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Synthesis and preliminary pharmacological evaluation of some cytisine derivatives

Caterina Canu Boido, Fabio Sparatore *

Dipartimento di Scienze Farmaceutiche, Università di Genova, Viale Benedetto XV, 3-16132 Genoa, Italy

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Abstract

Thirty-one *N*-derivatives of cytisine were prepared in order to modify its pharmacological profile and to obtain compounds of potential therapeutic interest either at a peripheral or central level, particularly as nicotinic ligands with improved ability to cross the blood–brain barrier. Actually, with the introduction of different kinds of substituent on the basic nitrogen of cytisine a variety of activities were observed, both in vivo (analgesic, dopamine antagonism, antihypertensive, inhibition of stress-induced ulcers, antiinflammatory, protection from PAF-induced mortality, hypoglycemic) and in vitro (positive cardio–inotropic, β-adrenergic antagonism, α_1- and α_2-antagonism, inhibition of PAF-induced platelet aggregation). Six randomly selected compounds were tested for the ability to recognize a central nicotinic receptor and four of them exhibited K_i values in the range 30–163 nM.

Keywords: Cytisine derivatives; Quinolizidine derivatives; Nicotine receptors; Cardiovascular agents

1. Introduction

Pursuing our systematic research on the structural modification of quinolizidine alkaloids, in order to obtain compounds of pharmacological interest, we devoted our attention to cytisine present in several plants of the *Leguminosae* family (Cytisus, Sophora, Ulex, Baptisia, Genista and others).

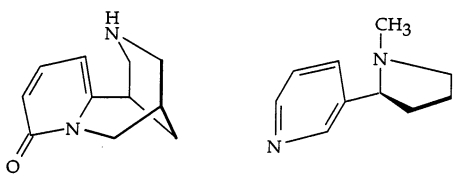
From a pharmacological point of view, cytisine strictly resembles nicotine (Fig. 1): it acts mainly at ganglionic level, exhibiting more stimulating than blocking effects; however, central effects are also shown. These two alkaloids are almost indistinguishable as far as peripheral effect is concerned but they differ somewhat for the central ones (kind of convulsions), which are related to cholinergic stimulation [1,2].

Such a pharmacological profile accounts for the present lack of therapeutic use of cytisine in western countries (in the past it was used as diuretic [3]), while in the former Soviet Union it is used as a respiratory analeptic for its stimulating activity on respiratory centers and on chemoreceptors in the aortic and carotid bodies [4]. Cytisine, at a dose of 1 mg e.v., is preferable to the widely used lobeline [5] by Russian pharmacologists.

Recent Japanese patents suggested hypoglycemic and antiinflammatory activities for cytisine and its *N*-methyl derivative [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 any other tested drug (nicotine, acetylcholine, carbachol as agonists and dihydro-β-erythroidine as antagonists).

Central nicotinic receptors are presently considered as a possible target for the development of drugs useful in cognitive and attention disorders, Alzheimer's disease and other CNS disfunctions [10], and several natu-



(-) 7R,9S-cytisine (-) S-nicotine

Fig. 1.

* Corresponding author. Fax +39-010-353 8358.
E-mail address: sparator@unige.it (F. Sparatore)

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ral compounds (anabaseine, arecoline, lobeline, anatoxine-a, *epi*-batidine) able to activate them, were added to nicotine and cytisine.

Many years ago Bovet and his collaborators [11] demonstrated that suitable doses of nicotine favored learning and memory in rats. More recently, beneficial effects of nicotine in both Alzheimer's and Parkinson's disease were asserted [12]. Lobeline also exhibited good activity in pharmacological models for learning and memory [13], and for analgesia [14], supporting that nicotinic receptors play a modulatory role in these processes.

The foregoing considerations justify the present research aimed at the structural modification of cytisine in order to obtain compounds of potential therapeutic interest either at peripheral or central levels with a particular concern for degenerative pathologies (Alzheimer's and related diseases).

The structural modifications of cytisine should increase, on the one hand, its passage through the blood–brain barrier (strongly limited by its relevant hydrophilic character), as well as reducing its affinity for ganglionic receptors. However, the selectivity for central nicotinic receptorial subtypes [10,15] should also be increased, eventually to the detriment of the potency.

Structural modification of cytisine was already attempted in the past, but was mainly aimed at improving its respiratory analeptic property [16,17] or obtaining local anesthetics [18].

The chemical modifications that can be operated on cytisine apply, either singularly or in succession, to secondary amino groups, conjugated double bonds and carbonyl groups; however, in order to maintain the central nicotinic activity, the cationic character should be secured, leaving the typical distance of the basic nitrogen from the carbonyl dipole unchanged, which must form a hydrogen bond with a donating group of the receptor [2,19,20].

In the present work, as a first possibility, the introduction of saturated or unsaturated alkyl or arylalkyl residues on the amino group was undertaken in order to increase the overall lipophilicity, but the introduction of more complex moieties to enable reinforcement or selection of the activities of cytisine was also considered.

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

In this study, cytisine displayed affinity for a number of receptors (cholecystokinin A, histamine H_3, kainate, muscarinic M_1 and M_2, *N*-methyl-D-aspartic acid NMDA, phencyclidine, serotonin $5HT_3$, thyrotropin-releasing hormone TRH, vasoactive intestinal polypeptide VIP) but the IC_{50} was always in the range 10^{-4}–10^{-5} M; thus, the very high affinity of cytisine for nicotinic receptors appears still more outstanding and quite peculiar.

On the whole, 31 *N*-substituted derivatives of cytisine were prepared (Fig. 2).

Of these, compounds **1**–**3** were intermediates for the preparation of other derivatives or could be of interest as nicotinic ligands. The ketone **3** corresponds to the alkaloid extracted from *Echinosophora koreensis* [21].

In the alkyl and arylalkyl derivatives **4**–**12** the steric hindrance around the cationic center and the lipophilicity were largely varied so that their influence on the affinity for nicotinic receptors in vitro and on the distribution pattern in vivo could be evaluated.

The arylalkyl derivatives could also be interesting as cardiovascular agents having some structural similarity with the aryl- and benzyl-sparteins prepared by Boido et al. [22] and found endowed with positive inotropic activity.

The presence of a phenylethanolamine or phenylethanonamine moiety in compounds **13** and **14** could give rise to some interaction with adrenergic receptors, influencing the sympathomimetic component of cytisine activity due to the stimulated release of adrenaline from adrenal medulla.

Compound **15** is structurally related to the classic butyrophenone antipsychotics, but still more to the recent derivatives having as basic head a bridged γ-carboline moiety characterized by simultaneous affinity for D_2 and $5HT_2$ receptors [23]. On the other hand, *p*-fluorobutyrophenone derivatives could also be of interest as HIV-1 protease inhibitors [24,25].

Compounds **16** and **17** represent examples of a set of compounds possessing two cytisine units connected through a polymethylene chain of growing length, which could generate selective ligands for nicotine receptors subtypes. 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, tubocurarine) nicotinic receptors. Moreover, alkanbiguanidinium compounds were described recently as being able to recognize selective nicotinic receptor subtypes [26].

Compound **16** and its homologs (where $n = 5$, 6 and 10) were already described [27], but, to the best of our knowledge, their biological activity was not described.

The aryl/heteroaryl–piperazinyl alkyl derivatives **18**–**24** and also, to some extent, compound **25** fall within the multitude of aryl piperazinyl derivatives that, depending on the nature of the aryl residue and of the moiety attached to the other piperazine nitrogens, can interact with serotonin and/or α_1-adrenergic receptors. Particularly, they closely resemble the 2-[4-arylpiperazinyl-1-alkyl]-perhydroimidazo[1,5-*a*]pyridines [28], which exhibited high affinity and selectivity for 5-HT_{1A}- versus α_1-receptor.

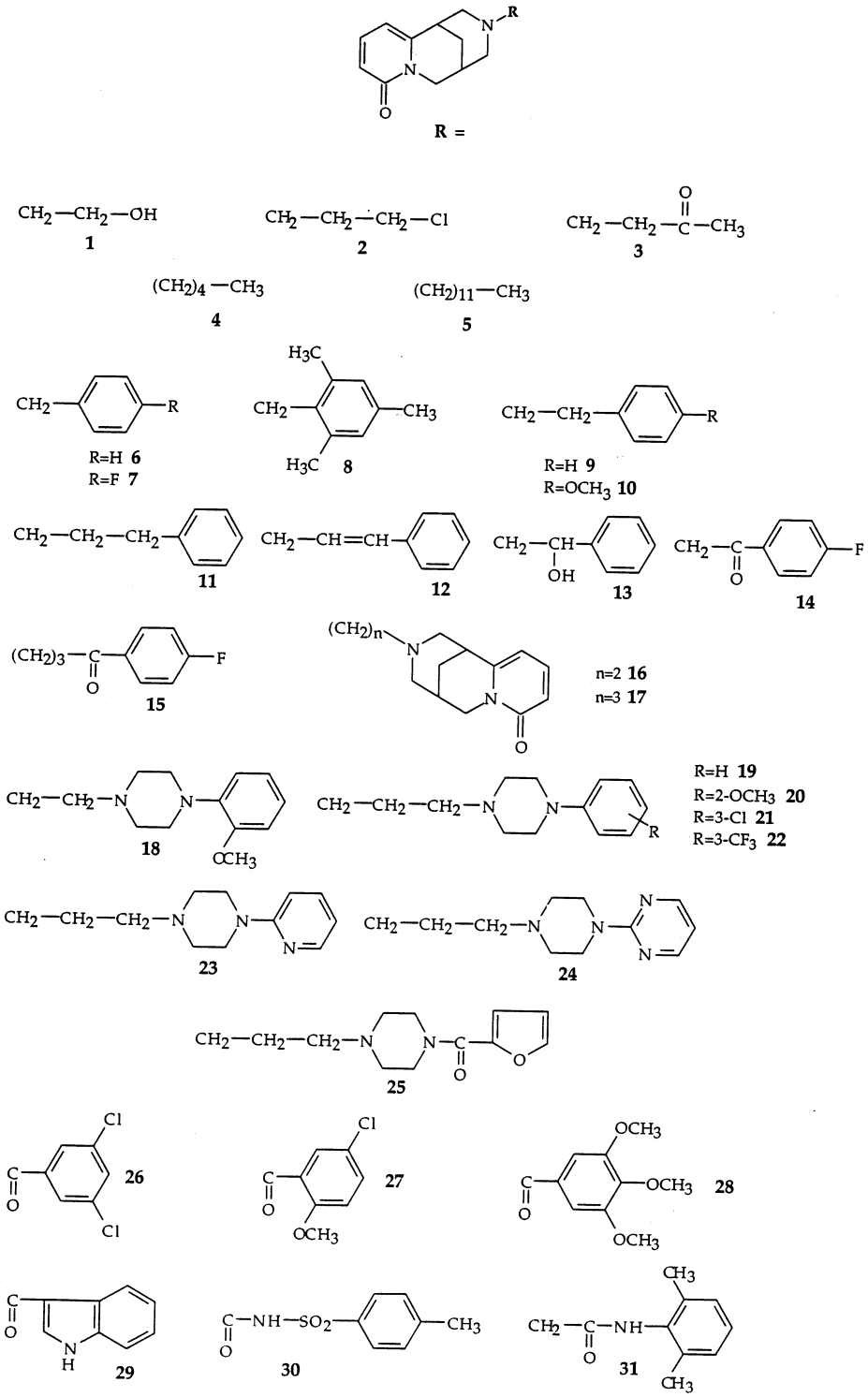

Fig. 2.

The aroyl derivatives **26**–**29** supplement the amido derivatives formerly studied as respiratory analeptics [16,17], but the acyl residues now utilized are commonly encountered among $5HT_3$ and $5HT_4$ agonists and antagonists, and also among sedative agents (trimetozine).

The superimposition of the terminal nitrogen of *p*-toluenesulphonylurea with the imino group of cytisine (**30**) could represent a symbiotic approach to enhance the hypoglycemic activity of cytisine.

Finally, compound **31** was also the result of a symbiotic approach to design an antiarrhythmic agent, taking into account that lidocaine and *N*,*N*′-disubstituted bispidines [29] share such activity.

Compound **31** is the 2,6-dimethylanilide of 12-cytisineacetic acid, an unusual amphoteric alkaloid isolated from *Euchresta japonica* [30].

2. Chemistry

For the preparation of compounds **1**, **2**, **4**–**12**, **14**–**18** and **31**, cytisine was reacted with the suitable halo derivatives; however, due to steric hindrance around the imino group, a thorough selection of experimental conditions is often very important for a successful alkylation.

N-(3-Chloropropyl)cytisine (**2**) was obtained without any particular problem, but all attempts to prepare *N*-(2-chloroethyl)cytisine by reacting cytisine with 1-bromo-2-chloroethane gave always the 1,2-bis-cytisinylethane (**16**). Therefore, compound **18** was obtained by reacting cytisine with 1-(2-chloroethyl)-4-(2-methoxyphenyl)piperazine.

For the synthesis of the piperazine derivatives **19**–**25**, the suitable aryl- or heteroaryl-piperazines were reacted with *N*-(3-chloropropyl)cytisine (**2**). All but one of the required piperazines were commercially available; *N*-furoylpiperazine was prepared as described in Ref. [31].

Compounds **3** and **13** were obtained by treating cytisine in dioxane with methylvinylketone and styrene oxide, respectively.

Finally, for the preparation of amides **26**–**30**, cytisine was treated with the suitable aroyl chlorides in acetone, or with *p*-toluenesulfonyl isocyanate in toluene.

The structures of the prepared compounds were supported from elemental analyses and spectral data.

The IR spectra of ketones **3**, **14**, **15** and amides **25**–**31** exhibited the expected peak for the carbonyl group, clearly separated from or as partially superimposed with that of α-pyridone ring occurring at 1650 cm^{-1} [2].

The NMR spectra did not exhibit any unusual features; thus, only the spectra of compounds **3** and **18** are described as examples (see Section 3).

3. Experimental

Melting points were determined by the capillary method (often sealed under vacuum) on a Büchi apparatus and are uncorrected.

The elemental analyses were performed at the Microanalytical Laboratory of the 'Dipartimento di Scienze Farmaceutiche' of Genoa University and the analytical results for the indicated elements were within $\pm 0.3\%$ of the calculated values.

IR spectra were recorded with a Perkin–Elmer Paragon 1000 PC spectrophotometer. ^1H NMR spectra were taken on a Varian Gemini 200 spectrometer, using $CDCl_3$ or DMSO-d_6 as solvent with TMS as internal standard.

In the NMR spectra, signals of the cytisine moiety protons are indicated as 'pyr' if pertinent to the α-pyridone ring and as 'bisp' if pertinent to the bispidine portion of the molecule.

*3.1. General method for the alkylation of cytisine (**1**, **2**, **4**–**12**, **14**, **31**)*

Cytisine (0.5–1 g, 2.63–5.26 mmol) was dissolved in dry acetone (10–20 ml) and the suitable aliphatic or arylaliphatic halide (1.32–2.63 mmol, ratio 2:1) was added. For the preparation of compound **2** a cytisine: 1-bromo-3-chloropropane ratio of 1:1 was used. The mixture was refluxed for a time varying from 1 to 4 h. In some cases, longer heating times were required (up to 20 h for 1-bromo-3-chloropropane); however, even after 36 h, the alkylation of cytisine with ethylene chlorohydrin was far from completion.

The alkylation progression was monitored through TLC on alumina using dry ether + 2% methanol as eluent.

After cooling, the cytisine hydrohalide was filtered and acetone was removed under reduced pressure. The residue, when taken up with ether, may crystallize; hence, it was partitioned between ether and acidic water. The acidic solution was basified and extracted with ether. This method was used to prepare compounds **1**, **2**, **4**–**9**, **11**, **12** and **31**. Generally, the crude products were crystallized from the solvents indicated in Table 1. In the case of compound **31**, purification was achieved through chromatography on alumina, eluting with ether + 2% methanol. By replacing acetone with ethylene glycol monomethylether, the method was also used to prepare compounds **10** and **14**.

*3.2. N-(2-Hydroxyethyl)cytisine (**1**)*

Since the yield of the cytisine alkylation with ethylene chlorohydrin was rather poor, the title compound was also prepared by the method reported by Ing and Patel [18]. Cytisine dissolved in chloroform was treated with ethylene oxide and heated for 4 h at 45°C in a closed tube. After removing the solvent, the residue was taken up with a little dry ether and formed crystals which melted at 83–85°C and corresponded to the hydrated *N*-(2-hydroxyethyl)cytisine $C_{13}H_{18}N_2O_2 \cdot H_2O$. Ing and Patel reported a m.p. of 73–74°C. The anhydrous compound was a glassy solid.

*3.3. N-(3-Oxobutyl)cytisine (**3**)*

Cytisine (2 g, 10.5 mmol) was dissolved in 7 ml of warm anhydrous dioxane and treated with 0.95 ml (11.4 mmol) of freshly-distilled methylvinylketone. The mix-

Table 1
Molecular formulae, melting points, recrystallization solvents and yields for compounds **1–31**

Comp.	Formula	M.p. (°C)	Solvent	Yield (%) [a]
1	$C_{13}H_{18}N_2O$ [b]	83–85	dry ether	75
2	$C_{14}H_{19}ClN_2O{\cdot}0.75H_2O$ [c]	oil		48
3	$C_{15}H_{20}N_2O_2$	115–118	dry ether	90
4	$C_{16}H_{24}N_2O{\cdot}HCl{\cdot}0.5H_2O$	250–260	isopropanol/ether	76
5	$C_{23}H_{38}N_2O{\cdot}HCl{\cdot}H_2O$ [d]	275–277	isopropanol/ether	57.3
6	$C_{18}H_{20}N_2O{\cdot}0.25H_2O$	142–143	dry ether	77.6
7	$C_{18}H_{19}FN_2O$	154–155	dry ether (washed)	46.4
8	$C_{21}H_{26}N_2O$	115–117	dry ether	47.5
9	$C_{19}H_{22}N_2O{\cdot}HCl{\cdot}0.5H_2O$	>250	absolute ethanol/dry ether	54.5
10	$C_{20}H_{24}N_2O_2$	100–101	dry ether	25
11	$C_{20}H_{24}N_2O{\cdot}HCl{\cdot}0.5H_2O$	>250	ethanol/ether	80
12	$C_{20}H_{22}N_2O$	86–87	ether	59
13	$C_{19}H_{22}N_2O_2$	144–146	ethanol	47.2
14	$C_{19}H_{19}FN_2O_2$	146–149	acetone	26
15	$C_{21}H_{23}FN_2O_2{\cdot}HCl{\cdot}1.5H_2O$	179–183	dry ether (washed)	28
16	$C_{24}H_{30}N_4O_2{\cdot}0.25H_2O$	182–185	acetone	68
17	$C_{25}H_{32}N_4O_2{\cdot}0.5H_2O$	138–141	ethyl acetate	22
18	$C_{24}H_{32}N_4O_2{\cdot}0.25H_2O$	153–154	dry ether	80
19	$C_{24}H_{32}N_4O$	oil	chromatography (dry ether+methanol)	53
20	$C_{25}H_{34}N_4O_2{\cdot}2HCl$	240	dry ether (washed)	56
21	$C_{24}H_{31}ClN_4O{\cdot}0.5H_2O$	oil	chromatography (ether)	45
22	$C_{25}H_{31}F_3N_4O{\cdot}H_2O$	oil	chromatography (dry ether+methanol)	26
23	$C_{23}H_{31}N_5O{\cdot}0.25H_2O$	oil	chromatography (dry ether+methanol)	52
24	$C_{22}H_{30}N_6O{\cdot}0.25H_2O$	oil	chromatography (dry ether+methanol)	30
25	$C_{23}H_{30}N_4O_3{\cdot}2HCl{\cdot}0.5H_2O$	>280	dry ether (washed)	58
26	$C_{18}H_{16}Cl_2N_2O_2$	212–213	ethanol	96
27	$C_{19}H_{10}ClN_2O_3{\cdot}0.25H_2O$	136–140	ethanol	54
28	$C_{21}H_{24}N_2O_5$	185–186	ethanol/dry ether	50
29	$C_{20}H_{19}N_3O_2$	290–292	ethanol	75
30	$C_{19}H_{21}N_3O_4S$	267	ethanol	34
31	$C_{21}H_{25}N_3O_2$	120–122	chromatography (dry ether+methanol)	26

[a] The yields are referred always to the free bases; the eventual conversion to the hydrochlorides gave yields superior to 90%.
[b] Ref. [18].
[c] Ref. [21].
[d] M.p. of free base: 40°C.

ture was stirred in a closed tube for 3 h at room temperature (r.t.) and then for 1 h at 60°C. After cooling, dry ether was added and the separated crystals were collected after standing in the refrigerator for some time.

^{1}H NMR [200 MHz, δ ppm, $CDCl_3$]: 7.24 (dd, $J = 9.1$ Hz, 6.9 Hz, 1H pyr), 6.38 (dd, $J = 9.1$ Hz, 1.4 Hz, 1H pyr), 5.93 (dd, $J = 6.8$ Hz, 1.3 Hz, 1H pyr), 3.96 (d, $J = 15.4$ Hz, 1H bisp), 3.81 (dd, $J = 15.4$ Hz, 6.4 Hz, 1H bisp), 2.9–2.8 (m, 3H bisp), 2.49–2.23 (m, 7H: 3H bisp + 2H N–*CH_2*–CH_2– + 2H CH_2–*CH_2*–CO), 1.91 (s, 3H, CH_3–CO), 1.9–1.7 (m, 2H bisp).

3.4. *N-[(2-Hydroxy-2-phenyl)ethyl]cytisine* (**13**)

Cytisine (1 g, 5.26 mmol) was dissolved in 7.5 ml of anhydrous dioxane; styrene oxide (0.7 ml, 6.14 mmol) was added and the mixture was refluxed for 6 h and then heated at 130–150°C for 12 h.

Dioxane was removed under reduced pressure and the residue was dissolved in a little warm ethanol. After cooling, some crystals were collected and the ethanol solution was added of dry ether that produced a further precipitation of the compound. The combined product was crystallized from ethanol to give 0.77 g of pure compound.

3.5. *N-{[4-(4-Fluorophenyl)-4-oxo]butyl}cytisine* (**15**)

To a solution of freshly distilled ω-chloro-4-fluoro butyrophenone (1.2 g, 6 mmol) in 10 ml of ethylene glycol monomethylether, 2.3 g (12.1 mmol) of cytisine dissolved in 10 ml of the same solvent were added. The mixture was refluxed for 7 h under nitrogen and then stirred overnight at r.t.

Cytisine hydrochloride was filtered and the solvent was removed under reduced pressure. The residue was partitioned between ether and acidic water. The acid solution was made alkaline and extracted with ether to

yield the crude compound **15**. Further extraction with dichloromethane allowed the recovery of unreacted cytisine.

The crude free base was purified by converting it into the hydrochloride.

3.6. 1,2-bis(N-Cytisinyl)ethane (16)

Cytisine (1 g, 5.26 mmol) was dissolved in dry acetone and added to 0.22 ml (2.6 mmol) of 1-bromo-2-chloroethane. The mixture was refluxed for 8 h and the solvent removed. The oily residue was treated with dilute sodium hydroxide solution and extracted with dichloromethane. After evaporation of the solvent, a solid was obtained that crystallized from acetone.

3.7. 1,3-bis(N-Cytisinyl)propane (17)

Cytisine (0.57 g, 3 mmol) was dissolved in 10 ml of diglyme and added to 0.4 g (1.5 mmol) of *N*-(3-chloropropyl)cytisine dissolved in a few milliliters of the same solvent. The solution was refluxed for 48 h. After cooling, the precipitate was filtered and the solvent was removed under reduced pressure. The oily residue was rinsed several times with dry ether to leave 0.14 (21.2% yield) of crystals that were further purified from ethyl acetate.

3.8. N-{2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl}-cytisine (18)

To a solution of 1-(2-chloroethyl)-4-(2-methoxyphenyl)piperazine (0.45 g, 1.76 mmol) in 15 ml of acetonitrile, 0.48 g of cytisine and 0.21 g of sodium hydrogen carbonate (2.52 mmol each) were added. The mixture was refluxed with stirring for 12 h. The inorganic material was filtered and the solvent removed. The residue was taken up with water and extracted with dichloromethane. The oily compound was chromatographed on silica gel (30 g) eluting with a mixture of dichloromethane:methanol (9:1), yielding 0.58 g of oil that crystallized from dry ether.

^{1}H NMR [200 MHz, δ ppm, $CDCl_3$]: 7.25 (dd, $J = 9.0$ Hz, 6.8 Hz, 1H, pyr), 7.1–6.8 (m, 4H, arom), 6.41 (dd, $J = 9.0$ Hz, 1.4 Hz, 1H, pyr), 5.96 (dd, $J = 6.8$ Hz, 1.3 Hz, 1H pyr), 4.05 (d, $J = 15.4$ Hz, 1H bisp), 3.90 (dd, $J = 15.4$ Hz, 6.5 Hz, 1H bisp), 3.86 (s, 3H, OCH_3), 3.05–2.87 (m, 7H: 5H bisp and 2H N–CH_2), 2.7–2.2 (m, 11H: 1H bisp and 10H –CH_2–N(CH_2–CH_2)$_2$–N–), 1.9–1.6 (m, 2H bisp).

3.9. N-[3-(4-Arylpiperazin-1-yl)propyl]cytisine (19–23)

To a solution of *N*-(3-chloropropyl)cytisine (**2**) (1–3 mmol) in 10–20 ml of toluene (for **19**, **20** and **22**) or acetone (for **21** and **23**), the suitable *N*-aryl-piperazine (2–6 mmol) was added and the mixture was generally refluxed for about 20 h, monitoring the reaction progression through TLC. For the preparation of compound **23**, 7 h of heating were sufficient, while for compound **19** the reflux was carried out for 32 h.

After cooling, the aryl-piperazine hydrochloride was filtered and the solvent was removed under reduced pressure; after taking up the residue with dry ether some more insoluble material was separated. The ether solution was concentrated and chromatographed on alumina ($\sim$20 g) eluting with dry ether or dry ether plus increasing amounts of methanol (0.5–2%).

3.10. N-{3-[4-(Pyrimidin-2-yl)piperazin-1-yl]propyl}-cytisine and N-[3-(4-furoyl piperazin-1-yl)propyl]-cytisine (24, 25)

To a solution of *N*-(3-chloropropyl)cytisine (**2**) (1–2 mmol) in 10–20 ml of acetonitrile, an equivalent amount plus a 20–30% excess of the *N*-(pyrimidin-2-yl)piperazine or *N*-furoylpiperazine [31], together with a large excess (2–6 mmol) of anhydrous sodium carbonate, were added. The mixture was refluxed for 48 and 7 h for **24** and **25**, respectively. The insoluble material was filtered and the solvent was removed. The residue was taken up with water and extracted with chloroform.

The solvent was evaporated and the residue was chromatographed on alumina, eluting with ether + 2% methanol (**24**) or dichloromethane + 1% methanol (**25**). The oily compound was analyzed either as such or after conversion to the dihydrochloride (**25**).

3.11. N-Aroylcytisines (26–29)

Cytisine (4–10 mmol) was dissolved in dry acetone (10–20 ml); an equivalent amount plus 20% excess of triethylamine was added, followed by an equivalent amount of the aroyl chloride. For the preparation of the amide **29**, no triethylamine was added, but a mixture of cytisine:aroylchloride (2:1 ratio) was used.

The mixture was refluxed for 1–2 h. After cooling, the triethylamine hydrochloride or the cytisine hydrochloride was filtered and the solvent was removed. The residue was rinsed with water, diluted sodium hydroxide solution and again with water and, finally, crystallized from the suitable solvent (see Table 1).

3.12. N-[N-(p-Toluenesulfonyl)carbamoyl]cytisine (30)

To a solution of cytisine (0.5 g, 2.6 mmol) in 25 ml of anhydrous toluene was dropped a suspension of 0.57 g (2.86 mmol) of *p*-toluenesulfonyl isocyanate in 15 ml of toluene. The mixture was refluxed for 1 h and then

stirred for 12 h at r.t. After removing the solvent, the residue was taken up with water adjusting the pH to around 7 and extracted with dichloromethane. After evaporation of the solvent, 0.8 g of solid were obtained.

4. Pharmacology

The prepared compounds were subjected to a wide pharmacological investigation in order to point out their central and/or peripheral activities. Due to the growing interest on the pharmacological properties of cytisine and other potential nicotine agonists, we deemed it worthy to describe the results so far achieved, though concerning only 23 of the 31 prepared compounds.

Initially, some of the compounds (cytisine plus **3**, **4**, **7**, **12**, **16**, **17**) were tested for their ability to recognize nicotinic receptors, while others (**5**, **9**, **12**, **13**, **15**, **20**, **26**, **29**, **31**) were subjected to a general screening concerning about sixty in vitro and in vivo tests.

On the basis of these results, some other compounds were selected for investigating single biological activities such as antihypertensive (**18**, **19**, **22**–**25**), cardioinotropic (**7**, **8**, **28**), antiinflammatory (**22**), passive cutaneous anaphylaxis (**24**) and hypoglycemic (**30**).

Other compounds are still under study and the corresponding results will be the object of a forthcoming report.

4.1. Materials and methods

Most of the basic compounds were tested as hydrochlorides, while a few (cytisine, **3**, **9**, **13**, **16**, **17**, **31**) were used as free bases, due to their sufficient solubility in water.

For in vivo tests, the substances were generally administered by the oral route using a gastric tube; they were prepared as aqueous solutions or finely homogenized suspensions in 'Tween 80' (2%). In a few cases the substances were introduced intraperitoneally (i.p.), dissolved in water. Groups of three or five animals (mice or rats) were used.

For in vitro assays, it was sometimes necessary to increase the solubility by means of dimethylsulfoxide at a concentration that would not interfere with the tests (0.1% for platelet aggregation and 0.5% for all others).

Doses (mg/kg) or concentration (μg/ml or μM) indicated in the methods were the highest utilized routinely depending on toxicity; when significant activity was detected, lower doses or concentrations were tested in order to define the minimal effective ones, and secondary tests were performed to have some insight for the possible mechanism of action.

The procedures used for most assays were already described [32–37]; the pertinent reference is indicated near each assay name in Tables, which are relative to the more significant ones only.

Procedures not previously described are outlined below.

4.2. α_2-Adrenoceptor antagonism (in vitro) [38,39]

The field stimulated (95% of maximum, 0.2 Hz) prostatic portion of rat vas deferens, bathed in physiological salt solution at 32°C, was used. If no significant response was elicited by the test substance (3 μg/ml), subsequent inhibition of clonidine (2 μg/ml) induced reduction in twitch responses by more than 50% indicated significant activity.

4.3. Central nicotinic acetylcholine receptor binding [9]

Brain cerebral cortices were removed from Wistar rats and a membrane fraction was prepared by standard techniques. Membrane preparation (600 μg) was incubated with [^{3}H]cytisine at a concentration of 2 nM for 75 min at 0°C. Non-specific binding was estimated in the presence of 100 μM nicotine. Membranes were filtered and washed three times with binding buffer and the filters were counted to determine the [^{3}H]cytisine bound. Compounds were screened at 10 μM.

4.4. Cholecystokinin A receptor binding [40]

A membrane preparation was isolated from guinea-pig pancreas using standard techniques. Membrane preparation (0.25 mg) was incubated with 0.2 nM [^{3}H]L-364718 (see Table 2) for 30 min at 37°C. Non-specific binding was estimated in the presence of 0.3 μM L-364718. Membranes were filtered and washed three times and filters were counted to determine the [^{3}H]L-364718 specifically bound. Compounds were screened at 10 μM.

4.5. Vasoactive intestinal peptide receptor binding [41]

Lungs were obtained from male guinea-pigs and a membrane fraction was prepared by standard techniques. Membrane preparation (0.2 mg) was incubated with 0.01 nM [^{125}I]VIP for 30 min at 22°C. Non-specific binding was estimated in the presence of 13.5 μM VIP. Membranes were filtered and washed three times and the filters were counted to determine the [^{125}I]VIP bound. Compounds were screened at 10 μM.

5. Results and discussion

The most significant results of the pharmacological investigations are collected in Tables 2–8; some additional results are mentioned within the following discussion.

Concerning the sought-after affinity for the nicotinic acetylcholine receptors, all the citysine derivatives

Table 2
Cytisine displacement of specific ligands from several receptors

Binding sites [a]	Preparation source	Ligand [b]	% inhibition at 10 μM conc.
Cholecystikinin A	guinea-pig pancreas	[^{3}H]L-364718	22
Histamine H_3	entire rat brain	[^{3}H]NAMH	17
Kainate	entire rat brain except cerebellum	[^{3}H]kainate	19
Muscarinic M_1	rat brain cortices	[^{3}H]pirenzepine	32
Muscarinic M_2	rat heart	[^{3}H]NMS	19
N-Methyl-D-aspartic acid (NMDA)	rat brain cortices	[^{3}H]CGS-19755	33
Phencyclidine	rat brain cortices	[^{3}H]TCP	23
Serotonin $5HT_3$	rabbit ileum muscularis	[^{3}H]GR-65630	18
Thyrotropin-releasing hormone (TRH)	rat brain	[^{3}H](Me) TRH	26
Vasoactive intestinal polypeptide (VIP)	guinea-pig lung	[^{125}I]VIP	18

[a] Cytisine at 10 μM conc. was found inactive for the displacement of the specific ligands from the following binding sites: adenosine A_1 and A_2, angiotensine II, bradykinin, cholecystokinin B, galanin, insulin, interleukin 1α, leukotriene B_4, neurokinin 1, neuropeptide Y, platelet activating factor, phorbol ester, serotonin $5HT_{1A}$, σ, sodium channel, thromboxane A_2, and tumor necrosis factor.

[b] L-364718 = 3-(*S*)-(−)1,3-dihydro-3-(2-indolecarbonylamino)-1-methyl-5-phenyl-2*H*-1[1,4]benzodiazepin-2-one; NAMH = α-methylhistamine; NMS = methylscopolamine; CGS 19755 = *cis*-4-phosphonomethyl-2-piperidine carboxylic acid; TCP = thienylcyclidine; GR-65630 = 1-(1-methylindol-3-yl)-3-(4-methyl-imidazol-5-yl)-1-oxo-propane; MeTRH = methyl thyrotropin-releasing hormone.

tested so far exhibited an affinity quite lower than that of cytisine itself (Table 3), with compound **17** being the most active ($K_i = 30$ nM). However, while the growing bulkiness of the substituents on the basic nitrogen was clearly detrimental for the affinity to the receptor, it is also responsible for a proportional increase in the lipophilicity which should affect favorably the passage of the blood–brain barrier.

Of the nine compounds (**5**, **9**, **12**, **13**, **15**, **20**, **26**, **29** and **31**) subjected to a general pharmacological screening, and therefore tested for toxicity (Table 4), only compounds **9**, **12**, **15** and **29** produced mortality in mice at the dose of 300 mg/kg p.o. or 100 mg/kg i.p. On the whole, with sublethal doses, the autonomic symptoms observed during a 72 h period from drug administration were rather modest.

Concerning the in vivo assays directed at showing any specific activity on the CNS (anticonvulsive, 5-methoxy-*N*,*N*-dimethyltryptamine potentiation, oxotremorine antagonism, tetrabenazine hypothermia antagonism), most of them gave negative results; however, some interesting responses were obtained concerning analgesia, dopamine antagonism and the inhibition of stress-induced ulcers.

N-Cinnamylcytisine (**12**) exhibited strong analgesic activity in the writhing test and against formalin, although only at doses approaching toxic, but the 2-methoxyphenylpiperazinylpropyl derivative **20** was still active as an analgesic at 30 mg/kg, while no toxicity was seen up to a dose of 300 mg/kg p.o. and 100 mg/kg i.p.

Compound **20** was also active in the apomorphine-induced climbing behavior, suggesting dopamine antagonistic activity. The observed response was practically superimposable with that of buspirone.

Taking into account that the arylpiperazinyl moieties, present in compound **20** and in buspirone, are valid templates for $5\text{-}HT_{1A}$ receptor affinity, further investigations are warranted to check the coexistence of affinity for serotonin and dopamine receptors in compound **20** and its analogs **18**–**24**.

On the other hand, the rather weak response of compound **15** in the climbing assay for dopamine antagonism was somewhat surprising, in spite of it being a *p*-fluorobutyrophenone derivative.

All the tested compounds except one (i.e. **31**) inhibited to some extent stress-induced ulcers in mice, suggesting that all were able to cross the blood–brain barrier. Compounds **9** and **12** exhibited significant activity, which was unrelated to anticholinergic activity since tremors, lacrimation and salivation induced by s.c. oxotremorine injection were not inhibited.

Compounds **15** and **20** both inhibited carrageenan-induced rat paw edema without inducing gastric irritation. Of interest, the complete failure to prevent arachidonic acid-induced platelet aggregation suggests that inhibition of the cyclooxygenase is not involved; this biological profile is rarely observed and may, therefore, warrant further investigation.

Table 3
Displacement of [^{3}H]cytisine from central nicotinic acetylcholine binding sites by cytisine and compounds **3**, **4**, **7**, **12**, **16** and **17**

Comp.	IC_{50} (nM)	K_i (nM)
Cytisine	5	3
3	265	163
4	70	43
7	1600	985
12	6700	4100
16	156	96
17	48	30

Table 4
Maximum tolerated dose in mice for compounds **5**, **9**, **12**, **13**, **15**, **20**, **26**, **29** and **31** (n.t. = not tested)

Administration route	Dose (mg/kg)	No. of animals dead (and effects) after treatment with compounds								
		5	**9**	**12**	**13**	**15**	**20**	**26**	**29**	**31**
Oral	300	0/3	2/3	3/3	0/3 irritation; slight increase in touch sensitivity	1/3	1/3	0/3 no effect	0/3 no effect	0/3 no effect
	100	n.t.	1/3	0/3 no effect	n.t.	0/3 no effect	0/3 slight decrease in abdominal tone and limb tone	n.t.	n.t.	n.t.
	30	n.t.	0/3 no effect	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Intraperitoneal	100	0/3 slight decrease in limb tone and grip	3/3	3/3	0/3 slight decrease in limb tone and grip	3/3	0/3 slight tremor and behavioral depression; slight muscular relaxation and ataxia; slight respiratory fastening; vasodilation	0/3 slight decrease in spontaneous activity; slight muscular relaxation and ataxia	0/3 no effect	0/3 slight decrease in abdominal tone and limb tone
	30	no effect	0/3 slight muscular relaxation; slight decrease in limb tone	0/3 slight decrease in limb tone	no effect	0/3 slight decrease in limb tone and grip	slight muscular relaxation; slight respiratory fastening	slight decrease in abdominal tone; slight decrease in limb tone	n.t.	no effect
	10	n.t.	slight decrease in limb tone	no effect	n.t.	no effect	slight decrease in limb tone	no effect	n.t.	n.t.
	3	n.t.	no effect	n.t.	n.t.	n.t.	no effect	n.t.		n.t.

Table 5
Some in vivo activities of compounds **5**, **9**, **12**, **15** and **20** [a,b]

Test [c]	Route; dose (mg/kg)	Effect	Response to compound [d]					Reference drug	Route; dose (mg/kg)	Effect
			5	**9**	**12**	**15**	**20**			
Dopamine antagonist [34] (mice)	i.p.; 30	[e]	0	17	0	17	67	buspirone	i.p.; 25	67
	10		n.t.	n.t.	n.t.	n.t.	17	trifluoroperazine	i.p.; 1	50
Phenyl quinone writhing [33] (mice)	p.o.; 100	[f]	40	n.t.	80	43	100	aspirin	p.o.; 50	68
	30		n.t.	36	20	n.t.	47			
Formalin analgesia [37] (mice)	p.o.; 100	[g]	n.t.	n.t.	100	n.t.	96	aspirin	p.o.; 50	60
	30		n.t.	n.t.	38	n.t.	64			
	10		n.t.	n.t.	n.t.	n.t.	0			
Antiinflammatory [32] (rats)	p.o.; 100	[h]	0	n.t.	n.t.	34	40	aspirin	p.o.; 150	40
	30		n.t.	0	0	8	0	phenylbutazone	p.o.; 50	33
Passive cutaneous anaphylaxis [34] (rats)	p.o.; 100	[i]	0	n.t.	n.t.	0	67	promethazine	p.o.; 10	73
	30		n.t.	0	0	n.t.	0			
Antiulcer [32] (stress induced in rats)	p.o.; 30	[j]	13	50	50	25	13	chlorpromazine	p.o.; 20	71
	10		n.t.	13	13	n.t.	n.t.	omeprazole	p.o.; 10	63
Antiulcer [33] (ethanol induced in rats)	p.o.; 100	[j]	38	n.t.	n.t.	25	0	carbenoxolone	p.o.; 300	65
	30		n.t.	38	13	n.t.	n.t.			
Hypoglycemic [32] (mice)	p.o.; 100	[k]	0	n.t.	25	0	26	phenformin	p.o.; 100	35
	30		n.t.	0	3	n.t.	3			
Hypoglycemic [32] (fasted mice)	p.o.; 100	[l]	n.t.	n.t.	31	n.t.	0	glibenclamide	p.o.; 3	22
	30		n.t.	n.t.	19	n.t.	n.t.			

[a] Only assays for which at least one compound gave a significant or boderline response are reported.

[b] Compounds **13**, **26**, **29** and **31** were also tested for all the indicated assays and found inactive; moreover, compounds **22**, **24** and **30** were tested for antiinflammatory, passive cutaneous anaphylactic and hypoglycemic activity, respectively, and were found to be inactive or very weakly active.

[c] References for the methods employed.

[d] n.t., not tested.

[e] % Reduction of apomorphine-induced climbing behavior.

[f] % Inhibition of number of writhes.

[g] % Reduction of induced paw licking time recorded during the following 20–30 min period after formalin injection (tested only compounds active in the preceding assay).

[h] % Inhibition of rat paw edema 3 h after carrageenan administration.

[i] % Inhibition of PCA blue-colored wheals.

[j] % Reduction of scores attributed to gastric ulcerative lesions.

[k] % Reduction of tolerance curve after a glucose load (1 g/kg s.c.).

[l] % Reduction of glycemia in fasted mice 1 h after drug administration (only compounds active in the preceding assay were tested).

Table 6
Antihypertensive activity in spontaneously hypertensive rats [32,33] of compounds **9**, **12**, **13**, **18–20** and **24** (n.d. = not determined)

Comp. [a]	Dose p.o. (mg/kg)	% Variation of blood pressure [b] after		
		1 h	2 h	4 h
9	30 [c]	−5	−3	−3
12	30 [c]	4	−8	2
13	100	−5	−4	−5
18	100	−2	−8	−6
19	100	−27	−20	−18
	30	−21	−20	−19
	10	−20	−18	−15
	3	2	0	−4
20	100	−36	−38	−34
	30	−18	−12	−12
	10	−5	−3	−2
24	100	−19	−17	−12
	30	−8	−6	−16
	10	n.d.	n.d.	−4
Reserpine	25	−20	−24	−26
Clonidine	0.1	−22	−22	−20

[a] In addition to the above, compounds **5**, **15**, **22**, **23**, **25**, **26**, **29** and **31** were also tested for antihypertensive activity and found to produce <5% variation of blood pressure in each of the three measures. A variation ≥10% is considered highly significant.

[b] The variation of blood pressure was associated with only a negligible (<5%) variation of heart rate; compound **20** at 100 mg/kg produced an 8% reduction of heart rate. A variation ≥20% is considered highly significant.

[c] Higher doses were not used because of toxicity (see Table 4).

According to a Japanese patent [7], cytisine and its *N*-methyl derivative are endowed with antiinflammatory activity; nevertheless, the observed activity of compounds **15** and **20** can be hardly ascribed to the mere presence of the cytisine moiety in their structure, since the remaining tested derivatives (including compound **22**, which is almost identical to **20**) were completely devoid of antiedema action. Thus, the entire structures of compounds **15** and **20** are responsible for their action.

It is worth noting that compound **20** (but not **15**) exhibited antiallergic activity in the passive cutaneous anaphylaxis test [34] in rats as well as moderate antihistamine activity, inhibiting by 40% the histamine-induced wheal in rats. These activities may relate to the observed in vitro antihistamine and antiserotonin activities (Table 8), which also might be, at least in part, responsible for the antiedema effect.

A significant reduction of glycemia was produced by compounds **12** and **20** in mice after a glucose load and also by compound **12** in fasting mice. Both compounds were inactive in mice previously treated with streptozotocin, indicating that they favor the release of insulin, as do the well-known sulfonylureas. Therefore, an attempt was made to improve this action by joining the cytisine moiety with a *p*-toluenesulfonylamidocarbonyl residue. The obtained compound **30** was completely inactive at a dose of 30 mg/kg p.o., while killing the mice at 100 mg/kg p.o., thus resulting in the most toxic of the cytisine derivatives tested so far.

Although cytisine and *N*-methylcytisine seem endowed with hypoglycemic activity [6], once more the observed activity of **12** and **20** must rely on their entire peculiar structures, since all the remaining tested compounds were completely lacking activity.

Among the previously cited nine compounds subjected to a general screening, compound **20** exhibited a very strong and long-lasting antihypertensive activity in rats, while compounds **9**, **12** and **13** showed only modest activity. Therefore, six more compounds structurally related to **20** (**18**, **19** and **22–25**) were tested, and two of them (**19** and **24**) were found to exert a good dose-related and long-lasting activity. Table 6 indicates that compound **19** was the most potent, but **20** was the most active. Compound **20** exerted a moderate bradycardic effect (−8% heart rate) at the highest dose employed (100 mg/kg), but this was not seen at a lower dose (30 mg/kg), still resulting in a strong antihypertensive response.

The antihypertensive activity was related neither to ganglionic blockade (pupil dilation test in mice [35]) nor to α_1- or α_2-adrenoceptor antagonism (inhibition of nor-epinephrine-induced mydriasis in mice [35] and reversal of clonidine-induced bradycardia in rats [37]), although α_1- and α_2-adrenoceptor antagonisms were observed in vitro (Table 7). Further β-adrenergic antagonism and negative chronotropic activity were shown in vitro on isolated atria, but calcium antagonism, potassium channel activation, or angiotensin inhibition were not observed on atria or ileum; thus, the mechanism of the antihypertensive action remains somewhat intriguing.

Still concerning the cardiovascular system, an interesting inotropic activity was observed in vitro on isolated guinea-pig left atria for compounds **9** and **15** (Table 7). This activity was not blocked by propranolol; moreover, a lack of reduction of spontaneous tone in guinea-pig trachea was observed (Table 8). Therefore, neither adrenergic stimulation nor phosphodiesterase inhibition seem to be involved in the inotropic activity. Inotropic activity is not commonly found in *p*-fluorobutyrophenone derived drugs, but it was observed by Platou et al. [42] in melperone.

Both compounds **9** and **15**, but particularly the latter, exhibited negative chronotropic activity in spontaneously beating guinea-pig right atria. It is worth noting that compound **13**, differing from **9** by the presence of an hydroxyl group on the phenylethyl moiety, was completely devoid of inotropic and chronotropic activities on isolated atria.

Table 7
In vitro tests related to cardiocirculatory activity for compounds **9**, **12**, **15** and **20**

Test [a]	Conc. (μg/ml)	Effect	Effects produced by compound [b]				Reference drug	Conc. (μg/ml)	Effect
			9	**12**	**15**	**20**			
Inotropic effect [33] (guinea-pig left atria)	10	[c]	+48	−7	+44	−27	trequinsin	10	+48
	3		+27	n.t.	+27	n.t.			
Chronotropic effect [33] (guinea-pig right atria)	10	[c]	−21	−16	−11	−12	quinidine	10	−20
	3		−15	−6	−3	−7	diltiazem	10	−18
	1		−7	n.t.	n.t.	n.t.	mecamylamine	10	−14
Ca^{2+} antagonism [33] (guinea-pig ileum)	3	[d]	26	10	23	5	cinnarizine	1	82
β-Adrenergic antagonism [35] (guinea-pig left atria)	10	[e]	n.t.	8	20	83	practolol	0.5	72
	3		12	n.t.	n.t.	56			
	1		n.t.	n.t.	n.t.	0			
α_1-Antagonism [37] (rat vas deferens prostatic portion)	3	[f]	18	0	0	71	prazosin	0.01	58
	1		n.t.	n.t.	n.t.	57			
	0.3		n.t.	n.t.	n.t.	25			
α_2-Antagonism (rat vas deferens prostatic portion)	3	[g]	0	0	0	58	yohimbine	0.2	62
	1		n.t.	n.t.	n.t.	21			
Angiotension I inhibition [33] (guinea-pig ileum)	3	[h]	0	14	0	20	[sar[1],thr[8]]ang. II	0.05	65

[a] References for the methods employed (when no reference is cited, the method is as described in this report).
[b] Compounds **5**, **13**, **26**, **29** and **31** were also assayed using the indicated tests, but did not produce any significant response (compounds **7**, **8** and **28** were also tested for inotropic effects but only exhibited weak negative effects). n.t., not tested.
[c] % Variation in contractile force or rate.
[d] % Inhibition of Ca^{2+}-induced contractions.
[e] % Reduction of isoproterenol-induced positive inotropic effect.
[f] % Reduction of phenylephrine-induced contractile response.
[g] % Inhibition of clonidine-induced reduction in twitch response.
[h] % Reduction of contractile response.

Compound **15** exhibited a good inhibition of PAF-induced platelet aggregation, while compounds **9** and **13** had only a moderate activity; the remaining compounds were inactive. Moreover, compound **15** (at 30 mg/kg) protected 60% of mice from PAF-induced mortality. This action is quite unusual, and its presence beside antiinflammatory activity not related to cyclooxygenase inhibition makes compound **15** worthy of further investigations, particularly those aimed at identification of histoprotective antiiflammatory agents.

Finally, compound **9** increased the electrically stimulated guinea-pig ileum contractions at concentrations ≥ 3 μg/ml (Table 8), but inhibited them at much higher concentrations (30 μg/ml). Such behavior is suggestive of a gastroprokinetic activity which will be checked in vivo.

In conclusion, the introduction of different kinds of substituents on the basic nitrogen of cytisine gave rise to several new interesting activities, while an appreciable degree of affinity for central nicotinic receptors was sometimes retained.

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Table 8
Some in vitro activities of compounds **5**, **9**, **12**, **13**, **15** and **20** (n.t. = not tested)

Test [b]	Conc. (μg/ml)	Effect	Effects produced by compound						Reference drug	Conc. (μg/ml)	Effect
			5	**9**	**12**	**13**	**15**	**20**			
Platelet aggregation induced by:											
PAF [33]	10	[c]	8	24	8	31	67 [l]	15	RP-48740 [m]	5	54
	3		n.t.	n.t.	n.t.	n.t.	23	n.t.			
ADP [33]	100	[c]	[d]	[d]	[d]	[d]	26	[d]			
Tracheal relaxation [33]	30	[e]	7	25	9	25	12	0	theophylline	30	60
LTD_4 antagonism [34]	3	[f]	30	0	25	0	0	11			
Bradykinin antagonism [33]	3	[f]	25	0	23	0	13	10			
Substance P antagonism [33]	3	[f]	15	15	10	20	0	3			
Cholecystokinin antagonism [33]	3	[f]	31	12	26	0	0	0			
Ileum electric stimulation increase [33]	30	[g]	n.t.	− [h]	n.t.	n.t.	n.t.	n.t.	metoclopramide	0.3	+
	10		n.t.	+	n.t.	n.t.	n.t.	n.t.			
	3		−	+	−	−	−	−			
	1		n.t.	−	n.t.	n.t.	n.t.	n.t.			
Antihistamine [34]	10	[i]	[j]	[j]	[j]	[j]	[j]	90	terfenadine	25	73
	3							68			
	1							31			
Antiserotonin [34]	100	[k]	[j]	[j]	[j]	[j]	[j]	80	promethazine	0.1	56
	30							55			
	10							10			

[a] Compounds **26**, **29** and **31** were also tested in the indicated assays but were found inactive or very weakly active.
[b] References for the methods employed.
[c] % Inhibition of rabbit platelets aggregation induced by the indicated agent.
[d] Only compounds active in the preceding assay were tested.
[e] % Inhibition of guinea-pig tracheal tone relative to relaxation induced by 0.3 μg/ml epinephrine.
[f] % Inhibition of contractile response of guinea-pig ileum to various agents.
[g] % Increase of contractions by >15% is recorded as significant (+).
[h] At 30 μg/ml a significant (>50%) decrease of contractions was observed.
[i] % Inhibition of histamine-induced guinea-pig ileum contraction.
[j] Tested only compounds active in PCA assay (see Table 7).
[k] % Inhibition of serotonin-induced rat aortic strip contractions.
[l] Compound **15** at 30 mg/kg p.o. reduced PAF-induced mortality in mice by 60%.
[m] 3-(3-Pyridyl)-1*H*,3*H*-pyrrolo[1,2-*c*]thiazole-7-carboxamide.

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