
- Olincy, A., Leonard, S et al. (1999) Decreased bombesin peptide response to cigarette smoking in schizophrenia. Neuropsychopharmacology 20(1): 52-9
- Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit composition, pharmacology, and function. Update Pharmacol Rev 60: 243–260
- Paleari L, Sessa F, et al. (2009) Inhibition of non-neuronal alpha7-nicotinic receptor reduces tumorigenicity in A549 NSCLC xenografts. Int J Cancer 125: 199–211
- Picciotto, MR, Kenny PJ (2013) Molecular mechanisms underlying behaviors related to nicotine addiction. Cold Spring Harb Perspect Med 3(1): a012112
- Pillai S, Rizwani W et al. (2011) ID1 facilitates the growth and metastasis of non-small cell lung cancer in response to nicotinic acetylcholine receptor and epidermal growth factor receptor signalling. Mol Cell Biol 31(14): 3052-67
- Pillai S, Trevino J et al. (2015) β-arrestin-1 mediates nicotine-induced metastasis through E2F1 target genes that modulate epithelial-mesenchymal transition. Cancer Res 15;75(6): 1009-20
- Quick MW, Lester RA (2002) Desensitization of neuronal nicotinic receptors. J Neurobiol 53(4): 457-478
- Quik M, Bordia T et al. (2015) Nicotine and Nicotinic Receptor Drugs: Potential for Parkinson's Disease and Drug-Induced Movement Disorders. Int Rev Neurobiol. 124: 247-71
- Rayes D, De Rosa MJ et al. (2009) Number and localization of agonist binding sites required to activate homomeric Cys-loop receptors. J Neurosci 29, 6022-6032

- Remsberg CM, Yáñez JA et al. (2008) Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity. Phytother Res 22(2): 169-79
- Rezvani K, Teng Y et al. (2007) Nicotine regulates multiple synaptic proteins by inhibiting proteasomal activity. J Neurosci 27: 10508–19
- Rezvani K, Teng Y et al. (2010) The ubiquitin–proteasome system regulates the stability of neuronal nicotinic acetylcholine receptors. J Mol Neurosci 40: 177–84
- Rockville MD (2010) How tobacco smoke causes disease: the biology and behavioral basis for smoking-attributable disease: a report of the Surgeon General. Dept. of Health and Human Services, Public Health Service, Office of Surgeon General.
- Roy HK, Olusola BF et al. (2002) AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis. Carcinogenesis 23(1): 201-5
- Saccone S (2007) Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. Human Molecular Genetics 16(1): 36-49
- Saccone NL, Wang JC et al. (2009) The CHRNA5-CHRNA3-CHRNB4 Nicotinic receptor subunit gene cluster affects risk for nicotine dependence in African-Americans and in European-Americans. Cancer Research 69(17): 6848-6856
- Sallette J, Pons S et al. (2005) Nicotine upregulates its own receptors through enhanced intracellular maturation. Neuron 46: 595–607
- Sassi N, Mattarei A et al. (2014) Cytotoxicity of mitochondria-targeted resveratrol derivatives: Interactions with respiratory chain complexes and ATP synthase. Biochimica et Biophysica Acta 1837(10): 1781-1789
- Saxena G, Patro IK, et al. (2011) Induced impairment in memory and neuronal mitochondrial function: A protective role of nicotinic receptor. Behav Brain Res 224(1): 50–7
- Schaal C and Chellappan SP (2014) Nicotine-mediated cell proliferation and tumor progression in smoking-related cancers. Molecular Cancer Research 12(1): 14-23
- Schuller HM (2007) Nitrosamine as nicotinic receptor ligands. Life Sci 80(24-25): 573-95
- Schuller HM (2009) Is cancer triggered by altered signaling of nicotinic acetylcholine receptors? Nature Reviews Cancer 9(3): 195-205
- Sgard F, Charpantier E et al. (2002) A novel human nicotinic receptor subunit, alpha10, that confers functionality to the alpha9-subunit. Mol Pharmacol 61(1): 150-9

- Shen B, Shi MQ et al. (2012) Correlation between polymorphisms of nicotine acetylcholine acceptor subunit CHRNA3 and lung cancer susceptibility. Mol Med Rep 6(6): 1389-92
- Sheng S, Qiao M et al. (2009) Metastasis and AKT activation. J Cell Physiol 218(3): 451-4
- Shin VY, Jin HC et al. (2008) Nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induce cyclooxygenase-2 activity in human gastric cancer cells: Involvement of nicotinic acetylcholine receptor (nAChR) and beta-adrenergic receptor signaling pathways. Toxicol Appl Pharmacol 233(2): 254-61
- Sinkus ML, Lee MJ et al. (2009) A 2-base pair deletion polymorphism in the partial duplication of the alpha7 nicotinic acetylcholine gene (CHRFAM7A) on chromosome 15q14 is associated with schizophrenia. Brain Res 1291: 1-11
- Son CD, Moss FJ et al. (2009) Nicotine normalizes intracellular subunit stoichiometry of nicotinic receptors carrying mutations linked to autosomal dominant nocturnal frontal lobe epilepsy. Mol Pharmacol. 75(5): 1137-48
- Sullivan LB and Chandel NS (2014) Mitochondrial reactive oxygen species and cancer. Cancer Metab 2: 17
- Sun H, Ma X (2015) α5-nAChR modulates nicotine-induced cell migration and invasion in A549 lung cancer cells Exp Toxicol Pathol 67(9): 477-82
- Sunday ME. (1996) Pulmonary neuroendocrine cells and lung development. Endocr Pathol 7: 173–20
- Taly A, Corringer PJ et al. (2009) Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system. Nat Rev Drug Discov 8: 733-750
- Tanibuchi Y, Wu J et al. (2010) Characterization of [(3)H]CHIBA-1001 binding to alpha7 nicotinic acetylcholine receptors in the brain from rat, monkey, and human. Brain Res 1348: 200-8
- Tapia L, Kuryatov A et al. (2007) Ca2+ permeability of the (alpha4)3(beta2)2 stoichiometry greatly exceeds that of (alpha4)2(beta2)3 human acetylcholine receptors. Mol Pharmacol 71(3): 769-76
- Tournier JM, Maouche K, et al. (2006) alpha3alpha5beta2-Nicotinic acetylcholine receptor contributes to the wound repair of the respiratory epithelium by modulating intracellular calcium in migrating cells. Am J Pathol 168(1): 55-68
- Tsurutani J, Castillo SS et al. (2005) Tobacco components stimulate Akt-dependent proliferation and NFkappaB-dependent survival in lung cancer cells. Carcinogenesis 26(7): 1182-95
- US Food and Drug Aministration. (2014) FDA Warns of Health Risks Posed by ECigarette

- Vachhani P and Chen H (2016) Spotlight on pembrolizumab in non-small cell lung cancer: the evidence to date. OncoTargets and Therapy 9: 5855-5866
- Vallejo YF, Buisson B et al. (2005) Chronic nicotine exposure upregulates nicotinic receptors by a novel mechanism. J Neurosci 25: 5563–72
- Wallace DC (2005) A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu. Rev. Genet *39*, 359–407
- Wang F, Gerzanich V et al. (1996) Assembly of human neuronal nicotinic receptor alpha5 subunits with alpha3, beta2, and beta4 subunits. J Biol Chem 271(30): 17656-65
- Wang JM, Zhang L et al. (2002) A transmembrane motif governs the surface trafficking of nicotinic acetylcholine receptors. Nat Neurosci 5(10): 963-70
- Wang Y, Xiao C et al. (2014) The duplicated α7 subunits assemble and form functional nicotinic receptors with the full-length α7. J Biol Chem 289(38): 26451-63
- Wei MC, Zong WX et al. (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292(5517): 727-30
- Weiss RB, Baker TB et al. (2008) A candidate gene approach identifies the CHRNA5-A3-B4 regions as a risk factor for age-dependent nicotine addiction. PLoS Genet 4: e1000125
- Wessker I, Kirkpatrick CJ (2008) Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. British Journal of Pharmacology 154: 1558-1571
- West KA, Brognard J et al. (2003) Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. J Clin Invest 111(1): 81-90
- Whiteaker P, Christensen S et al. (2007) Discovery, synthesis, and structure activity of a highly selective alpha7 nicotinic acetylcholine receptor antagonist. Biochemistry 46(22): 6628-38.
- Williams DK, Wang J et al. (2011) Positive allosteric modulators as an approach to nicotinic acetylcholine receptor-targeted therapeutics: advantages and limitations. Biochem Pharmacol 82(8): 915-30
- WHO Report on the Global Tobacco Epidemic, 2008: The MPOWER package. Geneva, World Health Organization, 2008
- Wu CH, Lee CH et al. (2011) Nicotinic acetylcholine receptor-based blockade: applications of molecular targets for cancer therapy. Clin Cancer Res 17(11): 3533-41
- Xu L, Deng X (2006) Protein kinase Ciota promotes nicotine-induced migration and invasion of cancer cells via phosphorylation of micro- and m-calpains. J Biol Chem 281(7): 4457-66

- Yang JS, Seo SW et al (2012) Rational engineering of enzyme allosteric regulation through sequence evolution analysis. PLoS Comput Biol 8: e1002612
- Young GT, Zwart R et al. (2008) Potentiation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site Proc Natl Acad Sci U S A. 2008 105(38): 14686-91
- Yu W, Mechawar N et al. (2011) Alpha7 Nicotinic receptor activation reduces beta-amyloid-induced apoptosis by inhibiting caspase-independent death through phosphatidylinositol 3-kinase signaling. J Neurochem 119: 848–58
- Zhang S, Togo S et al. (2010) Distinct roles of cholinergic receptors in small cell lung cancer cells. Anticancer Res 30(1): 97-106
- Zhang C, Ding XP et al. (2016) Role of α7-nicotinic acetylcholine receptor in nicotine-induced invasion and epithelial-to-mesenchymal transition in human non-small cell lung cancer cells. Oncotarget. doi: 10.18632/oncotarget.10498
- Zhao-Shea R, Liu L et al. (2011) Nicotine-mediated activation of dopaminergic neurons in distinct regions of the ventral tegmental area. Neuropsychopharmacology 36, 1021–1032
- Zoli M, Pistillo F et al. (2015) Diversity of native nicotinic receptor subtypes in mammalian brain. Neuropharmacology 96: 302-311