LG 30435, a new potential antiasthmatic agent

F. Berti, L. Daffonchio, F. Magni, C. Omini, G. Rossoni, A. Subissi*

ABSTRACT: LG 30435 is a new quaternary phenothiazine derivative with H1-antihistaminic and antimuscarinic properties. The ability of LG 30435 to prevent changes in respiratory mechanics, induced by different mediators and the immunological reaction, was monitored together with biological and radiolmmunological determination of circulating thromboxane-A1 (TxA1) in anaesthetized guinea-pigs. LG 30435 dose-dependently reduces the bronchoconstriction and TxA1 generation caused by different stimuli such as histamine, acetylcholine, leukotriene C4 (LTC4) and PAF-acether. In addition i.v. and aerosol administration of LG 30435 causes a dose-dependent reduction of the increase in airway resistance and TxA1 generation induced by ovalbumin challenge in actively sensitized animals. LG 30435 infused at different concentrations through the isolated guinea-pig lungs inhibits the TxB2 generation caused by different anaphylactic mediators, but not by arachidonic acid. These data, which further substantiate the bronchodilator activity of LG 30435 against a variety of stimuli and demonstrate its protective properties on lung anaphylaxis, suggest that this compound has a potential therapeutic value in the treatment of asthma.

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Both mediator release and the responsiveness of airway smooth muscle to mediators are of importance in the clinical manifestation of asthma. Therefore, compounds capable of controlling the effects of neuronal and immunological agents that lead to obstruction and hyperreactivity of airway smooth muscles may have therapeutic value. LG 30435, 1-methyl-3-(lOH-phenothiazine-10-ylmethyl)-1-azoniabicyclo (2.2.2) octane iodide, is a new quaternary phenothiazine derivative with H1-antihistaminic and antimuscarinic properties and a pharmacological profile suggesting therapeutic potential in human airway diseases [1]. Thus, LG 30435 has activity against PAF-acether (PAF), inhibiting PAF-induced platelet aggregation and bronchoconstriction [2], which may be of particular value since it has been suggested that PAF plays a role as a putative mediator of anaphylaxis and inflammation, together with its ability to induce airway hyperreactivity [3]. We now have new evidence that LG 30435 counteracts the effects of different autacoids in the airways, including the ability of such agents to amplify their pulmonary actions via activation of the eicosanoid system with preferential generation of thromboxane A2 (TxA2). Furthermore, LG 30435 possesses antiallergic activity in passive anaphylaxis reactions of lung and skin [4]. This observation prompted us to investigate the protection by LG 30435 against pulmonary responses induced by antigen challenge in actively sensitized guinea-pigs.

Institute of Pharmacological Sciences, University of Milan, Via Balbarezzi 9, 1-20133 Milan, Italy.

* Dept of Pharmacology, Laboratori Guidotti S.p.A., Via Livemose 402, I-56010 S. Piero a Grado, Pisa, Italy.

Correspondence: A. Subissi, Dept of Pharmacology, Laboratori Guidotti S.p.A., Via Livemose 402, I-56010 S. Piero a Grado, Pisa, Italy.

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Materials and methods

Bronchopulmonary effects of autacoids in the guinea-pig

Male Hartley strain guinea-pigs (300-400 g) were anaesthetized with urethane (1.2 g kg-1 i.p.), paralysed with pancuronium bromide (2 mg kg-1 i.v.) and prepared for recording pulmonary mechanics and arterial blood pressure [5]. Dynamic compliance (Cdyn), lung resistance (Rt.) and transpulmonary pressure (Ptp.) were recorded using Hewlett-Packard instruments.

Guinea-pigs were also arranged for extracorporeal circulation following the blood-bathed organ technique [6], in order to detect circulating thromboxane A2-like activity (TxA2-la). Blood derived from the left carotid artery superfused a helical strip of rabbit aorta (RbA) which was maintained overnight under Krebsbicarbonate buffer superfusion, containing a mixture of receptor antagonists [7] and indomethacin (1 µg ml-1) in order to increase both the specificity for TxA2-la and the sensitivity of the RbA. Aliquots (0.2 ml) of arterial blood were also taken for radiolmmunological determination of TxB2 [8].

Bronchospasm was induced by i.v. injections of histamine (50 nmol·kg-1), acetylcholine (ACH, 50 nmol·kg-1), PAF (0.2 nmol·kg-1) and leukotriene C4 (LTC4, 1.6 nmol·kg-1). The effects of these doses on Rt. and Cdyn were of comparable magnitude. In experiments with PAF,
changes of blood pH, carbon dioxide tension (Pco₂) and oxygen tension (Po₂) were conducted utilizing a blood/ 
ph/gas analyser (IL-1302). Different doses of LG 30435 
(0.01–0.1 μmol·kg⁻¹) in the case of histamine and ACh, 
0.1–3 μmol·kg⁻¹ in the case of LTC₄ and PAF) were 
injected 3 min before mediator challenge and pulmonary 
changes were evaluated at the peak effect. Antagonistic 
activity of LG 30435 was expressed in terms of median 
effective dose (ED₅₀), which was calculated from dose-
effect regression function [9]. In order to avoid tachy-
phylaxis of PAF, this autacoid was injected only once in 
groups of control and LG 30435-treated animals.

Active lung anaphylaxis in the guinea-pig

Male Hartley strain guinea-pigs (250–350 g) were 
actively sensitized to ovalbumin (100 mg·kg⁻¹ i.p. plus 
100 mg·kg⁻¹ s.c.) [10]. Twenty one days after ovalbumin 
treatment the animals were anaesthetized with urethane 
(1.2 g·kg⁻¹ i.p.) and resistance to lung inflation was evalu-
ated by the KONZEIT and RÖSSLER technique [11] using 
Hewlett-Packard instruments. Systemic blood pressure 
(carotid artery) was also recorded and respiration was 
fully arrested with pancuronium bromide (2 mg·kg⁻¹ i.v.). 
The animals were arranged for extracorporeal circulation 
as previously described and blood pH, Po₂, and Pco₂ were 
also monitored. The anaphylactic reaction was induced 
with ovalbumin (5 mg·kg⁻¹ i.v.) in control guinea-pigs 
and in groups of animals treated with graded doses of 
LG 30435 (0.1–3 μmol·kg⁻¹ i.v.) three min before antigen 
challenge. The severity of the bronchoconstriction 
was evaluated [12] and the protective activity of LG 30435 
was expressed as percentage of maximal bronchocon-
striction in control animals.

In another series of experiments the anaphylactic 
reaction was triggered in actively sensitized guinea-pigs 
by ovalbumin inhalation (aerosol, 10 mg·ml⁻¹ × 5 s) 
according to the ovalbumin macroshock procedure [13]. 
Bronchoconstriction and systemic blood pressure were 
measured as reported above. LG 30435 was administered 
by aerosol (0.65 and 6.5 mM for 30 s) 3 min before 
antigen challenge.

Guinea-pig perfused lungs

Lungs removed from both normal and ovalbumin sen-
sitized guinea-pigs (300–400 g) were perfused through 
the pulmonary artery with Krebs-bicarbonate solution at 
a flow rate of 10 ml·min⁻¹ [14]. The pulmonary outflow 
superfused a set of RbA in cascade to monitor TxA₂-la 
during the immunological and autacoid response of the 
pulmonary tissues [6]. As reported above, the bioassay 
tissues were treated with a mixture of receptor antago-
nists and indomethacin to increase their specific response 
[7]. The resting tension of the RbA was 1 g and tension 
changes were measured with isometric transducers. Histamine (27 nmol), PAF (0.2 nmol), arachidonic acid 
(16 nmol) and ovalbumin (1 mg, in sensitized tissues) 
were injected as bolus through the pulmonary artery, and 
lung formation of TxA₂ was evaluated as the maximal 
increase of RbA tension in g. Aliquots of perfusates (1 
min sample) were collected before and after challenge, 
in order to evaluate the concentration of TxB₂ [8].

Different concentrations of LG 30435 (0.01–0.1 μmol 
against histamine; 0.3–3 μM against PAF; 1–10 μM 
against ovalbumin; 0.1–1 mM against arachidonic acid) 
were infused through the lungs for 15 min before agonist 
or antigen challenge. The antagonistic activity of this 
compound on TxB₂ generation was expressed as IC₅₀ 
evaluated from the dose-effect regression function [9].

Drugs

The following drugs were used: histamine dihydro-
chloride (Sigma Chemical Co., St. Louis, MO, USA); 
acetylcholine bromide (Sigma); LTC₄ (OncPharma-
cutical Co., Ltd, Osaka, Japan); PAF-accepter (C₁₄ , 
Bachem Inc., Torrance, CA, USA); arachidonic acid 
sodium salt (Sigma); ovalbumin (Sigma, grade II) and LG 30435 
(Laboratori Guidotti S.p.A., Pisa, Italy). LTC₄ and PAF-
accepter were dissolved in ethanol and appropriately 
diluted with normal saline or Krebs-bicarbonate solution. 
All the other compounds were dissolved in normal saline 
or Krebs-bicarbonate solution.

Results

Bronchopulmonary effects of autacoids in the guinea-pig

The intravenous injection to anaesthetized guinea-pigs 
of histamine, LTC₄ and PAF brings about a severe bron-
chospasm with an increase in Rt and a decrease in Cldyn 
which are associated with a rise of the blood levels of 
immunoreactive TxB₂ (fig. 1a, figs. 2b and c). There-
fore, these results suggest that TxA₂ may take part in the 
overall effect observed with the above autacoids in 
anaesthetized guinea-pigs. On the contrary, the cardio-
pulmonary activity of ACh which (fig. 2a) is not accompa-
nied by TxB₂ formation indicating that the modifications 
of respiratory mechanics are mediated exclusively by 
muscarinic receptor activation.

LG 30435 dose-dependently antagonizes the changes 
in Rt and Cldyn caused by ACh, histamine and LTC₄; 
moreover the increase in blood TxB₂, induced by histo-
mine and LTC₄ is significantly reduced (fig. 2). How-
ever, LG 30435 is more potent in antagonizing histamine 
than ACh or LTC₄; ED₅₀ values and 95% confidence 
limits being 19 (17–22) nmol·kg⁻¹, 32 (29–36) 
nmol·kg⁻¹ and 180 (156–207) nmol·kg⁻¹, respectively. At 
higher doses, LG 30435 counteracts the bronchoconstric-
tion induced by PAF (ED₅₀; 284 (236–343) nmol·kg⁻¹), 
and the concomitant increase in blood TxB₂, is also dose-
dependently decreased (fig. 1a). The severity of the 
pulmonary effects of PAF correlates well with the modifi-
cations of blood pH, Pco₂ and Pco₂. In fact, according 
to the dose used, LG 30435 maintains the values of blood 
P (data not shown) and blood gases (fig. 1b) closer to 
control levels.
Fig. 1. - Protective effects of LG 30435 against the bronchoconstriction and the release of TxB₂ (a) and the blood changes of Pco₂ and Po₂ (b) induced by PAF (0.2 nmol·kg⁻¹ i.v.) in the anesthetized guinea-pig. LG 30435 was administered 3 min before PAF at the doses indicated (μmol·kg⁻¹ i.v.). Values are mean ± SEM of at least 6 experiments. RL: lung resistance; Cldyn: dynamic compliance; Pco₂: carbon dioxide tension; Po₂: oxygen tension; TxB₂: thromboxane B₂.

Fig. 2. - Protective effects of LG 30435 against the bronchoconstriction induced by acetylcholine (ACh) (a), histamine (H) (b) and leukotriene C₄ (LTC₄) (c) and against the release of TxB₂ induced by H (b) and LTC₄ (c) in the anesthetized guinea-pig. LG 30435 was administered 3 min before the challenge at the doses indicated (μmol·kg⁻¹ i.v.). Values are mean ± SEM of at least 5 experiments. RL: lung resistance; Cldyn: dynamic compliance; TxB₂: thromboxane B₂.

Fig. 3. - Protective effects of LG 30435 in actively sensitized anesthetized guinea-pigs from the anaphylactic reaction caused by antigen challenge (ovalbumin (OA) 5 mg·kg⁻¹ i.v.). A: Vehicle or B=LG 30435 (3 μmol·kg⁻¹ i.v.) was injected 3 min before the antigen challenge. AR: airway resistance; RbA: strip of rabbit aorta monitoring TxA₂-like activity in superfusing blood; BP: blood pressure; TxA₂: thromboxane A₂.
Lung anaphylaxis in the guinea-pig

The administration of ovalbumin (5 mg·kg\(^{-1}\) i.v.) to actively sensitized guinea-pigs brings about a sustained and irreversible bronchoconstriction and a progressive fall in systemic blood pressure, associated with a prompt tension increase in RbA, indicating the release of TxA\(_2\)-like during the immunological response (figs. 3 and 4). A marked reduction of blood pH and Po\(_2\), and increase in Pco\(_2\), accompanies the pulmonary dysfunction and the animals die in less than 10 min. Graded doses of LG 30435 (0.1–3 μmol·kg\(^{-1}\) i.v.), administered 3 min before ovalbumin challenge, cause a dose-dependent protection against the anaphylactic response (ED\(_{50}\): 599 (548–559) nmol·kg\(^{-1}\)) and all of the animals, except those treated with the lowest dose, survive the anaphylactic shock.

Fig. 4. – Protective effects of LG 30435 in actively sensitized anasthetized guinea-pigs from the anaphylactic reaction caused by antigen challenge (ovalbumin (OA) 5 mg·kg\(^{-1}\) i.v.). LG 30435 was administered 3 min before the challenge at the doses indicated (μmol·kg\(^{-1}\) i.v.). Values are mean ±SEM of at least 7 experiments. Raw: airway resistance; RbA: strip of rabbit aorta monitoring TxA\(_2\)-like activity in superfusing blood; TxB\(_2\): thromboxane B\(_2\).

Fig. 5. – Protective effects of LG 30435, administered by aerosol against the increase in pulmonary inflation pressure (Pip) evoked by aerosolized ovalbumin (OA, 10 mg·ml\(^{-1}\) for 3 s) in actively sensitized anasthetized guinea-pigs. LG 30435 (0.65 and 6.5 mM for 30 s) was given 3 min before OA. *p<0.01 by Student’s t-test.

Fig. 6. – Prevention by LG 30435 of TxA\(_2\) generation induced by histamine (H) (a), PAF (b), arachidonic acid (AA) (c) and ovalbumin (OA) (d) in isolated perfused guinea-pig lungs. LG 30435 was infused through the lungs for 15 min at the concentrations (μM) indicated before the releasing challenge. Values are mean ±SEM of at least 6 experiments. RbA: strip of rabbit aorta monitoring TxA\(_2\)-like activity in superfusing blood; TxB\(_2\): thromboxane B\(_2\).
The protective activity of LG 30435 on anaphylactic bronchoconstriction correlates well with the reduction in release of TxB$_2$ (fig. 4, ED$_{50}$ 730 (665-802) nmol·kg$^{-1}·h^{-1}$) and the modification of blood pH and blood gases (data not shown).

The challenge of actively sensitized guinea-pigs with ovalbumin given by aerosol (10 mg·ml$^{-1}·5·s$) induces an immediate and reversible bronchoconstriction. The administration of LG 30435 by aerosol 3 min before antigen is also effective in this model; in particular almost complete protection from bronchoconstriction is achieved at the dose of 6.5 mM for 30 s (fig. 5).

**Guinea-pig perfused lungs**

The bolus injection of histamine and PAF through the lungs causes generation and release in the pulmonary effluent of a significant amount of TxA$_2$-la, as indicated by the increase of RbA tension, and of TxB$_2$ immunoreactivity in the perfusate.

The bolus injection of ovalbumin (1 mg) through the lungs obtained from actively sensitized guinea-pigs also induces a fast and sustained increase in tension development of superfused RbA and in TxB$_2$ immunoreactivity in the pulmonary effluent (fig. 6).

The infusion of the lungs with graded concentrations of LG 30435 for 15 min inhibits, in a concentration-dependent manner, both the tension increase of RbA and the increase of perfuse TxB$_2$ caused by the two autacoids and by antigen challenge (fig. 6). The IC$_{50}$ s and 95% confidence limits, calculated for TxB$_2$ inhibition, are 0.035 (0.030-0.041) µM against histamine, 0.53 (0.45-0.61) µM against PAF and 2.9 (2.6-3.3) µM against ovalbumin. TxA$_2$-la or TxB$_2$, formation induced by arachidonic acid (17 nmol) administration to the lungs is poorly affected by LG 30435 even at the concentration of 1 mM (fig. 6).

**Discussion**

The present results underline the ability of LG 30435 to antagonize the pulmonary effects of various mediators believed to be involved in the clinical manifestations of asthma; they also suggest different sites of action, all useful in the control of reversible airway obstruction. The efficacy of LG 30435 in preventing bronchoconstriction induced by ACh, histamine and leukotrienes is already known [1], but the ability of this new compound to inhibit arachidonic acid metabolism and generation of TxA$_2$ represents a new insight into its mode of action. This is important since the pulmonary effects of leukotrienes [15] and PAF [16] are amplified by the formation of TxA$_2$, and other bronchoactive eicosanoids. Another point of interest emerging from these experiments is the ability of LG 30435 to affect the pulmonary changes due to PAF. There are several reasons to believe that PAF is involved in human asthma: this alkylphospholipid not only provokes bronchoconstriction and inflammation in various animal species but also causes bronchial hyperreactivity [3, 17]. Therefore, inhibition of the acute respiratory smooth muscle response to PAF observed with LG 30435 in guinea-pigs represents a good starting point for further investigations concerned with immediate and late anaphylactic responses. In this regard the ability of LG 30435 to inhibit systemic anaphylaxis, decreasing both the immediate bronchoconstriction and the concomitant TxB$_2$ generation, as shown with the present experiments, reinforces the potential value of the compound in asthma therapy.

The experiments carried out with guinea-pig perfused lungs substantiate the results obtained with anaesthetized animals and demonstrate that LG 30435 consistently reduces the formation of TxA$_2$, induced by histamine, PAF and antigen challenge in the pulmonary tissues, contributing to the bronchodilator and anti-allergic properties of the compound.

Our data, taken together, suggest that the mode of action of LG 30435 in protecting guinea-pigs from anaphylactic shock may result from this complex pharmacological profile. In fact LG 30435 has pronounced peripheral antihistaminic and antimuscarinic activity and also antagonizes the pulmonary effects of LTC$_4$, and PAF, including those mediated through release of eicosanoids. On the other hand the phenothiazine nature of LG 30435 allows another possible interpretation of the mode of action of this compound.

Phenothiazines may selectively bind and inhibit the action of calmodulin [18, 19], an ubiquitous intracellular calcium-receptive protein involved in mechanisms of stimulus-response coupling (such as muscle contraction, mediator release and inflammatory cell recruitment) as well as in phospholipase A$_2$, activation [20]. The latter is a crucial point since not only eicosanoid, but also PAF biosynthesis, requiring PLA$_2$ activation [17], could be affected by LG 30435. Preliminary experiments show that this compound does bind to calmodulin, indicating a possible inhibitory interference of LG 30435 with calmodulin-activated phospholipase A$_2$. This is in agreement with the inhibition of TxA$_2$ release induced by different agents (histamine, LTC$_4$, PAF and antigen), which are believed to activate PLA$_2$, but the lack of effect on the conversion of exogenously administered arachidonic acid, suggesting that neither cyclo-oxygenase nor thromboxane synthase are affected by the compound.

In conclusion, the multiple actions shown by LG 30435 reinforce its potential therapeutic value, since the effects of several mediators and their resultant interactions contribute to the pathology of asthma, especially antigen-induced bronchospasm.

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RÉSUMÉ: LG 30435 est un nouveau dérivé quaternaire de la phénothiazine doué de propriétés anti-histaminiques (anti H1) et de propriétés anti-muscariniques. Nous avons étudié la capacité du LG 30435 pour prévenir des modifications de la mécanique respiratoire ainsi que la réaction inflammatoire induite par différents médiateurs. Nous avons également déterminé par voie biologique et radioimmunologique la thromboxane A2 circulant chez des cobayes anesthésiés. LG 30435 réduit de manière dose dépendante la bronchoconstriction et la production de TXA2 générés par différents stimuli comme histamine, acétylcholine, LTC4 et PAF acéthyle. Par ailleurs, l'administration intra-veineuse ou en aérosol de LG 30435 provoque une réduction dose-dépendante de l'augmentation de la résistance des voies aériennes et de la production de TXA2 induite par une provocation à l'ovalbumine chez des animaux activement sensitialisés. LG 30435, infusé à différentes concentrations dans les poumons isolés de cobayes, inhibe la production de TXB2 provoquée par différents médiateurs de l'anaphylaxie mais pas celle due à l'acide arachidonique. Ces données qui confirment l'activité broncho-dilatatrice de LG 30435 contre une variété de stimuli démontrent sa valeur protectrice sur l'anaphylaxie pulmonaire et suggèrent que ce composé pourrait avoir une valeur thérapeutique potentielle dans le traitement de l'asthme.

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