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Cytisine derivatives as ligands for neuronal nicotine receptors and with various pharmacological activities

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Abstract

Neuronal nicotinic acetylcholine receptors (nAChRs) form a family of ACh-gated cation channels made up of different subtypes. They are widely distributed in peripheral and central nervous systems and are involved in complex cerebral processes as learning, memory, nociception, movement, etc. The possibility that subtype-selective ligands be used in the treatment of CNS disorders promoted the synthesis of a large number of structural analogues of nicotine and epibatidine, two very potent nAChR agonists. Pursuing our long standing research on the structural modification of quinolizidine alkaloids, we devoted our attention to cytisine, another very potent ligand for many nAChR subtypes. Thus a systematic structural modification of cytisine was undertaken in order to obtain compounds of potential therapeutic interest at peripheral as well as central level, with a particular concern for achieving nAChR subtype selective ligands. Up to the present more than 80 cytisine derivatives, mainly of *N*-substitution and a few by modifying the pyridone ring, have been prepared. The biological results, which concern so far about an half of the prepared compounds, indicate that the introduction of a nitro group in position 3 of the pyridone nucleus further enhances the high affinity of cytisine, while the introduction of substituents on the basic nitrogen, though reducing in different degrees the affinity, gives rise to compounds with a higher selectivity for central ($\alpha_4\beta_2$) versus gangliar (α_3-containing) receptor subtype. On the other hand, the analgesic, antihypertensive and inotropic activities found in some *N*-substituted cytisines, represent an attractive starting point for the development of more active compounds.

Keywords: Cytisine derivatives; Quinolizidine alkaloids; Neuronal nicotinic acetylcholine receptors

1. Introduction

Pursuing our long standing systematic research on the structural modification of quinolizidine alkaloids, in order to obtain compounds of pharmacological interest, we recently devoted our attention to cytisine (Fig. 1), an alkaloid mainly obtained from seeds of *Laburnum anagyroides*, but present also in several other plants of the *Leguminosae* family, to which it confers toxicity.

From a pharmacological point of view, cytisine strictly resembles nicotine: it acts mainly at ganglionic level, exhibiting more stimulating than blocking effects; however, central effects are also shown which are related to cholinergic stimulation [1,2].

At the present time, cytisine lacks any therapeutic application in Western countries (in the past it was used as diuretic [3]), while in the countries of the former Soviet Union it is preferred to lobeline as a respiratory analeptic for its stimulating activity on respiratory centers and on chemoreceptors in the aortic and carotid bodies [4,5].

Recent Japanese patents suggested hypoglycemic and antiinflammatory activities for cytisine and its *N*-methylderivative [6,7].

Recent in vitro research [8,9] has demonstrated a very high affinity of cytisine to cerebral nicotinic receptors ($K_d < 1$ nM), which is superior to that of nicotine, acetylcholine, carbachol (as agonists) and dihydro-β-erythroidine (as antagonist).

Neuronal nicotinic acetylcholine receptors (nAChRs) are widely expressed in the vertebrate nervous system, where they act as *postsynaptic receptors exciting neurons*

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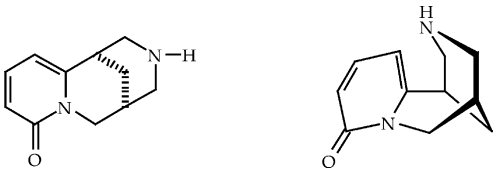


Fig. 1. Structure of (−)7R,9S-cytisine.

or as *presynaptic receptors modulating the release of many neurotransmitters* [10,11].

These receptors form a family of ACh-gated cation channels made up of different subtypes, each of which has a specific anatomical distribution in the central and peripheral nervous systems. This heterogeneity is based on the 12 genes cloned so far, that codify for the corresponding nAChR subunits (α_2–α_{10}; β_2–β_4), and the fact that the subtypes can be generated by the homopentameric or heteropentameric assembly of neuronal α and β subunits. The diversity of nAChRs and the number of potential nicotinic receptor subtypes is also increased by the 'in vivo' existence of receptor combinations consisting of up to four different subunits [12,13].

Recent studies have shown that an acetylcholine binding pocket is situated on each of the two α subunits of the pentameric nicotine receptor, in a region at about 30 Å above the cell membrane. These pockets are connected to the water filled channel vestibule by narrow, 10–15 Å long, tunnels by which ACh might gain access to them [14,15]. However, on the basis of the crystal structure of the molluscan ACh-binding protein, the last possibility is considered unlikely by Brejc et al. [16]. These authors suppose that the most likely access routes to the ligand-binding sites are from above or below the double cysteine-containing loop C. These binding sites may be allosterically coupled with a non competitive inhibitor (NCI) site, which is positioned within the central transmembrane domain of the ion channel, at the level of the extracellular surface of the bilayer, with a tranverse distance from agonist sites in the range of 20–30 Å [17].

nAChRs are known to be involved in a number of CNS diseases including degenerative ones such as Alzheimer and Parkinson diseases, Tourette's syndrome, schizophrenia and some forms of epilepsy [18]. Moreover they have also been associated with some lung tumors and autoimmune diseases [19,20].

The possibility that subtype-selective ligands be used in the treatment of central nervous system disorders promoted the synthesis of a large number of structural analogues of nicotine and epibatidine [21,22], two very potent natural nAChRs agonists endowed, however, with many undesirable side effects not easily surmontable. Epibatidine is a very potent analgesic agent, which might be useful in opiate resistant chronic pain, but it is an extremely toxic compound, about 100 times more toxic than nicotine in mice [23]. Thus nicotine and epibatidine may be questionable models for developing new drugs, though some success has recently been achieved with ABT-594 (a 2-chloro-5-pyridylether), which exhibited a quite improved therapeutic profile as an analgesic with respect to epibatidine [24] (Fig. 2).

On the other hand cytisine, in spite of its high affinity for many nAChR subtypes and the capability to discriminate among some of them, has not received much attention as a model for structural analogues or derivatives able to interact, directly or allosterically, with one or more receptor subtypes. Cytisine drawbacks are mainly related to its low lipophilicity, which could hamper the crossing of the blood brain barrier, as shown by Reawill et al. [25], and also to its high affinity for α_3-containing (gangliar) subtypes, though lower than that for $\alpha_4\beta_2$ subtype. However, cytisine's long half-life in vivo, compared with that of nicotine, should also be taken into account [26].

In addition to all the classical in vivo pharmacological activities of nicotinic agonists, cytisine stimulates the evoked neurotransmitter release [27,28], has potent antinociceptive activity [29,30] and modulates locomotor activity at high doses [31].

Cytisine appears to be a potent iron chelator, clearly inhibiting the hydroxyl free radical formation in the Fenton reaction. Such a property, its activity as a dopamine releaser and the fact that it has been shown to reduce MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced dopamine depletion in vivo, suggest that cytisine (or better some of its derivatives) may be useful in treating Parkinson disease by chelating iron and thus preventing cell death [32].

The foregoing considerations prompted us to undertake a systematic structural modification of cytisine, in order to obtain compounds of potential therapeutic interest either at peripheral or central level, with a particular concern for new nAChR subtype selective ligands.

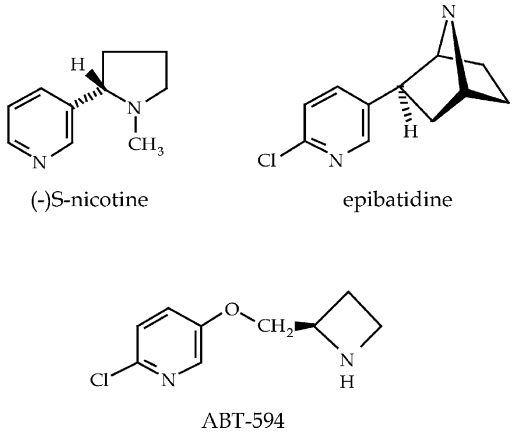


Fig. 2. Structures of some potent nAChRs ligands.

The structural modifications of cytisine should promote the passage through the blood brain barrier (limited, but not precluded, by its relevant hydrophilic character) and reduce the affinity for ganglionic receptors; the selectivity for central receptorial subtypes should be sought after even if with detriment of the potency.

The chemical modifications that can be operated on cytisine apply, either singularly or in succession, to the secondary amino group and the pyridone ring (electrophilic substitutions, additions to the conjugated double bonds, etc). However, in order to maintain the central nicotinic activity, it is generally claimed that the cationic character of the molecule should be secured, leaving unchanged the typical distance between the basic nitrogen and the carbonyl dipole, which must form a hydrogen bond with a donating group of the receptor [2,33–36].

Anyhow, it must be emphasized, given the number of nAChR subtypes and the diverse effects of different ligands on each subtype, that there are more than one, and perhaps several, different nicotinic pharmacophores [21].

Structural modifications of cytisine were already attempted in the past, but were mainly aimed at improving its respiratory analeptic properties [37,38] or obtaining local anesthetics [39].

Only quite recently, while our research program was developing, several (mainly halogenated) cytisine derivatives were claimed as useful agents for the treatment of neurodegenerative diseases, for use in addiction therapy (smoking cessation) and for in vivo studies of nicotinic receptors using positron emission tomography [40–44].

2. Results and discussion

Up to the present, we have prepared more than 80 cytisine derivatives [45–48], mainly of *N*-substitution and a few by modifying the pyridone ring. A small part of these compounds were already described by other authors, but their pharmacological activities were not studied.

As a first possibility, the introduction of saturated, unsaturated, or differently functionalized aliphatic or arylaliphatic residues on the amino group was undertaken with the aim to increase the overall lipophilicity and modify the ligand distribution pattern in vivo, as well as the discriminating capability towards nicotinic receptor subtypes.

The *N*-(ω-haloaliphatic) derivatives could represent irreversible antagonists, useful for studies on receptor functions.

The introduction of more complex moieties was also considered to enable reinforcement or selection of cytisine activities not related to nicotine receptor agonism/antagonism (as the mentioned hypoglycemic and antiinflammatory ones [6,7]), or to give rise to new activities which could be envisaged taking into account the cytisine molecular structure, which, for example, embodies a 'bispidine' scaffold that characterizes several antiarrhythmic agents [49–51].

In order to address the choice of such moieties, the capability of cytisine to displace specific ligands from 28 different receptors was preliminarily explored.

In this study, cytisine displayed only very modest affinity for some receptors (cholecystokinin A, histamine H_3, kainate, muscarinic M_1 and M_2, *N*-methyl-D-aspartic acid NMDA, phencyclidine, serotonin 5-HT_3, thyrotropin-releasing hormone (TRH), vasoactive intestinal peptide, (VIP)) inhibiting the ligand binding just for a 17–33% at 10 μM concentration. On the other hand, at the same concentration, cytisine failed completely in displacing the specific ligands from the remaining receptors assayed (adenosine A_1 and A_2, angiotensine II, galanin, insulin, interleukin 1_α, leukotriene B_4, neurokinin 1, neuropeptide Y, platelet activating factor, phorbol esters, serotonin 5-HT_{1A}, sigma, sodium channel, tromboxane A_2, tumor necrosis factor). Therefore, the very high affinity of cytisine for nicotinic receptors appears still more outstanding and quite peculiar.

The prepared compounds, depending on the nature of substituents, may be assembled in eight groups (Figs. 3–10).

1) Compounds with *N*-non carbon substituents;
2) compounds with *N*-aliphatic (eventually functionalized) substituents;
3) compounds with *N*-arylaliphatic (eventually functionalized) substituents;
4) compounds bearing a second basic moiety;
5) compounds bearing a second cytisine unit, linked through an aliphatic or a xylylene moiety;
6) compounds with *N*-heteroaryl substituents;
7) compounds with *N*-acyl or *N*-aroyl substituents;
8) compounds substituted on the pyridone ring.

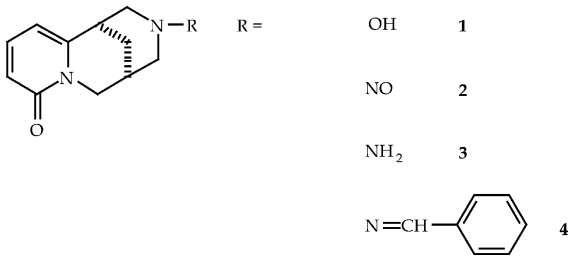


Fig. 3. First group of cytisine derivatives with *N*-non carbon substituents (and their derivatives).

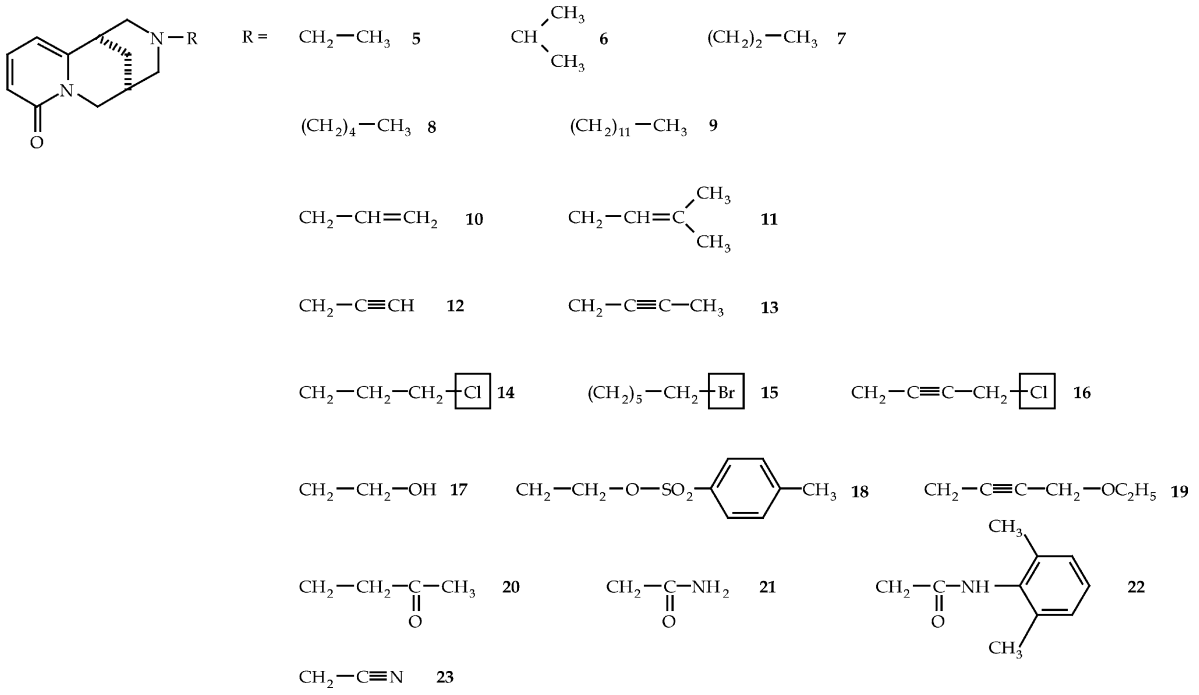


Fig. 4. Second group of cytisine derivatives with *N*-aliphatic substituents.

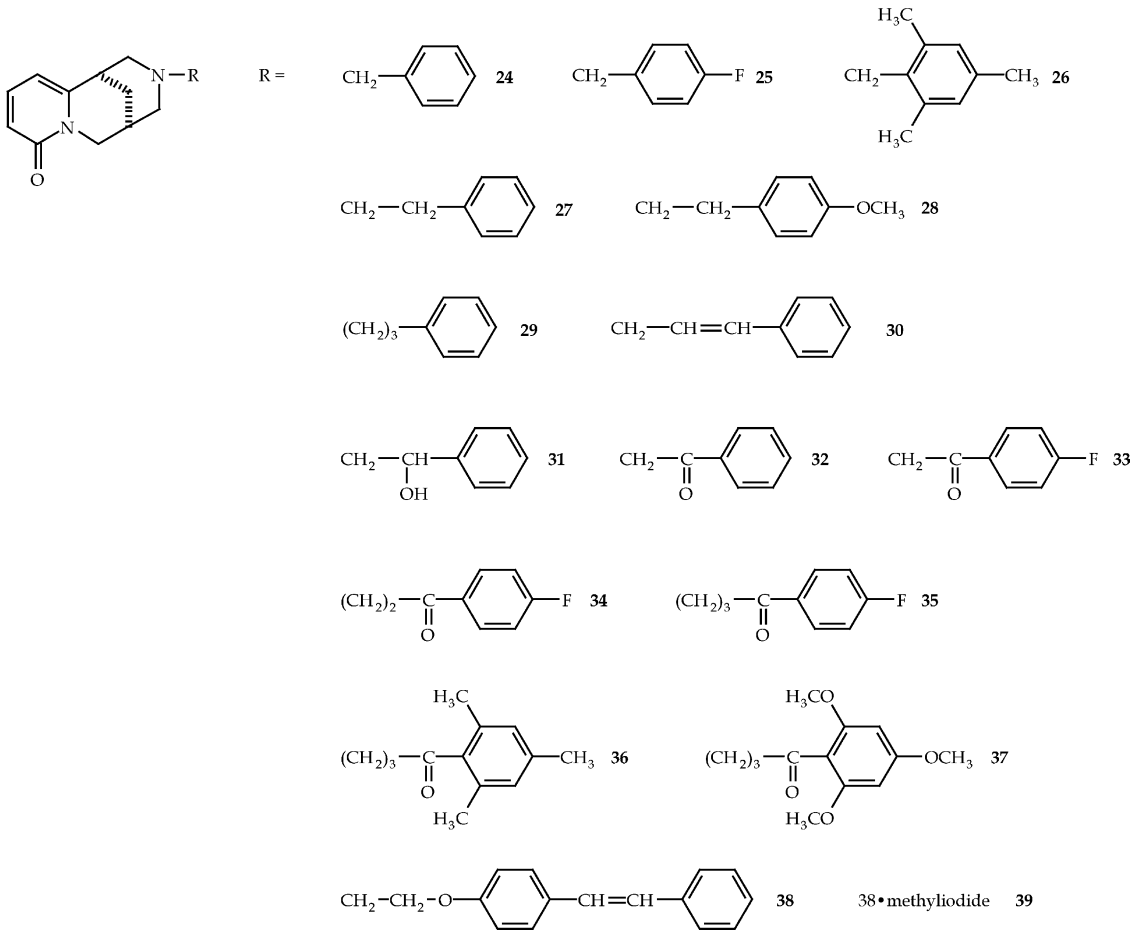


Fig. 5. Third group of cytisine derivatives with *N*-arylaliphatic substituents.

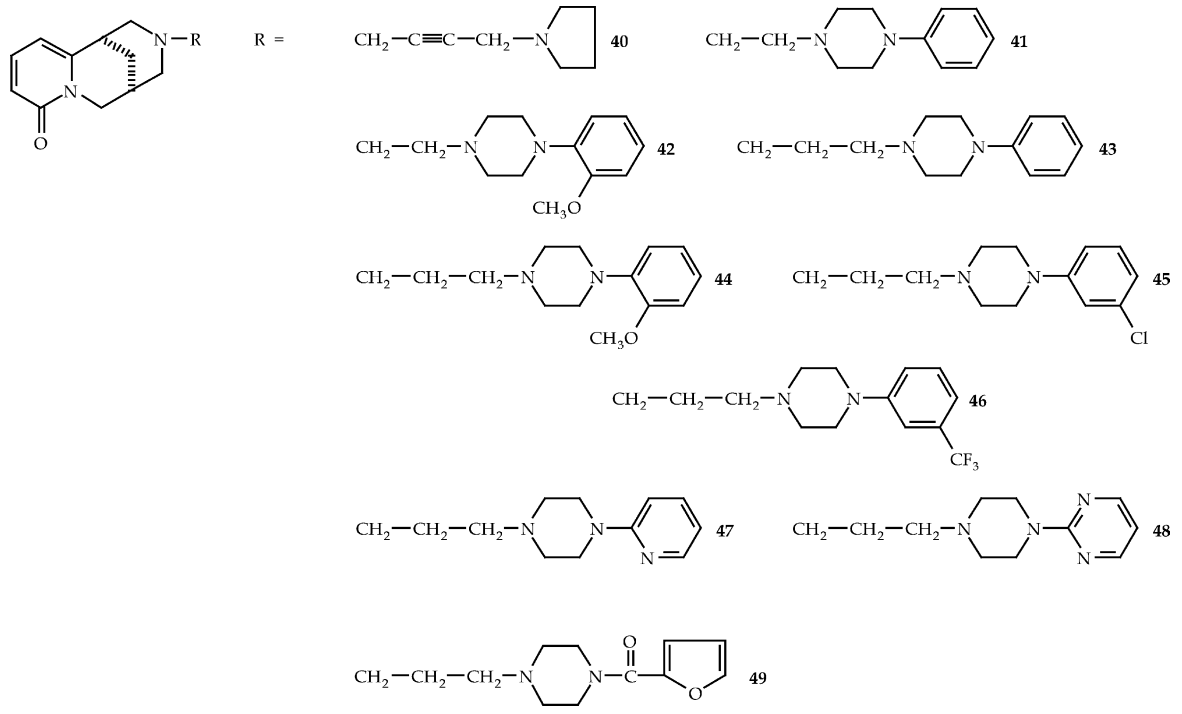


Fig. 6. Fourth Group of cytisine derivatives bearing a second basic moiety.

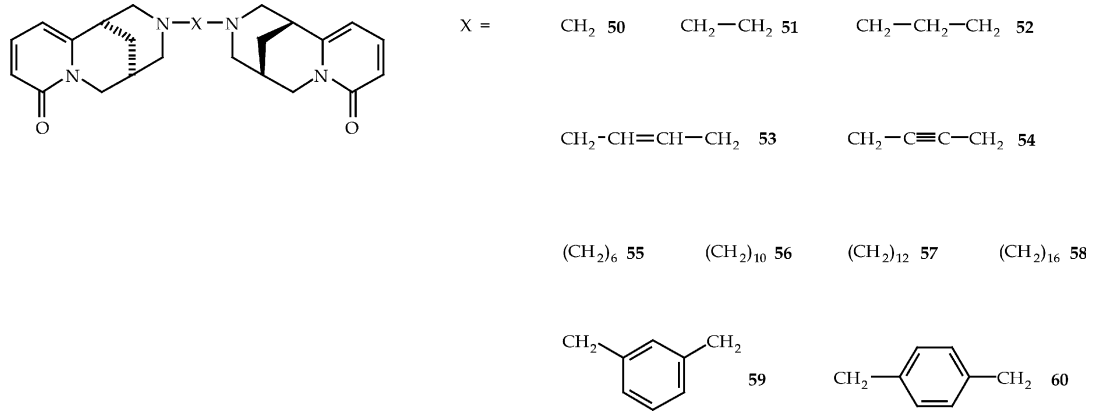


Fig. 7. Fifth group of cytisine derivatives bearing a second cytisine unit.

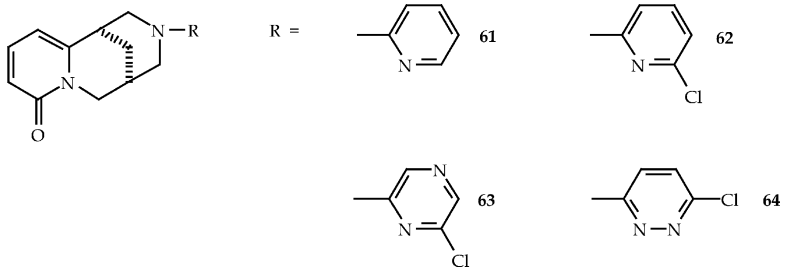


Fig. 8. 6th Group of cytisine derivatives with *N*-heteroaryl substituents.

The biological results so far available concern more than an half of the prepared compounds, which, however, were not all studied to the same extent.

Thirty compounds (**1**–**3**, **6**, **8**, **10**–**12**, **20**, **21**, **25**, **30**, **35**, **40**, **51**, **52**, **54**–**58**, **62**–**64**, **66**, **70**), selected as representative of the different kinds of structural modification effected on cytisine, have been assayed for affinity to nicotine receptors, through the displacement of [^{3}H]cytisine from rat cerebral cortices preparations.

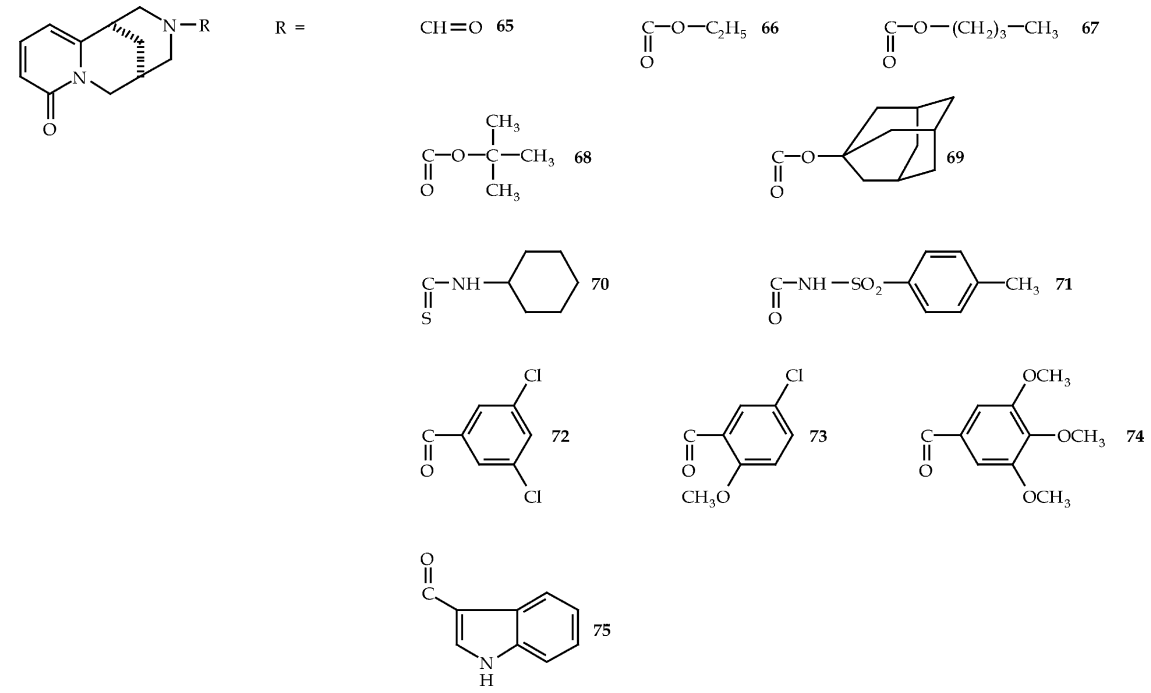


Fig. 9. 7th Group of cytisine derivatives with *N*-acyl or *N*-aroyl substituents.

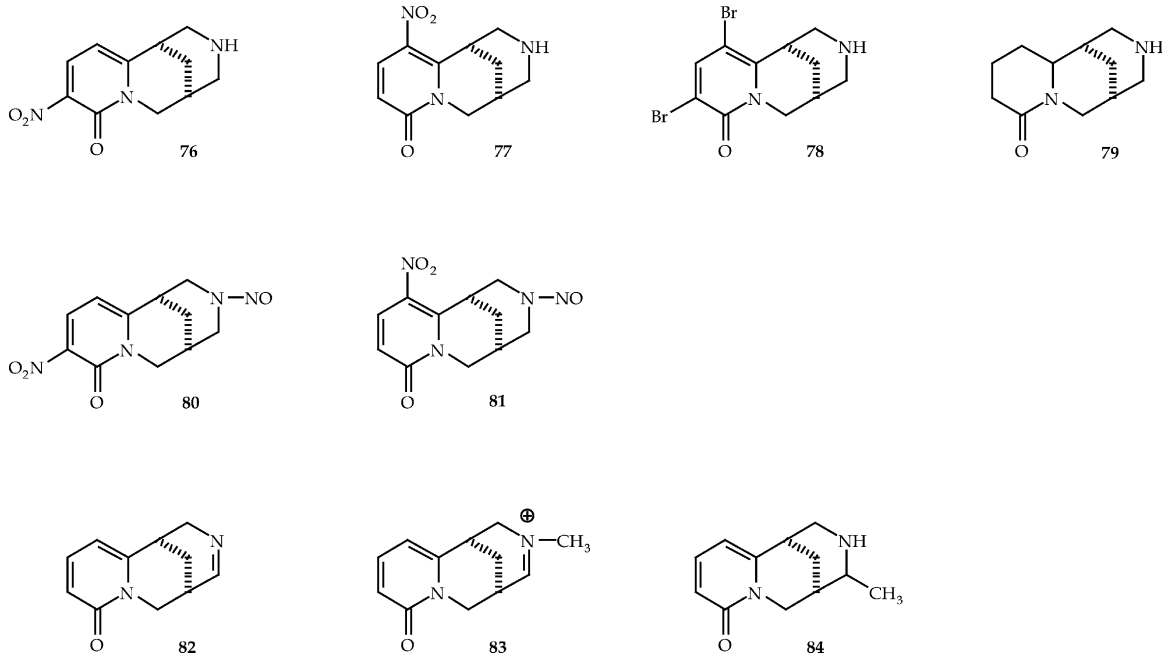


Fig. 10. 8th Group of cytisine compounds with substituents on pyridone ring or other structural modifications.

The affinity of eight compounds (**8**, **20**, **25**, **51**, **62**–**64**, **69**) for $\alpha_4\beta_2$, α_3 and α_7-containing receptor subtypes was investigated through the displacement of [^{3}H]epibatidine or [^{3}H]α-bungarotoxin [46], while functional studies with the same compounds are in progress.

Nine compounds (**9**, **22**, **27**, **30**, **31**, **35**, **44**, **72**, **75**) were subjected to a general pharmacological screening concerning about sixty in vitro and in vivo assays; on the base of the corresponding results, ten other compounds (**25**, **26**, **42**, **43**, **46**–**49**, **71**, **74**) were selected for investigating single or more than one pharmacological activities.

In the present survey the most interesting results of the pharmacological screening [45] are briefly summarized, while the results of the binding assays [46–48] are discussed in some more details.

2.1. Pharmacological screening results

Compounds exhibiting the most significant activities are collected in the Fig. 11.

N-Phenethylcytisine (**27**) and *N*-cinnamylcytisine (**30**) inhibited the stress induced ulcer in mice; this property, that is shared to some extent by most tested compounds, support a valid crossing of the blood brain barrier as a function of the lipophilicity of the substituent introduced.

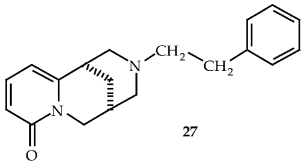
Omitting the discussion of some other minor in vivo activities (as antiinflammatory, anti PAF, hypoglycemic and dopamine antagonistic), are worthy of mention the *analgesic activity* (writhing test, formaline algesia) of compounds **30** and **44** and the strong and long lasting *antihypertensive activity* (in spontaneously hypertensive rats) of the same compound **44** (2-methoxyphenylpiperazinylpropyl-cytisine) and other analogous *N*-(arylpiperazinyl)alkylderivatives.

The inhibitory effects of naloxone and mecamylamine on the analgesic activity are under investigation to define if the activity is related to interaction with nicotinic rather than opioid receptors; the inhibition of cyclooxygenase has been already excluded.

The antihypertensive activity of compound **44** does not seem related either to ganglionic blockade (pupil dilation test in mice), or to α_1-adrenoreceptor antagonism (inhibition of noradrenaline-induced mydriasis in mice). α_1-Antagonism could have been expected since many arylpiperazine derivatives are endowed with α_1 and 5-HT_{1A} receptors affinity. Moreover, calcium antagonism, potassium channel activation and angiotensin inhibition were not observed in vitro on guinea pig atria and ileum, thus the mechanism of this activity remains somewhat intriguing.

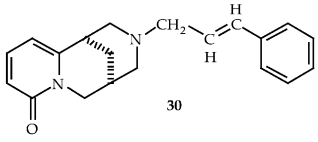
Compound **44** resulted as the most active, reducing for 38% the blood pressure at the oral dose of 100 mg/kg, while the analogue lacking the methoxy group (**43**) was the most potent, being still able to reduce for 20% the blood pressure at the dose of only 10 mg/kg.

An interesting inotropic activity was observed in vitro on isolated guinea pig left atria for compounds **27** and



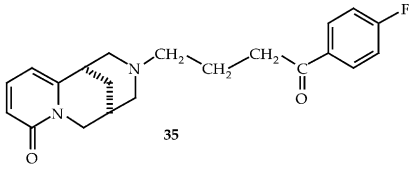
27

- inhibits stress induced ulcers
- positive inotropic activity (*in vitro*)



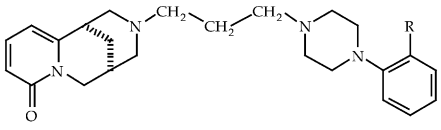
30

- inhibits stress induced ulcers
- analgesic activity
- hypoglycemic activity



35

- positive inotropic activity (*in vitro*)
- antiinflammatory activity
- inhibits PAF induced platelet aggregation
- protects mice from *i.v.* PAF induced mortality



R=OCH_3, **44**

- dopamine antagonist (apomorphine induced climbing test)
- analgesic activity
- antiinflammatory activity
- hypoglycemic activity
- antihypertensive activity (in SHR); the most active: at 100 mg/kg *p.o.* 38% blood pressure reduction

R=H, **43**

- antihypertensive activity (in SHR); the most potent: at 10 mg/kg *p.o.* 20% blood pressure reduction

Fig. 11. Pharmacological activities of some cytisine derivatives.

35 (*N*-[3-(4-fluorobenzoyl)propyl]cytisine). This activity is not related either with adrenergic stimulation (it is not blocked by propranolol) or phosphodiesterase inhibition (lack of reduction of spontaneous tone in guinea pig trachea). As far as we know, inotropic activity was not observed in 4-fluorobutyrophenone antipsychotics, but it is present in the antiarrythmic melperone (4′-fluoro-4-[4-methylpiperidino]-butyrophenone) [52]. Very strong inotropic activity was found by us [53] in some arylsparteines which exhibit some structural similarity with the relevant cytisine derivatives.

2.2. Binding assay results

First of all it must be emphasized that the results of binding assays are to be considered with care, being the expression of the average affinity to the subtype population present in the rat brain membrane preparation, even if the $\alpha_4\beta_2$ subtype is definitely prevailing. Moreover, one cannot meaningfully compare the K_i values of a series of compounds, when it is not known which are agonists and which are antagonists. The size and chemical nature of substituents introduced on cytisine may give rise to molecules which could still act as an agonist (even partial) or, extending outside the agonist volume, could act as competitive antagonist or, yet, by binding to the NCI site, as non-competitive antagonist.

Taking into account these cautions, the binding assay results, concerning so far 30 compounds, are illustrated.

The K_i values vary in the range between 1.2 and 4850 nM; for a single compound (cytisin-12-ylacetamide, **(21)**) it was not possible to measure the K_i, since it exhibited only 43% inhibition of [^{3}H]cytisine binding at the highest (10 μM) concentration tested.

The observed K_i values are not homogeneously spread within the cited limits, but are concentrated in four sub-ranges. Six compounds have K_i between 1.2 and 43.5 nM; 11 between 96 and 423 nM; six between 740 and 1230 nM and five between 2270 and 4850 nM. The structures of compounds which are included in each of these groups are quite different, making practically impossible to find a unifying structural feature to which relate the particular level of affinity.

However, when the tested compounds are assembled by structural similarity, largely different K_i values are still observed in each group, but it is possible to envisage reasonable explanations for most of them (Table 1).

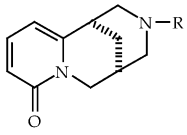
(a) The introduction of a substituent on the amine nitrogen always brought about a decrease of affinity, in respect to cytisine ($K_i = 2.4$ nM); such a decrease can be insignificant as in the case of *N*-aminocytisine ($K_i = 3.6$ nM), or very high as for cytisin-12-ylacetamide ($IC_{50} > 10$ μM).

With the passage from the strongly basic secondary amino group to a tertiary one, the formation and stability of the cationic head (which is commonly considered a peculiar feature of nicotinic ligands) will be clearly influenced by the *field*, *resonance* and *steric* effects produced by the aliphatic, arylaliphatic or heteroaromatic substituents which were introduced. With the introduction of acyl or aroyl groups (or isosteric moieties), the nitrogen basicity should be abolished or largely compromised.

The ensuing different degree of protonation at the cellular pH value and the substituent volume will also hinder to different extents the approaching of the nucleophilic or cationic nitrogen to the corresponding binding site of each nicotine receptor subtype, thus modifying the discriminating capability of the new ligand with respect to cytisine.

The combined effects of the above discussed factors may explain the observed large variation of K_i values,

Table 1
Results of binding assays (displacement of [^{3}H]cytisine from rat brain preparation)



Group	R =	K_i (nM)
	H (cytisine)	2.4
1st	NH_2	3.6
	NO	39
	OH	423
2nd	$(CH_2)_4CH_3$	43.5
	$CH_2–C\equiv CH$	101
	$CH_2–CH=C(CH_3)_2$	120
	$CH_2–CH_2–CO–CH_3$	163
	$CH_2–CH=CH_2$	200
	$CH(CH_3)_2$	2270
	$CH_2–CO–NH_2$	$> 10 \times 10^3$ [a]
3rd	$CH_2–p–C_6H_4F$	983
	$CH_2–CH=CH–C_6H_5$	4100
4th	$CH_2–C\equiv C–CH_2–N(CH_2)_4$	131
5th	$CH_2–C\equiv C–CH_2–$(N12)cyt [b]	25
	$(CH_2)_3–$(N12)cyt	30
	$(CH_2)_2–$(N12)cyt	96
	$(CH_2)_6–$(N12)cyt	313
	$(CH_2)_{12}–$(N12)cyt	1040
	$(CH_2)_{16}–$(N12)cyt	1093
	$(CH_2)_{10}–$(N12)cyt	1230
6th	2-Cl-pyrid-6-yl	740
	2-Cl-pyrazin-6-yl	840
	3-Cl-pyridazin-6-yl	4815
7th	$CS–NH–C_6H_{11}$	2300
	$COOC_2H_5$	4600
8th	H; 3-nitrocytisine	1.2
	H; 5-nitrocytisine	328
	H; 3,5-dibromocytisine	200
	H; tetrahydrocytisine	138

[a] IC_{50}.
[b] cyt, second cytisine unit.

though the steric, more than electrostatic interactions, seem to modulate the binding of cytisine derivatives to nAChRs, as resulted from a comparative molecular field analysis (CoMFA) performed by Nicolotti at al. [47].

The good affinity of *N*-pentylcytisine ($K_i = 43.5$ nM) suggests that the alkyl substituent, even if containing five carbon atoms, does not represent a significant obstacle to the interaction of the tertiary amino group with the receptor. In other words, near to the nitrogen binding site, the receptor should contain a pocket able to receive a even large moiety which could eventually contribute to the binding through hydrophobic bonds. This consideration could also justify the higher affinity of *N*-prenylcytisine ($K_i = 120$ nM) respect to *N*-allylcytisine ($K_i = 200$ nM).

On the other hand, when the aliphatic chain possesses a branching very close to the nitrogen, an overwhelming hindrance to its interaction (perhaps more nucleophilic than ionic) with the receptor will be encountered. Indeed, *N*-isopropylcytisine ($K_i = 2270$ nM) while easily salifiable, meets great difficulties to form quaternary ammonium salts, behaving as a Hünig base.

Anyway several cases should be pointed out, for which the foregoing arguments do not apply consistently and deserve further investigation.

Thus the non basic *N*-nitrosocytisine ($pK_a = 3.87$) exhibited a good affinity ($K_i = 39$ nM), while the *N*-hydroxycytisine, whose basicity is comparable to that of pyridine ($pK_a = 5.51$) showed only a weak affinity ($K_i = 423$ nM). The possible existence of a amphionic hybrid bearing a positive charge on cytisine nitrogen and the corresponding negative one on the oxygen of nitroso group, might explain only in part the observed affinity.

On the other hand cytisin-12-yl-acetamide, which is basic and bearing on the basic nitrogen a rather small substituent, exhibited a very poor affinity ($IC_{50} > 10$ μM), even worse than that of *N*-ethoxycarbonylcytisine ($K_i = 4600$ nM) completely devoid of basicity ($pK_a = -1.20$).

Even more, the *N*-(cyclohexylaminothiocarbonyl)-cytisine, which is non basic ($pK_a = 1.26$) and bearing a rather bulky substituent, showed a better affinity ($K_i = 2300$ nM) than that of the cited *N*-ethoxycarbonylcytisine. It is possible that the substituent bulkiness is not so important if some binding with accessory sites take place through the additional chemical functionalities present on the substituent itself.

The *N*-(cyclohexylaminothiocarbonyl)cytisine supplements the *N*-(arylaminothiocarbonyl)cytisines formerly studied [37] as respiratory analeptics: the stimulation of breathing is the results of a typical activity of nicotinic agonists on peripheral chemoreceptors and successively on medulla oblongata.

N-ethoxycarbonylcytisine, together with *n*-butoxy, *tert*-butoxy and adamantyloxycarbonylcytisine, were designed as possible prodrugs. The lack of nitrogen basicity and the increasing bulkiness of the *N*-substituents should hinder or totally prevent the direct interaction of these ligands with the peripheral receptors, while the simultaneously increasing lipophilicity should improve their crossing of the blood brain barrier, beyond which a even slow hydrolysis of the hindered ester function should take place. In this way an indirect selectivity of cytisine for central versus peripheral receptors would be gained.

The observed low, but not negligible, affinity displayed by the *N*-ethoxycarbonylcytisine was somewhat surprising and conflicting with our initial assumptions. Still more surprising was the affinity shown by the very cumbersome *N*-(adamantyloxycarbonyl)cytisine (**69**), which was able to displace [^{3}H]epibatidine from brain $\alpha_4\beta_2$ containing subtype with a $K_i = 2370$ nM [46]. The same compound displaced [^{3}H]epibatidine from α_3-containing subtype of rat superior cervical ganglia with $K_i = 247$ μM (Fig. 12).

Such a large difference suggests that the need for a cationic head in ligands for $\alpha_4\beta_2$-containing subtype is not so stringent as for other nicotinic receptor subtypes (α_3-containing) and a somehow polarized group (as an amidic one) could be sufficient for binding.

Moreover the existence of a bulk tolerating region should further distinguish central $\alpha_4\beta_2$ from peripheral α_3-containing receptor subtype allowing for compound **69** a two order of magnitude selectivity for central versus gangliar nicotinic receptor, apart from the eventual in vivo release of cytisine beyond the BBB.

A still higher selectivity was observed in the case of *N*-pentylcytisine with an affinity for α_3-containing subtype 200 times lower than for $\alpha_4\beta_2$ subtype [46].

Such supposed 'bulk tolerating region' seems to possess dimensional limits not allowing to receive substituents even not too big but *rigidly* bound to the cytisine backbone. Indeed the alkaloid *anagyrine*, which could be considered a cytisine derivative through a cyclic substitution involving position 11 besides the nitrogen atom, exhibited a $IC_{50} = 2096$ μM, that is 15 000 fold that found for cytisine (0.14 μM) in the same experimental conditions (porcine cerebral cortex preparation labeled with [^{3}H]nicotine) [54].

(b) A particular comment is deserved by the set of compounds possessing two cytisine units joined through an aliphatic or arylaliphatic moiety of increasing length. These compounds were designed with the aim of producing selective ligands for nicotinic receptor subtypes taking advantage of their structural diversity, though not always well defined.

It is well known that the elongation of the carbon atom chain joining two ammonium heads can shift the selective blockade from ganglionic (hexamethonium) to muscular (decamethonium) nicotinic receptors. Similarly some alkanbiguanidinium compounds are claimed

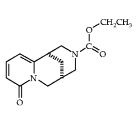
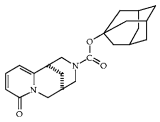
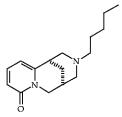
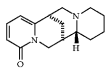
Binding assays conditions:					
• rat brain preparation labeled with [^{3}H]cytisine	4,6	nt	0,0435	0,0024	nt
• $\alpha_4\beta_2$ subtype (rat brain) labeled with [^{3}H]epibatidine	nt	2,37	0,0784	0,0015	nt
• α_3 containing subtypes (rat superior cervical ganglia) labeled with [^{3}H]epibatidine	nt	247	15,6	0,081	nt
• porcine brain preparation labeled with [^{3}H]nicotine	nt	nt	nt	0,140 [a]	2096 [a]

Fig. 12. Binding assays results, obtained in different experimental conditions [45–48,54]: K_i (μM); $a = IC_{50}$ (μM); nt, not tested.

as able to recognize selective subtypes of neuronal nAChRs [55].

The presence of two cytisine units in the same molecule could allow additional interactions with accessory binding sites or with the NCI site or, yet, could limit or prevent the ligand entrance into the binding pockets when the two units are too close each other.

In the case of a very long connecting chain (around 30 carbon atoms), the two cytisine units could involve simultaneously the binding pockets of the two α subunits of the same homo or heteropentameric receptor. In the last case the distance, center to center, is of about 42 Å [56]. The simultaneous occupancy of the two binding pockets is required for the opening of the ion channel.

The binding data so far available (Table 1), indicate that compounds with a connecting moiety of 3–4 carbon atoms display a good affinity ($K_i = 25–30$ nM), while those with two or six carbon atoms display a reduced affinity, with $K_i = 96$ and 313 nM, respectively. The further chain lengthening (from 10 to 16 carbon atoms) strongly decreased the affinity ($K_i = 1040–1230$ nM). Attempts to prepare compounds with 24 and 32 methylenes between the cytisine units are in progress.

The lower affinity of 1-(cytisin-12-yl)-4-(pyrrolidin-1-yl)-2-butyne ($K_i = 131$ nM) respect to 1,4-bis(cytisin-12-yl)-2-butyne ($K_i = 25$ nM) supports the assumption that the second cytisine unit could play a role in the binding to the receptor exceeding that of an additional basic head.

Functional studies, still in progress, and concerning so far only eight compounds [46], indicate that the introduction of substituents (alkyls, arylalkyls, heteroaryls, acyls) on cytisine secondary amino group, gives always antagonists or very weak partial agonists.

(c) Interesting results were obtained with compounds derived from the modification of the pyridone moiety of cytisine molecule.

The introduction of a nitro group in position 3 (close to the carbonyl group) enhanced the affinity for nAChRs ($K_i = 1.2$ nM), probably by extending the molecular portion able to accept a hydrogen bond. On the contrary the introduction of the same group in position five strongly reduced the affinity ($K_i = 328$ nM).

Such a two order of magnitude reduction of affinity might be related to the exceeding of receptor boundaries, which could be very exacting. One cannot exclude a competition between the carbonyl and the 5-nitrogroup with the receptor hydrogen bond site, which will impose an uncorrect collocation of the basic nitrogen in respect to the corresponding binding site.

Similar effects have been observed by other authors [43] for 3- and 5-bromocytisine.

In the 3,5-dibromocytisine the affinity enhancing effect of 3-substitution counterbalanced only partially the deleterious effect of 5-substitution, with a resulting $K_i = 200$ nM.

The other modification that was carried out on pyridone ring was the saturation of the two conjugated double bonds; the obtained *tetrahydrocytisine* exhibited a reduced but still good affinity for nAChRs ($K_i = 138$ nM). This result supports further the importance generally attached to the carbonyl group as a pivotal pharmacophore feature for high affinity to nAChRs.

Correspondingly, tetrahydrodesoxycytisine should exhibit a very poor affinity; the binding assays with the last compound, that is in preparation, will definitely prove this assumption.

The importance of carbonyl group is supported also by the strong differences of affinity which were observed

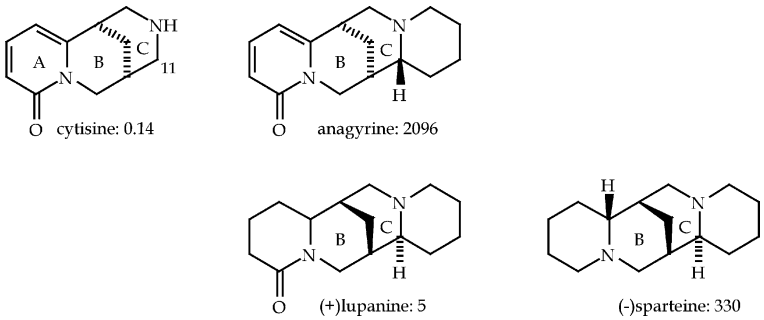


Fig. 13. Displacement of [^{3}H]nicotine from porcine brain preparations [54]: IC_{50} (μM). Effect of substitution on position 11 and of rings B/C configuration.

for *lupanine* and *sparteine* [54], though measured in different experimental conditions (porcine brain preparation labeled with [^{3}H]nicotine, instead of rat brain preparation labeled with [^{3}H]cytisine). The two alkaloids (Fig. 13), which possess the same configuration and differ only for the presence of a carbonyl in the former, exhibited $IC_{50} = 5$ and 330 μM, respectively.

By comparing the IC_{50} values of lupanine (5 μM) and the previously mentioned anagyrine (2096 μM), it is observed that the *correct configuration* of the whole molecule is of prevailing importance respect to the presence of pyridone nucleus and basic nitrogen even in the right distance conforming to the pharmacophore models. Thus one can anticipate that a synthetic cytisine, having the lupanine configuration of rings B and C, should be a better ligand than the natural (−)cytisine.

3. Conclusions

The study of cytisine derivatives, though in a initial phase, is disclosing interesting perspectives for developing new ligands for nAChRs and also for obtaining compounds endowed with different pharmacological activities not related to the interactions with these receptors.

The introduction of substituents in position 3 of pyridone nucleus further enhances the high affinity of cytisine, while the introduction of substituents on the basic nitrogen, though reducing in different degrees the affinity, gives rise to compounds with a higher selectivity for central ($\alpha_4\beta_2$) versus peripheral (α_3-containing) receptor subtypes.

On the other hand the analgesic, antihypertensive and inotropic activities found in some *N*-substituted cytisines represent an attractive starting point for the development of more active compounds.

The recent total syntheses of (±)cytisine effected by scientists of Pfizer Inc. [57,58] is certainly a prelude to intensive investigations in this field. Such syntheses allow an easier access to the alkaloid for preparing new *N*- or *C*-substituted derivatives, but also make possible the design of novel molecular templates to obtain cytisine analogues.

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