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CYTISINE AND CYTISINE DERIVATIVES. MORE THAN SMOKING CESSATION AIDS

Cecilia Gotti[1,2,3*], Francesco Clementi[1,2,3]

[1]*CNR, Institute of Neuroscience, Milan, Italy*

[2]*NeuroMi Milan Center for Neuroscience University of Milano Bicocca,Italy.*

[3]*Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Milan, Italy*

[*]Corresponding author at: CNR, Institute of Neuroscience c/o Università di Milano-Bicocca Edificio U28, Via Follereau 3, 20854 Vedano al Lambro(MB). cecila.gotti@in.cnr.it

Abstract

Cytisine, a natural bioactive compound that is mainly isolated from plants of the Leguminosae family (especially the seeds of *Laburnum anagyroides*), has been marketed in central and eastern Europe as an aid in the clinical management of smoking cessation for more than 50 years. Its main targets are neuronal nicotinic acetylcholine receptors (nAChRs), and pre-clinical studies have shown that its interactions with various nAChR subtypes located in different areas of the central and peripheral nervous systems are neuroprotective, have a wide range of biological effects on nicotine and alcohol addiction, regulate mood, food intake and motor activity, and influence the autonomic and cardiovascular systems. Its relatively rigid conformation makes it an attractive template for research of new derivatives. Recent studies of structurally modified cytisine have led to the development of new compounds and for some of them the biological activities are mediated by still unidentified targets other than nAChRs, whose mechanisms of action are still being investigated.

The aim of this review is to describe and discuss: 1) the most recent pre-clinical results obtained with cytisine in the fields of neurological and non-neurological diseases; 2) the effects and possible mechanisms of action of the most recent cytisine derivatives; and 3) the main areas warranting further research.

Abbreviations

AD: Alzheimer disease; ACh, acetylcholine ; ACHE, acetylcholinesterase ; AKT, protein kinase B ; AChR, acetylcholine receptor; mAChR, muscarinic acetylcholine receptor ; nAChR, neuronal nicotinic acetylcholine receptor; ARC, arcuate nucleus of the hypothalamus ; BBB, blood brain barrier; Bcl2, B-cell lymphoma 2; aBgtx, a-Bungarotoxin; BDNF, brain derived neutrofic factor ; DA, dopamine ; CC4, cytisine dimer1,2-bisN-cytisinylethane; CI, confidence interval; CNS, central nervous system; CPP, conditional place preference; CREB, cAMP-response element binding; EC_{50}, half maximal excitatory concentration ; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; HAAF, hypoglycemia-associated autonomic failure; IP_3, inositol trisphosphate; JAK2, Janus kinase 2; JNK, c-Jun N-terminal kinase; CHOP, C/EBP homologous protein ; hiPSC-CMs, human-induced pluripotent stem cell derived cardiomyocytes; Ki, inhibition constant ; KO, Knockout ; MDD, major depressive disorder; MLL, mixed-lineage leukemia; NAc, Nucleus Accumbens ; nAChR, neuronal nicotinic acetylcholine receptor; NF-kB, nuclear factor k-light-chain-enhancer of activated B cells; mAChR, muscarinic acetylcholine receptor; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase ; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NR-2B NMDAR, N-methyl-D-aspartate receptor containing the NR2B subunit; 6-OHDA, 6-hydroxydopamine ; PD, Parkinson disease; PI3K, phosphoinositide 3-kinase; PNS, peripheral nervous system; PTK, protein tyrosine kinase; RANK, receptor activator of nuclear factor -kB; RANKL, receptor

activator of nuclear factor -kB ligand ; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; STAT, signal transducer and activator of transcription

Keywords : Cytisine, varenicline, cytisine derivatives, neuronal nicotinic acetylcholine receptors, smoking cessation, reward

1. Introduction

Cytisine is the main alkaloid in the plants of the Faboideae sub-family of the Fabaceae family (Figure 1) , which includes the ornamental trees *Laburnum anagyroides* or *Cytisus laburnum* and *Laburnum alpinum Genista, Sophora* and a number of hybrids. Gaius Plinius Secundus, in its book of Naturalis Historia vol Xlll,73, reports that Cytisus has been well known from antiquity and cultivated in Greece for its beauty, and pharmaco-toxicological properties. The plants are now known as golden chain trees or golden rain acacias, they are common in northern Europe and the mountains of southern Europe and, since antiquity, all of their parts (but particularly the seeds) have been recognised as being poisonous and causing symptoms such as vomiting, pupil dilatation, tachycardia, prostration, torpor, excitement, delirium, hallucinations, muscle twitches, diarrhea, sweating, and even death due to respiratory paralysis[1-3]

Cytisine (Figure 2) was used in the USSR as a respiratory stimulant similar to lobeline and, since 1960, it has been used to treat nicotinism in Bulgaria and other eastern and central European countries [4]. In traditional Chinese medicine it is used to treat hepatitis and liver cancer exploiting a small but promising effect of cytisine on cell growth [5]. Very recently, a renewed interest in cytisine has arisen not only as an aid to smoking cessation and a possible drug for other pathologies, but also as a template for the synthesis of new cytisine derivatives that should be more target specific and have more appealing pharmacokinetics [4, 6].

The pharmacological effects of cytisine on skeletal muscles, respiration, heart and blood circulation, alimentary canal, salivary glands, eyes, uterus and urinary bladder of various animal species were first described in detail in a paper published in 1912 by Dale and Laidlaw [1], who compared them with the effects of nicotine. This seminal paper clearly established two important points: 1) the effects of cytisine and nicotine are very similar in terms of the quality of the responses obtained albeit with some differences in doses and between species; and 2) the two drugs compete with each other and have different potencies in different organs and/or different experimental settings. These first experimental findings amplified and rationalised later by various authors, clearly established the particular sensitivity of ganglionic cells to cytisine, and anticipated the discovery that it acts through nicotinic receptors that were discovered more than 80 years later.

The role of cytisine, particularly in smoking cessation has been the subject of many previous reviews [7-11] and so this review will concentrate on the literature of the last 10 years describing cytisine selective molecular targets,and its potential effects other than those in smoking cessation, and finally will provide a brief survey of new cytisine derivatives, their bioactivities and their possible use in the treatment of human diseases.

2. Major pharmacological targets of cytisine: neuronal nicotinic acetylcholine receptors

2.1 Nicotinic receptor structure

As we have pointed out before, since the early pharmacological studies on cytisine it appeared evident that its activity is very similar to that of nicotine, suggesting that the most relevant targets of the drug are cholinergic nicotinic acetylcholine receptors (AChRs). Acetylcholine (ACh) is released in both central and peripheral tissues and can control target cell activity via a wide range of mechanisms and signals through two families of receptors (neuronal nicotinic receptors,nAChRs and muscarinic acetylcholine receptors, mAChRs). Since the most important targets of cytisine are nAChRs we report here the most relevant findings on these receptors in order to better understand cytisine activity.

Brain and ganglionic nAChRs are a heterogeneous family of ubiquitously expressed pentameric ion channels, whose responses to endogenous ACh or choline ligands and exogenous nicotine are involved in a number of physiological processes and pharmacological effects [12-14]. Sixteen subunits (α1–α7, α9, α10, β1–β4, γ and δ and ε) have so far been identified in mammals that form receptors with a common basic structure, but different and specific pharmacological and functional properties. The α1, β1, γ ,δ and ε subunits form muscle-type receptors that play a major role in neuromuscular transmission, whereas the most widely expressed subtypes in the brain are heteromeric α4β2* (*means that additional subunits may be present) and homomeric α7 receptors, whereas α3β4* subtypes are most widely expressed in autonomic ganglia of the peripheral nervous system [14]. In addition to these major central and peripheral nAChR subtypes, many other native nAChR subtypes with more complex subunit compositions have been identified in the rodent mesotelencephalic, habenulo-interpeduncular and visual pathways including the α6β2β3* receptors that consist of the α6β2β3 and α4α6β2β3 subtypes [15].

The heteromeric α4β2 and α3β4 receptors may have the same subunit composition but different subunit stoichiometries (2α:3β or 3α:2β subunits), depending on the type of subunit in the fifth position [15, 16].

Homomeric nAChRs have five ACh orthosteric binding sites. Although it was thought that heteromeric receptors had two ACh binding sites at the α/β subunit interfaces, it has recently been discovered that the α4 β2 subtype with a stoichiometry of 3α:2β subunits has a third binding site at the α4/α4 interface, and the two stoichiometries of the α4β2 and α3β4 subtypes are differently sensitive to ACh (see Table 1). However, concentration-response studies of oocytes expressing uniform populations of $(\alpha4)_3(\beta2)_2$ or $(\alpha4)_2(\beta2)_3$ receptors have shown that the presence of an α4/α4 interface has a very limited effect on cytisine receptor activation [17]. In the α6β2* receptors the two orthosteric ACh binding sites are identical in the α6β2β3 subtype, but different in the α4α6β2β3 subtype, which has both an α6β2 and an α4β2 interface [18].

2.2 Cytisine binding to nAChRs

The earliest autoradiographic and ligand binding studies of rodent brain demonstrated that ^{3}H-cytisine binds with nM affinity to the same sites as those bound by ^{3}H-nicotine and ^{3}H-ACh, and that this binding was distinct from that obtained using the antagonist ^{125}I- αBungarotoxin that selectively binds α7or α9-containing receptors [19, 20]. Patch clamp electrophysiological measurements of heteromeric combinations of α3 or α4 and β2 or β4 subunits and various chimeric constructs have confirmed that cytisine potently binds β2-containing receptors (see Table 1), but primarily activates β4-containing receptors, and shown that, despite

the large difference in cytisine binding affinity between β2- and β4-containing receptors, maximal efficacy is fully determined by the β4 subunit [17].

These studies clearly indicate that cytisine binds with high affinity (low nM) to the α4β2, α3β2 and α6β2* subtypes, and with lower affinity to the α3β4, α7 and α1β1γδ subtypes, with a rank order of a α4β2= α6β2> α3β2>α3β4>α7>α1β1γδ [17, 21, 22]. On the other hand functional characterisations of the subtypes expressed in heterologous systems clearly show that cytisine is a very high affinity, partial agonist of the α4β2 subtype and a full agonist of the α3β4 and α7 subtypes [22-24].

The effect of cytisine on rat and human α4β2 and α7 subtypes is similar, but is species specific in the case of α3β4 subtype; in fact cytisine is as efficacious as ACh on the rat α3β4 subtype, but much less efficacious than ACh on the human subtype [25] (See Table 1).

Most neuronal nAChRs have a pre-synaptic and/or pre-terminal location, and modulate the release of almost all neurotransmitters but, in some brain areas, they have a somato-dendritic post-synaptic location and their activation mediates fast synaptic transmission, as in the case of autonomous ganglionic neurons [26]. Recent studies have also shown that nAChRs are found in a large number of non-neuronal cell types including endothelial cells, glia, immune cells, lung epithelia, and cancer cells where they regulate cell differentiation, proliferation and inflammatory responses [27].

By acting as a partial agonist of α4β2* and a nearly full agonist of α6β2*-containing receptors, cytisine mediates the release of dopamine from rodent striatal slices and synaptosomes [22, 28] with a potency on α6β2 subtype that is 15 times higher than on the α4β2 subtype (see Table 1) [28]. Moreover, by acting on α3β4*-containing receptors, cytisine modulates noradrenaline release from the hippocampus [22].

Nicotine activates nAChRs, but nicotine-bound nAChRs can also be desensitised and inactivated by nicotine sometimes regardless of nAChR activation (reviewed in [29]) . Moreover, chronic nicotine exposure leads to neural adaptations that may be due to nAChR activation and/or desensitisation and, in the latter case, can alter neuronal function by interrupting the transmission of endogenous ACh [30].

2.3 Cytisine regulation of nAChRs assembly and intracellular trafficking

nAChRs are multisubunit, multispan, integral membrane proteins, and their folding and assembly is a very inefficient process, with only a small portion of subunits forming functional pentamers. Among nAChRs the efficiency of assembly and trafficking varies widely depending on the nAChR subtypes and the cell type in which they are expressed (reviewed in [31])

Cell biology studies have shown that nicotine is a target-specific, pharmacological chaperone that increases surface expression of nAChR subtypes by facilitating receptor assembly, enhancing export of assembled pentamers from the endoplasmic reticulum (ER), and/or stabilizing assembled receptors, thus enabling more nAChRs to be inserted into the plasma membrane (see Fig 3). Most studies of the effects of nicotine have examined the α4β2 subtype, which is the most expressed in the brain and have shown that chronic nicotine exposure *in vitro* and *in vitro* up-regulates the $(\alpha4)_2(\beta2)_3$ stochiometry [32, 33] whereas chronic treatment with cytisine up-regulates the $(\alpha4)_3(\beta2)_2$ stoichiometry [34, 35] .

Relatively little is known about α3β4 receptors, and study performed by our group [36] have demonstrated that exposure to nicotine, cytisine or CC4 (a cytisine dimer) of HeLa cells expressing the α3β4 subtype induces the assembly of a pentameric receptor with a stoichiometry of $(\alpha3)_2(\beta4)_3$ subunits that it is less prone to proteasome degradation and can more easily exit from the ER and reach the plasmamembrane.

The SH-SY5Y human neuroblastoma cells express both homomeric (α7) and heteromeric nAChRs (α3β4* and α3β2*). Treatment of SH-SY5Y cells for 48 hours with 100 μM cytisine or CC4 leads to un upregulation of heteromeric receptors and to a lesser degree of homomeric α7 receptors [37]. In particular we found that cytisine and CC4 treatments led to a much higher level of assembled receptors containing the β2 subunit, in particular the α3β2β4 and α3β2 subtypes, and to a lesser degree to the upregulation of the α3β4 subtype and these receptors present in the plasmamembrane are functional. The increased level of the heteromeric receptors was due to a post-transcriptional mechanism and treatment of the cells with the cell impermeable antagonist d-Tubocurarine did not block the upregulation of the subtypes. These studies clearly indicate that cytisine and CC4 as well as nicotine could upregulate the plasmamembrane expression of functional α4β2 and α3β4 subtypes by modifying their assembly and intracellular trafficking.

From the above reported data we can conclude that cytisine can affect nicotinic transmission by acting both 1) at the cell surface as a high affinity, partial agonist of the α4β2 subtype, a nearly full agonist of α6β2*-containing receptors and a full agonist of the α3β4 and of α7 subtypes. 2) as an intracellular chaperon that modulates nAChR assembly and trafficing leading to an up regulation of cell surface receptors .

3. Cytisine toxicity and pharmacokinetics

3.1 Cytisine toxicity

Before describing the cytisine pharmacological activity it is worth considering its toxicity and pharmacokinetics, two properties that are relevant for the full understanding of its possible pharmaco-therapeutical activity.

It has been known since long time that all of the parts of plants containing cytisine are toxic, particularly the seeds [3].The lethal dose of *Laburnum anagyroides/alpinum*–seeds in sheep and horses is in the order of 0.5g/kg, whereas well-tolerated chronic ingestion in dogs and rats is between 0.45 and 0.9 mg/kg. The LD_{50} in rat per oral administration is in the order of 5-50 mg/kg but cytisine does not have embryotoxic or teratogenic activity in experimental animals.

In humans, particularly children [38], cytisine intoxication has effects on the gastrointestinal system (nausea, emesis, and bowel movements), the central nervous system (CNS) (drowsiness, fatigue, dizziness, delirium), and the motor system (muscle twitching and fasciculation, difficulty in walking). The ingestion of seeds of cytisine- containing plants (particularly *Spartium junceum* or Spanish broom) by children [39] or animals [40] can induce nicotine-like symptoms that usually fully recover, although some fatal cases have been reported [41]. In addition to its nicotinic-related toxicological effects, cytisine has moderate activity as an acetylcholinesterase (AChE) inhibitor [42]. In humans, cytisine may be safe when given in a single 4.5 mg dose, which leads to a mean plasma concentration of 50.8 ± 4.7 ng/mL: i.e. three times higher than the plasma concentration associated with the single administration of the recommended dose for nicotinism [43]. Moreover, It has been reported that cytisine induces partial seizures in mice [44], and more recently it has been shown that intraperitoneal injection of a subthreshold dose of cytisine significantly alters the protection provided by some antiepileptic drugs against seizures [45, 46].

3.2 Cytisine pharmacokinetics

Although suitable methods for detecting cytisine and other toxic plant alkaloids in body fluids and tissues have been described [47-49], and the use of cytisine in humans has been well established for a long time, its

identification and quantitative determination in biological samples and pharmaceutical formulations is still difficult [50]. Furthermore, there is still much to learn about its pharmacokinetic properties. However, studies of mice, rats, rabbits and humans [8, 21, 43, 49, 51-53] have provided some initial data.

On the basis of physico-chemical parameters, it can be argued that protonated cytisine is very hydrophilic, and more lipophilic in a basic environment and therefore easily soluble in gastrointestinal fluids, however its gastrointestinal permeability is limited, and it is the combination of these properties that contribute to its poor bioavailability [53]. In mice, peak blood level is reached two hours after administration, indicating an adsorption rate of 42%. In rabbits, oral biovailability is 34% and peak plasma concentrations are reached after 35 minutes [8]. After oral and intravenous administration in mice, the highest concentrations are found in the liver, bile, adrenal gland, and kidney [51]. Brain concentrations in rats are lower than those of equal doses of nicotine or varenicline, a cytisine analogue, because of its low lipophilicity and, probably, a still unknown brain efflux mechanism [21]. It does not reach in brain more than 30% of its plasma concentration, whereas the brain concentration of nicotine under the same conditions is 65% of its plasma concentration. These data indicate poor penetration of the blood- brain barrier (BBB). Cytisine is not metabolised but excreted unchanged in urine. Its $t_{1/2}$ in rabbits is in the order of 37-52 minutes.

Human data are very scanty but, by taking into account the experimental animal data, it is possible to foresee hypothetical values for the most common kinetic parameters such as its plasma half-life (average 4.8 hours), peak plasma concentration (1-2 hours post dosing) [43] clearance (2-5 mL/min/kg), volume distribution (1.6 L/kg), $t_{½}$ (3.6 hours), unbound brain concentration (2-10 nM) [21]. Cytisine is eliminated unchanged through the kidneys: after oral or intravenous administration, respectively 18% and 32% of the drug is found in urine after 24 hours [9].

One conclusion that can be drawn from these data is that one of the limitations affecting the clinical use of cytisine is its low brain penetration, much less than that of nicotine or varenicline, and so any new cytisine derivatives developed for the treatment of brain diseases needs to have better favourable pharmacokinetics than cytisine.

4. Main activities of cytisine in the nervous system and metabolism

In addition to acting on nAChRs, cytisine can also interfere with neuronal functions by modifying non-classical neural activities such as inflammation, immunity, and neuroprotection [54]. We here report the most relevant effects of cytisine on behavioural activities and its molecular mechanism of action.

4.1 Effects on nicotine addiction and smoking cessation

Tobacco smoking is the most important and dangerous preventable pathology in industrialized countries killing more than 6 million people/every year worldwide [7, 55, 56]. The carcinogenic potential of tobacco smoke is due to the presence of various carcinogens and other compounds whose highly addictive properties favour and maintain the smoking habit. Nicotine triggers the neurobiological and psychological effects associated with smoking dependence and addiction by interacting with nAChRs in the mesolimbic pathway [57] and, although it does not initiate tumorigenesis in humans or rodents, it does promote tumour growth and metastasis by inducing cell cycle progression, epithelial-to-mesenchymal transition, cell migration and invasion, angiogenesis, and the evasion of apoptosis in a number of systems (reviewed in [58-60]. These effects may facilitate the carcinogenic potential of tobacco smoke and represent a major limitation of

the long-term use of nicotine in replacement therapy. In addition nicotine has numerous activities on circulatory vasculature and on heart that lead to cardiovascular toxicity that is maintained at doses used in smoking prevention and nicotine has addictive properties that also can interfere with brain activities. To avoid all these effects nicotine replacement therapy, the most effective and widely adopted therapeutic approach to counteracting tobacco smoking (see recent reviews [61-64]) can be substituted by the use of drugs such as cytisine and cytisine analogues that interact with nAChRs [21, 65]). It is beyond the scope of this review to consider all of the aspects of nicotine replacement therapy [7, 62, 66] and so we will concentrate on the role of cytisine as an aid in inducing smoking cessation.

Cytisine has been used for this purpose in eastern and central Europe since 1960, and is currently marketed under the name of "Tabex" in 18 countries [2, 4, 7, 8, 67]. Cytisine is a partial agonist of α4β2 and a near full agonist of α6β2 nAChRs and at low doses can activate the nAChRs in the mesolimbic reward pathways, inhibit the sensory stimulation of nicotine and decrease withdrawal symptoms. Pre-clinical evidence from experimental models of smoking addiction indicates that it aids smoking cessation [2, 4, 8, 67] and a number of clinical studies have found that it is more effective than placebo [68] and superior to nicotine replacement therapy [10]. It has been successfully used as the main agent for smoking cessation in countries such as Poland [69], and meta- analyses of papers published over the last 40 years have confirmed these findings [70], although these have been criticised on methodological grounds [71]. One recent meta-analysis has found that the overall relative risk of successful continuous abstinence at the longest follow-up versus placebo was 1.74 (95% confidence interval [CI] 1.38-2.19) and that the most frequent adverse reactions are mild or moderate nausea, vomiting, dyspepsia, upper abdominal pain and dry mouth, with a relative risk versus placebo of 1.10 (95% CI 0.95-1.28) [7].

In the search for new partial α4β2 agonists for smoking cessation, a number of cytisine derivatives or analogues have been studied, and this has led to the introduction of varenicline (Fig 2B), which has all of the characteristics of cytisine but is more efficient and possibly has fewer side effects [6, 65, 72]. Two studies have compared the two partial agonists by analysing recent clinical trials and found that both are active and more effective than placebo, although cytisine may be more clinically effective than varenicline, whereas varenicline may have fewer side effects [73, 74]. However, the comparision of cytisine versus varenicline is not yet fully determined and one very recent randomised controlled study of indigenous Māori in New Zealand has confirmed that cytisine is as effective as varenicline in supporting smoking cessation but has significantly fewer side effects [75]. The greatest advantage of cytisine is that it is much cheaper than varenicline, but the latter has been investigated in more clinical studies using different scientific approaches. It is difficult for governments to choose which of the two to use in the first-line treatment of tobacco dependence, particularly because the decision may affect the lives of millions of people. There is therefore a need for further studies of the long-term effectiveness and safety of smoking cessation policies in the general population, as well as more consolidates cost effectiveness studies of smoking cessation.

However the recent report on in vitro cytisine cardiotoxicity [76], should be taken in consideration in the search for anti-smoking nicotinic drugs.

4.2 Effects on alcohol use disorder (AUD)

This is the clinical term for the misuse of alcohol, and includes the pathologies of alcohol abuse and alcohol dependence, which are both very difficult to treat and have, among others, devastating effects on brain

structure and functions. There is constant and persistent association between alcohol abuse and the use of nicotine, marijuana and cocaine, and epidemiological studies have shown that there is a close correlation between the use of alcohol and tobacco smoking [77-79] and that they share genetic and neural mechanisms. Furthermore, the relationship between depression and alcohol dependence [80] suggests the involvement of nAChRs [81, 82]. None of this is surprising because, among the other biological targets for obtaining drug-related reward effects, the direct and indirect effects of alcohol on the nAChRs in the dopaminergic mesolimbic system play a very important role [80, 83], though it is not yet clear which subtypes are involved in alcohol addiction and withdrawal or their mechanism of action (direct or allosteric activation, inhibition) [84, 85].The effects of alcohol are complex and depend on the receptor subtype, the tested dose, and the length of the alcohol chain (in general, short chain alcohols potentiate nAChR currents but, as the carbon chain lengthens, the currents are inhibited).

Antagonists of α7 receptors such as methyllycaconitine [81] have little or no effect on alcohol consumption in a number of animal models, but the results of experiments involving α7KO mice suggest that α7 receptors modulate various ethanol responses, including consumption, sedation, hypothermia, ethanol-induced locomotor stimulation, and neurotoxic effects [86]. Mecamylamine and other general antagonists of heteromeric nAChRs reduce alcohol drinking in animals [81, 87].

The most effective drugs in animal models of alcohol consumption and in human alcoholics are those that bind to α4β2 receptors such as varenicline, cytisine, lobeline, and sazetidine [81, 87-90]. Varenicline is not active in α4KO mice [91], and mecamylamine is not active in humans [92]. Dihydrobetaerythroidine, a selective α4β2 antagonist, does not decrease ethanol drinking in animals [93], indicating that α4β2 receptors are not the only ones involved, and inhibitors of α3β4 receptors, such as CP-601932, PF 4575180 [93] and 18-Methoxycoronaridine [94] are also very active in decreasing alcohol consumption. Experiments involving mice devoid of different nicotinic subunits partially confirm the results obtained using selective drugs, but there are some discrepancies and the relative roles of the different subtypes are not completely clear (for a review, see [84]). Demonstration of the involvement of nAChRs in alcohol intake, abuse and withdrawal has come from clinical evidence showing that varenicline, reduces alcohol self-administration in smokers [38, 88], and that cytisine and varenicline reduce alcohol consumption and seeking behaviour in animal models of alcoholism [89, 90, 95]. This effect is quite specific as nicotinic drugs do not decrease saccharine or sucrose consumption [89], although the effect has a bell-shaped curve indicating a gradual loss of effectiveness that is probably due to the development of tolerance.

Another feature of alcohol addiction is relapse and, in a mouse model of relapse, cytisine treatment reduces intake and preference after re-exposure to ethanol [95, 96].

The mechanisms underlying nAChR modulation of alcohol consumption are still unknown, but one important mechanism could be modulation of the expression and activity of the early genes triggering the gene expression patterns necessary for the neuro-adaptations leading to drug addiction [97, 98]. Chronic voluntary alcohol consumption increases the expression of the *delta* FosB transcription factor involved in promoting reward and progression to addiction, and exposure to cytisine decreases chronic voluntary alcohol consumption and the level of striatal delta Fos B [95].

In connection with cytisine effect on alcohol consumption, it is worth considering its protective effect on liver disease, particularly liver fibrosis, a factor that can potentiate its use in alcohol use disorders [99, 100].

4.3 Effects on reward and cognition

Cytisine is as active as nicotine at CNS level, although higher doses are required probably because of its poor penetration of the BBB [21], but the effects of the two substances are not the same [101]: cytisine reduces the pleasurable sensations that smokers get from cigarettes, reduces withdrawal symptoms in human smokers, and diminishes the dysphoric-like state associated with nicotine withdrawal in rodents [102]. Moreover, using the drug discrimination assay, a tool that directly assesses the agonist and antagonist properties of cytisine, it was found that the latter has weak nicotine-like discrimination stimuli being much less potent than nicotine in behavioural experiments [103]. Intracranial self-stimulation (ICSS) is a behavioural procedure used to study the abuse potential of drugs and in this procedure, unlike nicotine that diminishes the ICSS threshold thus indicating a potentiation of brain reward function, cytisine does not facilitate ICSS or the re-instatement of seeking behaviour [101]. These latter results indicate that cytisine does not potentiate reward functions in rodent and the different effects obtained by nicotine and cytisine are due to the different affinity and efficacy of the two compounds for nAChR subtypes present in different cerebral nuclei.

One important effect of cytisine binding to the α4β2 and α6β2 subtypes in mesolimbic nuclei is to modulate dopamine release. Moreover, as a partial agonist, it can interfere with nicotine binding to nAChRs and thus minimise the addictive effect of nicotine and attenuate withdrawal symptoms, which is why it has been widely used in the treatment of nicotinism [2]. Increased brain dopaminergic activity may also be responsible for enhanced cognitive activity, particularly the retention of avoidance training, learning and memory.

Over the last decade, zebrafish (*Danio rerio*) have become a valuable complementary model in neurobehavioural research as the learning and memory capabilities of teleosts are as complex as those of mammals and share homologous neural mechanisms [104]. The zebrafish cholinergic system is generally similar to that of other vertebrates having mAChRs and the full set of nAChRs. It has been shown that the effects of nicotine and other cognition-enhancing drugs on zebrafish are similar to those observed in rodents, monkeys and humans [105, 106]. Braida *et al*. [107] demonstrated that cytisine given alone improved learning and memory in zebrafish by acting on α4β2/α6β2 receptors, and that its dose–response curve was similar to that of the partial α4β2 agonist varenicline.

4.4 Effects on mood regulation

A number of studies have shown that tobacco smoke can modulate depressive symptoms in humans, and pre-clinical studies of animal models and human clinical trials have shown that nicotinic agents have antidepressant-like effects and that nAChRs are involved in controlling depression [54, 108-111]. Increased cholinergic signalling in the brain increases behaviours related to anxiety and depression in mice [110, 112], and decreasing cholinergic signalling trough nAChRs using partial agonists or antagonists can have anxiolytic and antidepressant effects [113-115].

Behavioural studies have shown that, being a partial agonist of α4β2 receptors, cytisine can limit cholinergic signalling and is particularly active in various rodent models of acute and chronic depression [82, 114, 116-118]. On the basis of these findings, a series of cytisine derivatives have been investigated and it has been found that the most active are those acting on α4β2 receptors [119, 120], although hippocampal α7 contributes to alleviating depression-like behaviours in some stress-induced depression-like phenotype [116].These compounds include varenicline that has a stronger antidepressant effect [121]. The mechanism

of cytisine's antidepressant activity is not clear but interactions between the nicotinic and aminergic systems may be involved as serotonin depletion prevents the antidepressant-like effects of cytisine and an 5-HT1A receptor agonist potentiates cytisine effect [122]. Moreover, cytisine has synergistic antidepressant activity with a number of selective serotonin reuptake inhibitors [117], and its effect on a number of animal models of depression depends on the expression of post-synaptic 5-HT1A receptors in the hippocampus [122]. Chronic cytisine treatment relieves depression-like behaviours in a mouse model of depression, and increases the number of 5-HT1A receptors and the levels of brain-derived neurotrophic factor (BDNF) and mTOR in the hippocampus and amygdala, which are normally decreased in depressive disorders [118, 123]. Recent studies [112] have also shown that signalling interactions between β2-containing nAChRs and α2 adrenergic receptors in the amygdala are critical for the control of anxious and depressive behaviours.

The findings of pre-clinical and clinical studies (reviewed in [54, 123] show that inflammation, with the up-regulation of pro-inflammatory cytokines and a decrease in BDNF levels, plays an essential role in the pathophysiology of major depressive disorder (MDD). They have also shown that α7 nAChRs are expressed by non-neuronal cells and that those present in microglial cells inhibit microglia activation and reduce inflammatory processes in CNS disorders. Moreover, in animal models, the positive allosteric α7 nAChR modulator PNU120596 has anti-inflammatory effects by deactivating microglial activation and reducing the other inflammatory markers associated with MDD-related symptoms [54].

As cytisine is a full agonist of α7 nAChRs, it cannot be excluded that the cytisine-induced relief of depression and increased levels of BDNF [118] may also be due to the activation of α7 receptors.

Despite the interesting preclinical studies that consistently have demonstrated antidepressant-like effects of cytisine and other cytisine-based partial α4β2 nAChR agonists, the clinical trials in humans were in general rather negative. Only some small clinical trials have found that in a particular type of depressive patients, those resistant to a SSRi treatment, the nicotinic antagonist mecamylamine can have significant effects [124]. Also, add-ons of nicotinic partial agonists to classical antidepressants show greater effects than monoaminergic compounds alone [125]. However, there is a need for more sophisticated clinical studies in order to determine whether there is a sub-set of depressive patients who may be more sensitive to nicotinic partial agonists and /or antagonists [82, 109, 126, 127].

4.5 Effects on food intake and body weight

Tobacco smoking in humans reduces food intake and weight gain, and its cessation leads to the return of the body weight of a typical non-smoker. Similar results have been obtained in animal studies showing that the chronic administration of nicotine by various routes affects food intake and fat storage by acting on nAChRs, and that the co-administration of nicotine and the BBB-permeable antagonist mecamylamine can prevent these effects.

Acute cytisine administration also reduces food intake in mice [108] and, when chronically delivered contingently or non-contingently at a high dose of 5 mg/kg, decreases weight gain and food intake in a manner that is comparable to the effect of nicotine even though it does not substitute nicotine as a reward (see above and [128]).

The acute effect of cytisine on food intake is abolished by abolishing the expression of β4-containing receptors by means of the viral-mediated knockdown of the β4 nAChR subunit in neurons of the arcuate nucleus of the hypothalamus (ARC) [108], a brain area that is known to affect appetite. The ARC is a central

node in the hypothalamus that regulates food intake, and stimulation of its two main cell populations (agouti-related peptide[AgRP]- and pro-opiomelanocortin[POMC]-expressing neurons) respectively induce hunger and satiety [129, 130]. Recent studies have shown that both cell populations express a large number of different nAChR subunits and similar receptor subtypes and, despite their different roles in regulating food intake, knocking down the β4 subunit in either cell type blocks or blunts the decreased feeding induced by the acute administration of cytisine or nicotine [131].

The relevance of β4-containing receptors in food intake is further supported by studies showing that selective targeting of the α3β4 subtype, using the positive allosteric α3β4 modulator levamisole [132], or the agonist 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) [133], prevented weight gain in mice fed with a high-fat diet [132] and lowered body weight in diet-induced obese mice [133].

Cytisine is a partial α4β2 agonist and, when given to animals, can decrease dopaminergic activity in the mesolimbic reward pathway [134], in which dopamine drives and maintains behaviours that perpetuate addiction to substances of abuse, and the consumption of sucrose, and alcohol. Consequently, cytisine may further contribute to weight loss by decreasing sugar consumption [134].

Although its exact mechanism of action is unknown, cytisine by acting on the β4-containing nAChR within the ARC decreases food intake in mice, but is not effective in reducing long term weight gain in smokers probably because is a less efficacious agonist on human β4-containing receptors [25](see Table1).

4.6 Effects on motor activity.

The complex control of movement requires inputs from both the central and peripheral nervous system, and the involvement of many neurotransmitter networks. Pre-clinical studies have demonstrated that the nicotinic cholinergic system plays an important role in regulating motor activity, and great expectations were raised by the discovery that, like nicotine, the direct injection of cytisine into the ventral tegmental area and the nigro-striatal pathway (two brain areas controlling movement) has pro-locomotion activity [135]. However, these expectations were disappointed when it was found that the peripheral, intra-peritoneal or subcutaneous administration of cytisine concentrations that block the effect of nicotine had no effect on locomotion [101, 114]. Consequently, more active cytisine derivatives that are more selective on the receptor subtypes in the locomotor pathway and have more favourable pharmacokinetic characteristics are now being investigated [136-138]. Furthermore, cytisine may facilitate locomotion by acting peripherally on the neuro-muscular junctions activating the negative-feedback exerted by pre-junctional nicotinic auto-receptors [139].

High cytisine levels at the neuro-muscular junctions (such as those reached in the case of cytisine poisoning) induce muscle weakness and possible respiratory muscle paralysis that may be caused by the inactivation of muscle AChR [8, 49].

4.7 Neuroprotective effects

Parkinson's disease (PD) is a neurodegenerative disorder in which the loss of nigrostriatal dopaminergic neurons depletes striatal dopamine (DA) levels, thus causing motor and cognitive dysfunctions. Epidemiological studies have shown that there is an inverse correlation between smoking and the incidence of PD [140] and studies of animal models with selectively lesioned nigrostriatal dopaminergic neurons and PD patients have shown that there is a significant decline in the number of nicotinic α4β2* and α6β2*

receptors in the substantia nigra and striatum, with little or no change in the number of α7 receptors [140, 141].

The epidemiological evidence on a possible protective effect of smoking in PD has prompted many researchers to investigate whether cytisine can prevent or attenuate symptoms in animal models of PD in the way that smoking seems to do in humans [142].

In mouse PD models of MPTP or 6-OHDA-induced DA denervation, Ferger *et al*. [143] and Abin *et al*. [137] (2010) found that cytisine treatment is neuroprotective, because it prevents the decrease in striatal tissue DA levels and increases the striatal release of DA by acting on α4β2* and α6β2* receptors. Ferger *et al*. [143] also showed that, as an iron chelator, cytisine decreases hydroxyl radical production in the brain and its toxic effects on neurons.

Very recently, Zarate *et al*. [144] have tested the effects of chronic cytisine treatment on 6-OHDA-lesioned mice and found that its protective effects include a reduction in PD-like behaviours and the loss of DA neurons, although these effects were only observed in female mice. This sex-specific effect was obtained using a cytisine concentration of 0.2 mg/kg, which does not activate nAChRs, and was potentiated by co-treatment with 17-β-estradiol. The authors hypothesised that the protective effect of cytisine was due to the inhibition of apoptotic ER stress signalling pathways in DA neurons due to the cytisine-mediated chaperoning of nAChRs, the up-regulation of ER exit sites, and a change in the outflux of proteins from the ER (see Figure 3).

Early indications that smoking may also correlate with protection and a delay in the onset of Alzheimer's disease (AD) [145] stimulated research into whether the nicotinic cholinergic pathway has a neuroprotective effect. However, more recent and more precise clinical and pre-clinical investigations have reached the opposite conclusion that cigarette smoking increases the risk of developing AD probably as a result of smoking-related cerebral oxidative stress, neuro-inflammation, and impaired neuro-protection [146]. However, the dilemma is not solved yet since these CNS alterations could be due to the toxic effects of the other compounds present in cigarette smoke and do not rule out the possible beneficial effects of a specific nicotinic stimulation.

Possible neuroprotective effects are also suggested by data showing that *in vitro* cytisine protects cortical neurons from the excitotoxicity of NMDA by reducing the number of surface NR2B-containing NMDA receptors and has an anti-apoptotic effect by regulating the Bcl-2 family of proteins [147]. Moreover, in a model of cerebral ischemia/reperfusion injury, cytisine significantly decreased infarct size and neuronal apoptosis, and improved histopathological lesions by promoting ERK and CREB phosphorylation and mRNA expression, suppressing the expression of NR2B, and down-regulating related genes [148].This and the data showing that cytisine reduces stress-related hydroxyl radicals [5, 143] need to be further investigated in order to establish whether other inter-related mechanisms underlie the effects of cytisine on CNS.

4.8 Effects on autonomic nervous system

As suggested by Dale and Laidlaw [1], cytisine activity in peripheral organs is due to the stimulation of nAChRs in the sympathetic autonomic ganglia and adrenal gland (mainly α3β2 and, to a lesser extent, α7 and β4-containing AChRs [26]), which increases blood pressure, blood glucose levels, and heart rate, slightly stimulates respiratory centres by activating the carotid sinus, and directly stimulates the respiratory centres themselves at low doses [26, 149]. At gastrointestinal level, it stimulates the contractility of the

intestinal musculature and increases the intestinal secretion underlying vomiting and diarrhea. Stimulation of the superior cervical ganglion dilates the pupil of the eye, retracts the nictitating membrane in cats, and broadens the palpebral fissure. All these effects are very similar to those of nicotine and are inhibited by the previous administration of hexamethonium; moreover, cytisine pre-treatment counteracts the effect of nicotine in peripheral ganglia, once again indicating that it is a partial agonist of nAChRs.

In mouse trachea, cytisine increases ciliary beat frequency and particle transport by stimulating α3β4* receptors in epithelial cells [150]. At high doses or after repeated administration, it desensitises nAChRs and therefore ganglionic stimulatory activity.

4.9 Effects on sugar metabolism

Hypoglycaemia triggers the release and turnover of noradrenaline and adrenaline by activating ganglionic nAChRs in the adrenal medulla [151], and recurrent hypoglycaemia can interfere with the normal counter-regulatory hormonal response that should defend against hypoglycaemia. Cytisine preserves this counter-regulatory response in an animal model of hypoglycemia-associated autonomic failure (HAAAF).

ACh released from the cholinergic interneurons of the nucleus accumbens (NAc) binds to nAChR, and modulates the release of DA from dopaminergic terminals and reinforced behaviours. Interestingly also increased sugar consumption, albeit indirectly, affects the release of DA in the NAc via nAChRs [152]. Inhibitors or partial inhibitors of nAChRs, such as cytisine, varenicline and mecamylamine, decrease sucrose consumption in a long-term paradigm measured using the intermittent-access two-bottle choice in experimental animals [134] and therefore could have a marginal effect on sugar metabolism.

In humans chronic nicotine smoking is associated with progression in insulin resistance [153] but recently it has been shown that chronic treatment with DMPP improves glycaemic tolerance in diet-induced obese mice [133]. This effect is specifically due to the agonism of β4-containing receptors that determines a robust increase in peripheral insulin sensitivity, with improved in vivo glucose clearance in skeletal muscle, heart and brown adipose tissue [154].

Pursuing a completely different pathway, Jin *et al,* [155] explored a drug-induced gene expression dataset, and found that cytisine together with a vitamin E analogue may help in the treatment of type 2 diabetes. and demonstrated that the treatment is effective in an experimental model of diabetes. These observations open up the possibility of developing new therapeutic uses of nicotinic drugs for major diseases such as diabetes and obesity, and should be borne in mind when using cytisine to induce smoking cessation in diabetic patients.

4.10 Effects on cardiovascular system

nAChRs are present in the sympathetic and parasympathetic ganglia, and their stimulation can increase or decrease heart rate and blood pressure, however cardiovascular control also involves central nAChRs primarily located in nuclei of the brainstem [156, 157].

In freely moving rats, subcutaneous injection of cytisine induces changes in sympathetic and parasympathetic cardiovascular activity: a small increase in arterial pressure similar to that obtained with lower concentrations of nicotine, and a non-significant reduction in heart rate [158]. However, these small cardiovascular effects are not blocked by peripheral (hexamethonium) or general (mecamylamine) anti-

nicotinic drugs, although it has previously been reported that the *in vivo* effects of cytisine are partially or fully attenuated by mecamylamine [103, 159].

On the contrary, the cardiovascular effects induced by varenicline, that increases blood pressure and decreases heart rate, are more similar to those induced by nicotine and are counteracted by nicotinic antagonists [158] probably because of drug binding to peripheral nAChRs.

The strong cardiac toxicity due to long and severe tobacco smoking could suggest that nicotinic agents could have a direct toxic effect on cardiomyocytes. Recently, the use of human-induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) has allowed to better evaluate the effects of drugs on cardiac function and structure. Using hiPSC-CMs Wang *et al.* [76], found that Cytisine, at a dose of 50 μM, has cytotoxic effect, impairs oxidative stability and increases lipid peroxidation. Moreover, by increasing oxidative stress and disrupting calcium homeostasis cytisine showed significant cardiotoxicity with functional changes of cardiomiocytes. These findings on direct toxicity exerted by nicotinic drugs on cardiomyocytes, can further arise the attention to the safety of a nicotinic approach for smoking cessation.

5. Other activities of cytisine outside the nervous system

5.1 Anti-proliferative effects

Over recent years, the search for possible drugs originating from the *Sophora* plant species used in traditional Chinese herbal medicine has led to the discovery of new pharmacological properties of cytisine and its derivatives [160]. Specifically, it was found that cytisine and its derivatives can affect many tumor-related processes, such as apoptosis, and various molecular targets or signaling pathways, such as reactive oxygen species (ROS), nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and PI3K/AKT/mammalian target of rapamycin (mTOR) pathways.

Some cytisine derivatives are slightly cytotoxic in some cancer cell lines [161] and cytisine inhibits the growth of lung cancer cell lines *in vitro* and suppresses lung tumour growth in mouse xenograft models [162]. It inhibits the proliferation of human lung cancer cells in a dose-dependent manner by inducing the excessive production of ROS, which leads to intracellular stress, mitochondria dysfunction, and unbalanced redox reactions, and eventually cell apoptosis [162].

Cytisine also induces apoptosis in HepG2 human hepatocellular carcinoma cells through the mitochondrial pathway by increasing the mitochondrial release of cytochrome C, up-regulating caspase-3 and down-regulating pro-caspase-3 [163]. Electron microscopy of Cytisine-treated HepG2 cells revealed apoptotic bodies, an expanded ER, swollen mitochondria, and significant vacuolisation. Cytisine also caused a dose-dependent increase in cytoplasmic Ca^{2+} levels and ER stress-mediated apoptosis by activating CHOP and JNK and up-regulating caspase-4, thus suggesting its possible role in the treatment of hepatic tumours.

Recent studies have shown that the mixed-lineage leukemia (MLL) histone methyltransferases act as epigenetic transcriptional regulators and methylate H3K4me3 genes. MLL makes a complex with menin, the product of the multiple endocrine neoplasia type 1 (MEN1) gene, and it has been found that when this complex is activated it promotes the development of human hepatocellular carcinoma in an H3K4me3-dependent manner [164]. Cytisine and six of its derivatives inhibit the menin–MLL interaction to some degree: in particular, Compound 1a (which bears the 5-phenylpentan-2-ol side chain) inhibits the expression

of H3K4me3 and has potent antiproliferative activity on hepatocellular carcinoma cells possibly by inhibiting the formation of the MLL-menin complex [165].

5.2 Anti-osteoclastogenesis effects

Another important effect of cytisine is its ability to decrease bone loss [166]. Post-menopausal osteoporosis, the most frequent disease occurring in older women, is due to an imbalance between osteogenesis and osteoclastogenesis. Osteoclasts are huge, multi-nucleated cells of monocyte/macrophage lineage that differentiate into mature osteoclasts primarily as a result of interactions with macrophage colony-stimulating factor (M-CSF) and the receptor activator of NF-κB ligand (RANKL) [167]. By interacting with RANK, RANKL stimulates intracellular pathways that are important for the maturation of osteoclasts. Cytisine treatment of the monocyte/macrophage RAW 264.7 cell line and bone marrow monocytes suppresses RANKL-induced osteoclastogenesis by inhibiting osteoclast-precursor maturation to osteoclasts and decreases bone absorption by impacting NF-κB, MAPKs and the PI3K/AKT signalling pathways. Cytisine also plays a positive role in decreasing bone loss in estrogen deficiency-induced osteoporosis mouse model, which suggests that it may have effects against osteolytic diseases and may be used to treat osteoporosis [166]. These effects may be of a large interest and could be exploited by further researches with more selective cytisine derivatives on cell survival.

6. New cytisine derivatives and their bioactivities

As we have pointed out in this review, the multiple pharmacological and toxicological activities of cytisine and its similar alkaloids found in several plants have been exploited since the antiquity in traditional medicine for their therapeutical effects. However, the presence of so many and sometimes contradictory activities has strongly hampered their large use in clinical practice. The effort was then put in the synthesis of new and more specific cytisine derivatives, but this new line of research was difficult in view of the complexity of the laboratory synthesis of these molecules.

As preclinical studies have shown [21] that the low brain concentration of cytisine, is due to its high hydrophilicity and poor brain penetration, and by the possible presence of an unidentified active efflux mechanism that excrete cytisine from the brain, the main original aims of modifying cytisine structure were to increase its lipophilicity (thus allowing greater BBB permeability), to decrease its possible excretion from the brain, to create more nAChR subtype-selective compounds for therapeutic purposes, but also to give rise to compounds with activities beyond those of cytisine itself.

Cytisine (see Figure 2A) is characterised by the presence of a basic secondary amino group incorporated into a bispidine scaffold and a quasi-aromatic pyridone nucleus, and the reactional capability of both makes it a popular scaffold for the synthesis of biologically active compounds and allows the formation of a large variety of derivatives.

Cytisine derivatives have previously been discussed in important reviews [6, 168, 169] and one more recent review [170] and so we will here concentrate on the more recent highly potent and specific derivatives, and those that have new activities.

6.1 Smoking cessation aid

Our group, together with the chemist group of professor Sparatore, has previously synthetised and pharmacologically characterised several cytisine derivatives with different substituents on the basic nitrogen [171, 172]. All these compounds had a rather poor affinity for the α7-containing receptors but some of them showed a nanomolar affinity towards the α4β2 subtype. Among all these different N- substituents we have characterised the cytisine dimer1,2-bisN-cytisinylethane (CC4, Figs.3 and 4A), which binds to the α4β2 and α6β2 subtypes with high affinity and selectivity, and to α3β4 and α7 subtypes with much lower affinity. CC4 is a partial agonist of all nAChR subtypes, although it is less efficacious on the α3β4 and α7 subtypes than cytisine. It is more lipophilic than cytisine, and *in vivo* studies in rats have demonstrated that it has reinforcing properties and, when co-administered with nicotine, it reduces the reinforcing properties of nicotine, which is in line with the fact that it reduces nicotine-induced DA release when co-administered with nicotine in an *in vitro* system [22].

The effect of CC4 and CC26 (another cytisine dimer,) (Fig 4A) on nicotine reward has been investigated in zebrafish by means of CPP. Both derivatives had reinforcing properties similar to that of nicotine and, when co-administered with the maximally effective dose of nicotine, significantly blocked nicotine-induced CPP [107] thus indicating that they may be active in inducing smoking cessation.

The C(10) (IUPAC numbering) functionalisation of (−)-cytisine by the group of Gallagher [173] is efficient and highly flexible as docking studies have shown that the C(10) substitution targets the complementary region of the receptor binding site, thus mediating subtype differentiation. C(10)-modified cytisine ligands retain affinity for α4β2 nAChRs, are partial agonists, and more selective for the α4β2 than the α3β4 or α7 subtypes: in particular, they are negligibly active on α7 receptors, which are considered off-target for smoking cessation. The most active and selective compound was that with the C(10) methyl analogue (Fig 4B) which has been used to identify a conserved arginine residue in the β3 strand that, in α7 nAChRs, not only suppresses the agonism of 10-methylCytisine but also modulates agonist binding and channel function [174].

6.2 Nootropic activity

Makara *et al.* [175] have screened the nootropic effects of 10 new derivatives of (-)-cytisine on the learning, memory and cognitive capacities of laboratory animals under stress conditions, and found that compound 2b (Fig 4C) improves cognitive function in rats. The molecular docking of this compound in the active sites of ionotropic glutamate receptors suggests an interaction between them that may also be responsible for the nootropic activity of compound 2b.

The synthesised (—)-Cytisine derivatives N-allylCytisine-12-carbamide,cCytisine-12-carbamide and N-1-adamantylCytisine-12-thiocarbamide are weak AChE inhibitors [176].

6.3 Anti-cancer activity

There is growing evidence that many herbal medicines have anti-cancer activity, and these are considered important alternatives to conventional cancer treatments [177]. Given continuous interest in the development of agents that are active on breast cancer cells, cytisine/pterocarpan-derived compounds have been biomimetically synthesised even though they are naturally present in extracts of *Sophora tonkinensis*. The most interesting is compound 4 (Fig 4D), which is more cytotoxic against MDA-MB-231 breast cancer cells and differently toxic for cancer and normal cells [160].

Naturally occurring isoflavones present in plants belonging to the Fabaceae family have a long history in the treatment of human diseases. Cytisine-linked isoflavonoids have been synthesised and, when tested on PC-3 prostate and LS174T colon cancer cells, they inhibited proliferation by inhibiting the bi-functional peroxisomal enzyme hydroxysteroid 17β-dehydrogenase-4 (HSD17B4), which is overexpressed in prostate and colon cancer tissues [178]. A new compound cytisine N-methylene-(5,7-dihydroxy-4_-methoxy)-isoflavone (Fig 4E) has been isolated from the *Sophora alopecuroides* plant used in Chinese herbal medicine, and preliminary pharmacodynamic studies have shown that it inhibits breast cancer cell metastases [179].

6.4 Anti-liver fibrosis activity

Liver fibrosis is a histological hallmark of liver injury, and the persistent liver fibrosis that leads to cirrhosis, hepatoma and liver failure is characterised by the excessive deposition of extracellular matrix proteins. New cytisine derivatives with various substituents at the 12N-position of cytisine have been synthesised, of which those with the 12N-benzyl being the more active and the dichlorobenzyl 5f (Fig 4F) the most active. Preliminary data indicate that it targets the PI3K/Akt/Smad pathway, and is thus extensively active against a number of fibrogenetic proteins in human hepatic stellate LX2 cells [99].

6.5 Anti-plasmodium, anti-fungal, and antiviral activities

New chloroquine analogues (cytisinyl derivatives) are active against *Plasmodium falciparum* at nM concentrations, have very limited cytotoxicity against human cell lines, and non-hemolytic activity at concentrations of up to 200 μM [180].

Eleven cytisine-containing 1,3-oxazoles have been synthesised and evaluated for their activity against *Candida* spp. Compounds 10 and 11 (Fig 4G) were most active against *Candida albicans* and the fluconazole-resistant *Candida krusei* strain. Docking studies suggest that they are potential inhibitors of *Candida* glutathione reductase and have considerable anti-fungal properties [181].

Zhang *et al*. [182] have demonstrated that a dimer of matrine and (−)-Cytisine (Fig 4H) inhibits the expression of hepatitis B DNA virus in HepG2.2.15 cells.

The pandemic caused by SARS-CoV-2 has turned attention to the anti-viral activity of cytisine derivatives that are active against influenza viruses. The 9-carboxamides of methyl cytisine (compounds 13 and 14) are non-toxic against HEK293, but active against influenza H1N1 and pdm09 VIRUS (Fig 4I) and compound 23 (Fig 4I) against the human parainfluenza virus type3 ([183].

6.6 Pesticidal agents

Huang *et al*.[184] have prepared a series of N-acyl/sulfonyl derivatives of 5(3,5)-(di)halogenocytisines/cytisine as botanical pesticides by structurally modifying cytisine, and tested their pesticidal activity against three seriously aggressive crop insects: *M. separata*, *T. cinnabarinus* and *S. avena*. The compound with the strongest pesticidal activity was 5f (3,5-dichloroCytisine) (Fig 4L) , but all of the compounds had potent acaricidal activity.

7. Conclusion and future research

Cytisine is an alkaloid present in a number of plants that binds to nAChRs with high affinity and potency, but has a structure that also allows albeit weaker interactions with other receptor types that may be responsible for its non-nicotinic effects in animals and humans. The fact that most of its effects on the nervous systems are due to interactions with nAChRs is clearly demonstrated by the ability of nicotinic antagonists to interfere with its activity or its lack of effects in mice devoid of specific nAChR subunits, but much less is known about its mechanisms of action outside the nervous systems.

Cytisine is one of the most promising smoking cessation treatments as it is cheap and rapidly reduces the rewarding effect of nicotine and attenuates nicotine withdrawal symptoms. It also seems to be a valid and appropriate candidate for the treatment of alcohol addiction, although more studies are required to clarify the mechanisms underlying its anti-alcohol effects. Another important field that merits further study is the mechanism underlying the way in which it significantly reduces depression-like behaviours in pre-clinical models of major depressive states and, as stress and depression are associated with neuronal atrophy and decreasing synaptic connections, the way in which chronic cytisine treatment improves depression- and stress-induced cognitive, behavioural and biochemical alterations.

One particular point of interest that could lead to practical consequences is its effect on Ca^{2+} metabolism in different cell types: it is known that it induces neuroprotection by reversing intracellular Ca^{2+} overload and balancing Bcl-2 and Bax expression levels in neurons [147], but by increasing intracellular Ca^{2+} levels in hepatoma Hep H2 cells [163] it induces apoptosis. Furthermore, the anti-cancer effects are mainly due to its ability to increase cell apoptosis and/or induce cancer cell death by means of mitochondria-generated ROS (particularly in the case of cytisine derivatives that have been synthetised with the aim of increasing specificity or membrane permeability). An effect that can merit further studies is that on osteolytic diseases that could be of interest if combined with the antismoking therapy.

When considering approaches to drug effects it is important to distinguish active and passive targeting: the former exploits specific interactions with specific surface-exposed targets, whereas the latter includes interactions or effects due to the physico-chemical characteristics of a compound. Modifying the structure of cytisine may reinforce some of the non-nicotinic affinities of the original molecule and/or give rise to molecules with previously unknown activities: for example, studies on the effects of cytisine on fibrosis or its, anti-plasmodium, anti-fungal and anti-viral activity have never tested whether these can be blocked by nicotinic antagonists, and we do not know whether they are due to the nicotinic or other effects of the new compounds.

Much more research is required to define the targets of cytisine derivatives and their *in vivo* mechanisms of action: for example, animal models such as pregnant mice or developing offspring can also allow studies of multi-organ diseases.

Last but not least, there is a need for clinical studies in order to establish the safety and therapeutic effects of cytisine in humans, and compare these with those of its possible clinical alternatives. As an important medicinal herb, cytisine may be particularly attractive to smokers, alcohol users and/or depressed people looking for 'natural' medicines.

Declaration of Competing Interest

The two authors declare that they do not have conflict of interest, with no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted.

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Figure 1 Two varieties of *Laburnum* taken from from '*Hortus Eystettensis* of **Basilius Besler** (1561–1629) in which he describes the flowers present in the Botanical Garden of Johann Konrad von Gemmingen (1593/95–1612), Prince-Bishop of Eichstätt, Bavaria.

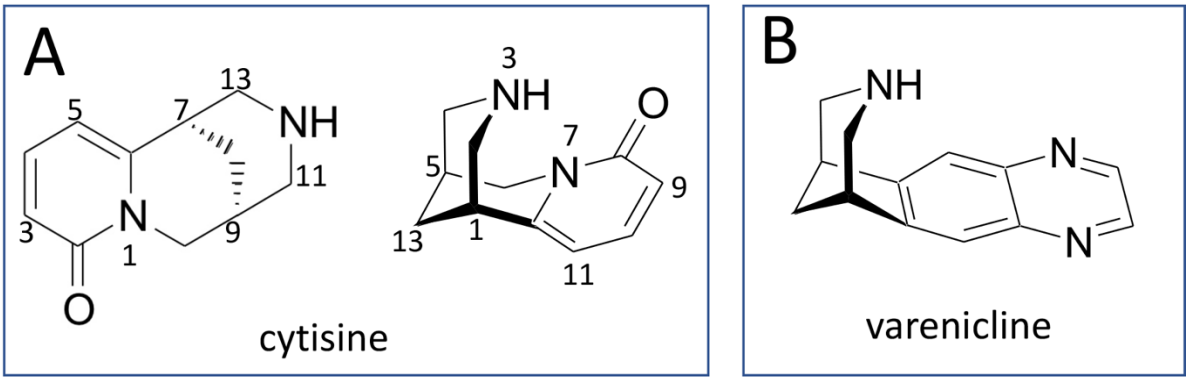

Figure 2 A) Structure of cytisine with traditional numbering (left); three-dimensional structure with IUPAC numbering (right) . **B)** Structure of varenicline.

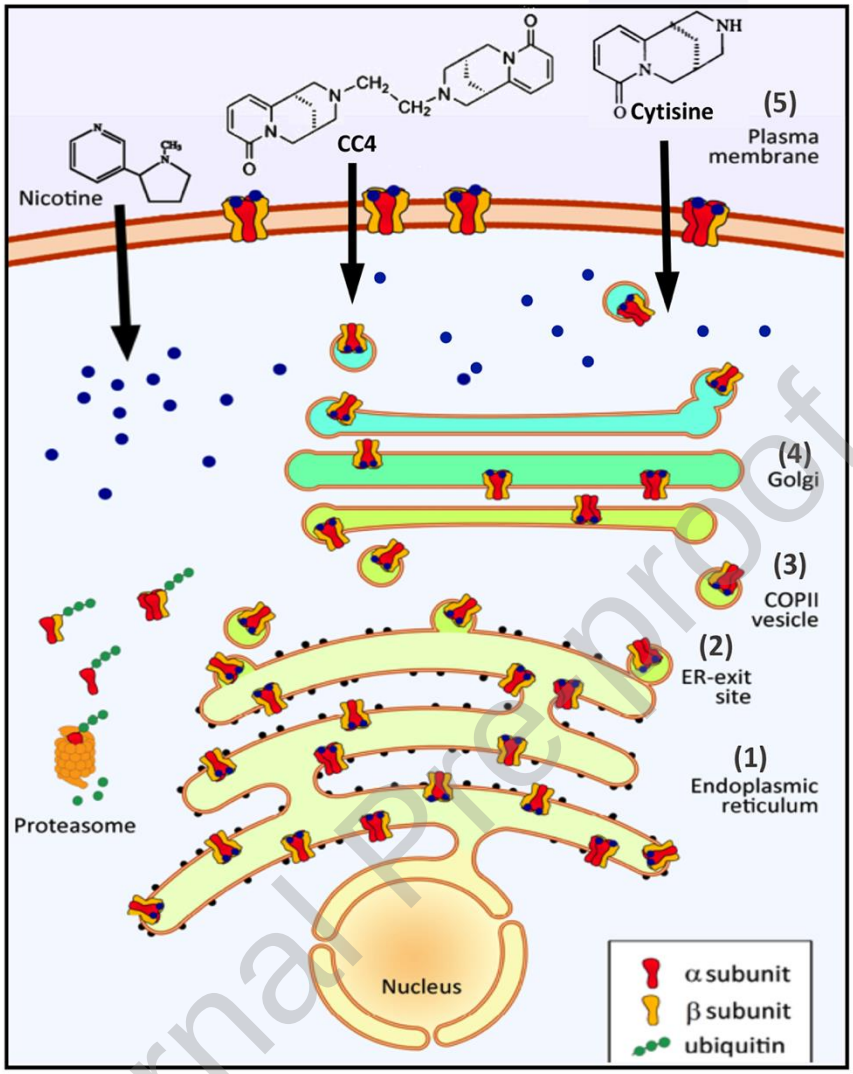

Figure 3: Pharmacological chaperoning of nAChRs. A scheme of the nAChR subtypes synthesis and trafficking in cells is shown. Pentameric α4β2 or α3β4 receptors assemble in the endoplasmic reticulum (ER) **(1)** and concentrate in ER exit sites **(2).** nAChRs traffic from the ER to the trans Golgi network via COPII vesicles **(3)** and then to Golgi intermediate compartment **(4)** and to plasmamembrane **(5).** Nicotinic drugs (blue dots) can modify the nicotinic response by a modification of receptor function by a direct interaction with the binding sites of nAChRs at the plasma membrane **(5)** or by changing the number of plasma membrane nAChRs through a modification of the intracellular receptor traffic (right part of the figure). In the latter case nicotine up-regulates cell surface receptors by increasing the ER assembly and transport of the $(\alpha4)_2(\beta2)_3$ receptors to the plasma membrane while cytisine and CC4 enhance the export of assembled α3β4 subtype pentamers from the ER, and/or stabilise assembled receptors, thus enabling more nAChRs to be transported at and inserted into the plasma membrane.

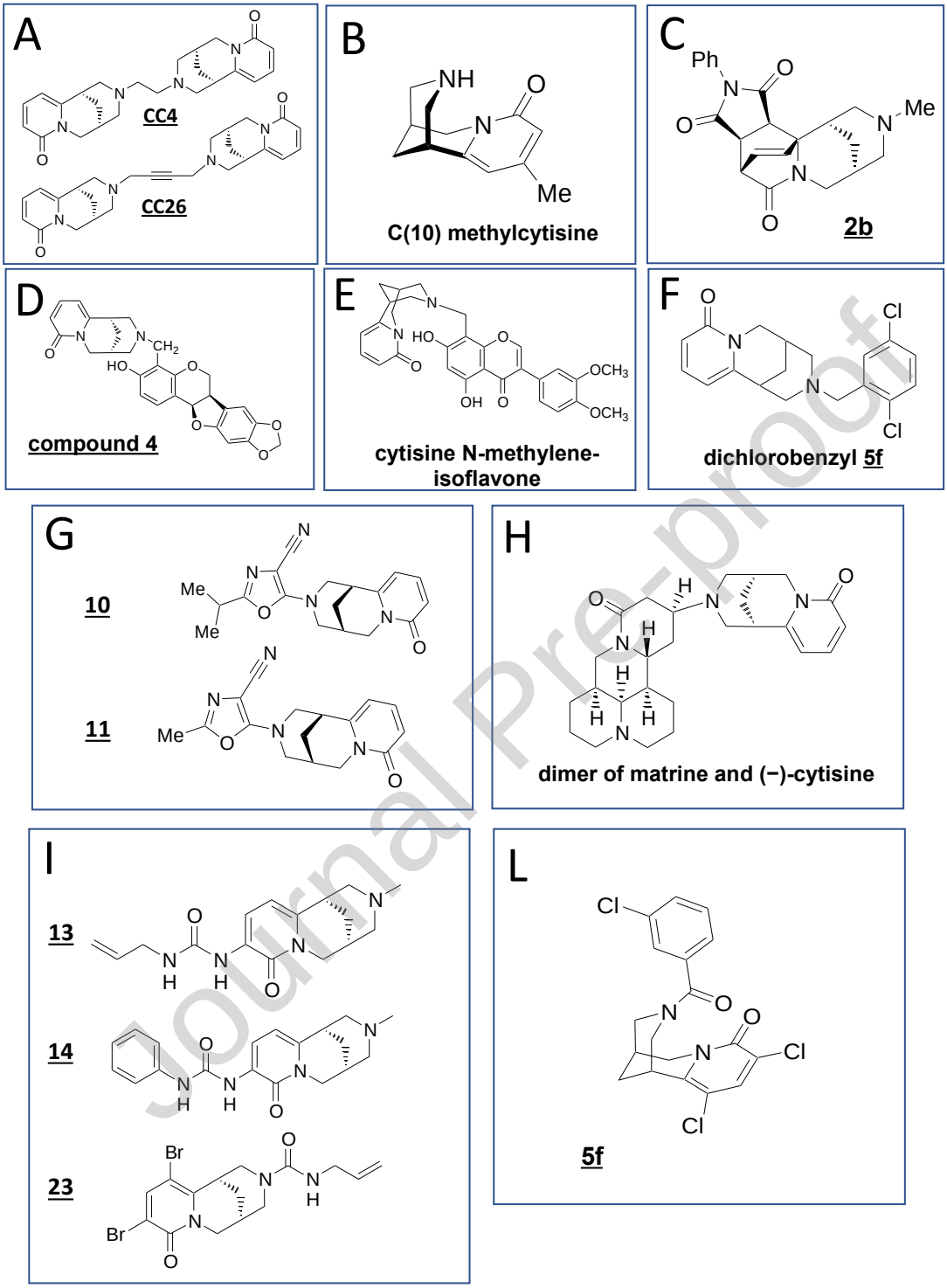

Figure 4: Chemical structure of cytisine derivatives

A ref [107]; **B** ref [173]; **C** ref [175]; **D** ref [160]; **E** ref [179]; **F** ref [99]; **G** ref [181]; **H** ref [182]; **I** ref [183]; **L** ref [184].

Table 1. Affinity, efficacy and potency of cytisine for α4β2, α6β2, α3β4 and α7 subtypes

	Ki, nM	Native, α:β ratio, concatamer	Efficacy, % of max ACh	EC_{50}, μM
α4β2(R)	2.1[1], 0.49[2], 0.19[3]	native[1,2,3]	0.11[2]	29.5[2]
α4β2(M)	0.55[2], 0.2[4]	native[2,4]	0.33[5]	0.47[5]
α4β2(H)	1.3[6]	1 : 1 [1]	0.09[1]	11.6[1]
$(α4)_2(β2)_3$(H)	-	1 : 10 [6]	0.02[6]	ND[6]
$(α4)_3(β2)_2$(H)	-	10 : 1 [6]	0.19[6]	5.3[6]
α6β2(R)	2.1[1],0.65[3]	native[1, 3]		
α6β2(M)	0.8[4]	native[4]	0.85[5]	0.031[5]
α3β4(R)	122[7]	native[7]		
	-	1 : 1 [8]	1.10[8]	520[8]
α3β4(H)	285[1], 103[6]	1 : 1 [1]	0.76[1]	19[1]
		1 : 1 [8]	0.59[8]	890[8]
		4 : 1 [9]	0.48[9]	180[9]
$(α4)_3$ $(β2)_2$(H)	-	concatamer	0.34[8]	1750[8]
$(α4)_2$ $(β2)_3$ (H)	-	concatamer	0.16[8]	214[8]
α7 (R)	228[1],331[2]	native[1,2]	-	-
α7 (H)	601[7]	-	0.83[1],0.97[7]	98[1],110[7]

The affinities (Ki values) were obtained from binding studies in native or transfected subtypes. The potencies (EC_{50} values) were determined by functional studies (electrophysiological experiments or release experiments(5)) on native or transfected subtypes. The efficacies (% to max ACh) values were defined as the fraction of the max response to ACh.

Native: Receptors present in native tissues.

α:β ratio: heterologously expressed heteromeric receptors obtained by transfection in cells or injection in oocytes of different α:β subunit ratios

Concatamer: heterologously expressed pentameric receptor with linked subunits with defined subunit ratios and assembly orders .

R=rat, M= mouse, H=human

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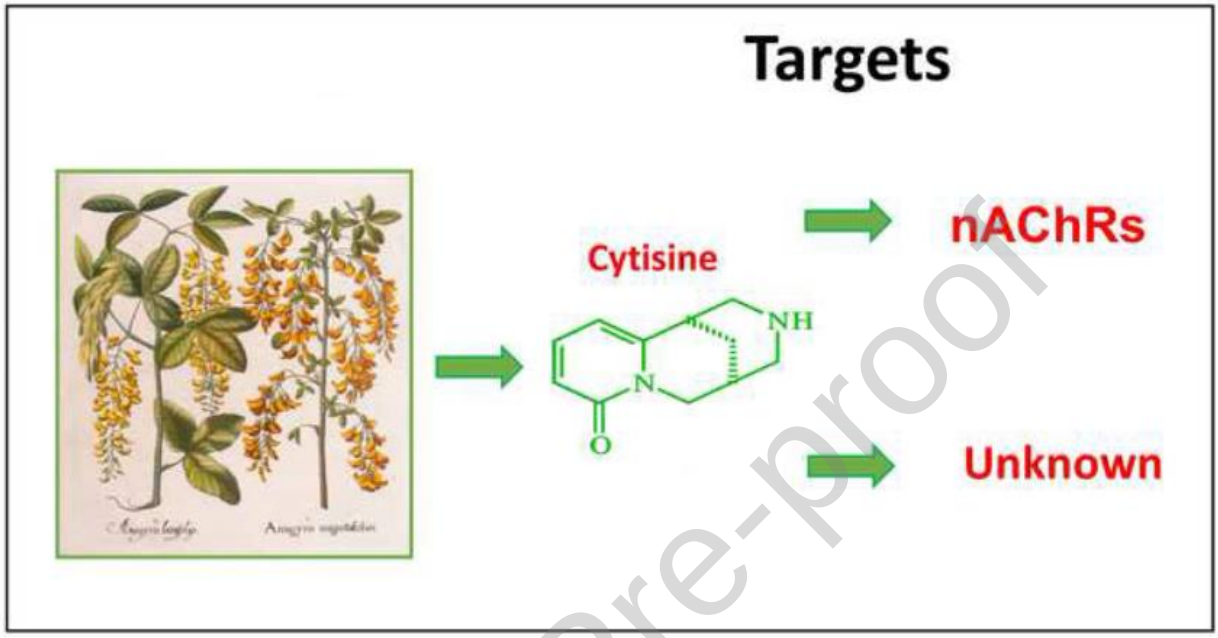

Graphical abstract