Korean J Physiol Pharmacol
Vol 10: 317−321, December, 2006

Glycyrrhizin and Morroniside Stimulate Mucin Secretion from Cultured Airway Epithelial Cells

Ho Jin Heo[1], Hyun Jae Lee[1], Cheolsu Kim[1], Kun Ho Son[2], Young Choong Kim[3], Young Sik Kim[3], Sam Sik Kang[3], Yang Chun Park[4], Yun Hee Kim[4], Un Kyo Seo[5], Jeong Ho Seok[1], and Choong Jae Lee[1]

[1]Department of Pharmacology, College of Medicine, Chungnam National University, Daejeon 301-131, [2]Department of Food Science an Nutrition, Andong National University, Andong 760-749, [3]Department of Pharmaceutical Sciences, College of Pharmacy, Seoul Nationa University, Seoul 110-799, [4]Department of Oriental Medicine, College of Oriental Medicine, Daejeon University, Daejeon 300-716 and [5]Department of Oriental Medicine, College of Oriental Medicine, Dongguk University, Gyeoungju 780-714, Korea

In this study, we investigated whether glycyrrhizin, prunetin and morroniside affect mucin secretion from cultured airway epithelial cells and compared the possible activities of these agents with the inhibitory action on mucin secretion by poly-L-lysine (PLL) and the stimulatory action by adenosine triphosphate (ATP). Confluent primary hamster tracheal surface epithelial (HTSE) cells were metabolically radiolabeled using ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of each agent to assess the effects on ^{3}H-mucin secretion. The results were as follows: 1) glycyrrhizin and morroniside increased basal mucin secretion from airway; 2) prunetin did not affect basal mucin secretion; 3) glycyrrhizin did not inhibit ATP-induced mucin secretion. We conclude that glycyrrhizin and morroniside can increase basal mucin secretion, by directly acting on airway mucin-secreting cells and suggest that two compounds be further investigated for the possible use as mild expectorants during the treatment of inflammatory airway diseases.

Key Words: Airway, Mucin, Glycyrrhizin and morroniside

INTRODUCTION

Mucus in the airway plays a pivotal role in defense mechanisms against various pathogens through a mechanism called the mucociliary clearance. The protective function of mucus is due to the viscoelastic property of mucous glycoproteins or mucins (Ellis, 1985). Mucins are high molecular weight glycoproteins produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. Therefore, any abnormality in the quality or quantity of mucins not only causes altered airway physiology, but also impairs host defenses, often leading to serious airway pathology as exemplified in chronic bronchitis, cystic fibrosis, asthma, and bronchiectasis (Newhouse & Biennenstock, 1983; Kim et al, 1997). To remove an excess mucus from the airway, there could be two ways; i) getting rid of the mucus by physical means, that is to say, aspiration after dilution of mucus, and ii) suppression of secretion and/or production of mucus by pharmacological means. However, clinically, the physical method induces irritation of airway luminal wall and leads to hypersecretion of mucus through a reflex mechanism. Thus, the pharmacological means to inhibit mucin secretion and/or production has become an important approach to regulate the hypersecretion of airway mucus (Mutschler & Derendorf, 1995; Lee et al, 2002). Therefore, we suggest it is valuable to find the possible activity of controlling (inhibiting) the excess mucin secretion by the components from medicinal plants that have been used for the management of airway diseases in oriental folk medicine. We have tried to investigate the possible activities of some natural products on mucin secretion from cultured airway epithelial cells using a primary hamster tracheal surface epithelial (HTSE) cell culture - an established in vitro model for secretory cell metaplasia (Wasano et al, 1988). As a result of our trial, we previously reported that several natural compounds affected mucin secretion from HTSE cells (Lee et al, 2003; Lee et al, 2004a; Lee et al, 2004b). Glycyrrhizae Radix and Corni Fructus have been used for controlling airway inflammatory diseases in oriental medicine and their components, glycyrrhizin, prunetin and morroniside were reported to have various biological effects including anti-inflammatory effect (Jang, 2003). However, to the best of our knowledge, there are no reports about the effect of the aforementioned compounds on airway mucin secretion. Thus, in the present study, we investigated whether glycyrrhizin, prunetin and morroniside affect mucin secretion from cultured airway epithelial cells and tried to compare the possible activities of these agents with the inhibitory action on mucin release by PLL, a non-steroidal polycationic inhibitor of mucin secretion (Ko et al, 1999) and the stimulatory action by ATP, a stimulator

Corresponding to: Choong Jae Lee, Department of Pharmacology, College of Medicine, Chungnam National University, 6, Munhwa-dong, Joong-gu, Daejeon 301-131, Korea. (Tel) 82-42-580-8255, (Fax) 82-42-585-6627, (E-mail) LCJ123@cnu.ac.kr

ABBREVIATIONS: PLL, poly-L-lysine; ATP, adenosine triphosphate; HTSE, hamster tracheal surface epithelial; PBS, phosphate-buffered saline.

of mucin secretion (Kim et al, 1997).

METHODS

Materials

All the chemicals and reagents used in this experiment were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified. Glycyrrhizin, prunetin and morroniside were isolated, purified and identified by analytical chemists in Andong National University (Gyeongbuk, Korea) and Research Institute of Natural Products of Seoul National University (Seoul, Korea).

Primary hamster tracheal surface epithelial (HTSE) cell culture

Tracheas were obtained from male Golden Syrian hamsters, 8 weeks of age (Harlan Sprague Dawley, Indiana, USA). HTSE cells were harvested and cultured on a thick collagen gel substratum as previously reported (Wasano et al, 1988). Briefly, animals were euthanized in a CO_2 chamber and the tracheas were exposed under aseptic conditions. The tracheas were canulated using a polyethylene tube through which the tracheal lumen was filled with 0.1% pronase (Type XIV) prepared in Ca^{++}, Mg^{++} free Minimum Essential Medium (MEM, GIBCO) and incubated at 4°C for 16 h. The luminal contents were flushed, and cells were washed twice with MEM containing 10% fetal bovine serum by centrifuging at 200×g. The washed cell pellets were dissociated in a growth medium containing Medium 199 and Dulbeccos Modified Eagles medium (DME) (1 : 1) supplemented with insulin (5 μg/ml), epidermal growth factor (12.5 ng/ml), hydrocortisone (0.1 μM), fetal bovine serum (5% v/v, Hyclone, Logan, UT, USA), sodium selenite (0.01 μM), retinoic acid (0.1 μM), Penicillin G (100 U/ml, GIBCO), Streptomycin (100 μg/ml, GIBCO), and Gentamicin (50 μg/ml) ("complete" medium). At this stage, most of the cells were in small aggregates and plated at a density of 10^4 cells/cm^2 into tissue culture dishes containing a thick collagen gel (0.15 ml/cm^2) using collagen type I (Regenmed, Seoul, Korea). Cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 and culture medium was changed on day 1, 3, 5 and 7.

Metabolic labeling of mucins and treatment of cultures

Mucins were metabolically radiolabeled for 24 h by incubating confluent cultures (24 well plate, 5×10^5 cells/well) with 0.2 ml/well of the "complete" medium containing 10 μCi/ml of [6-^{3}H] glucosamine (39.2 Ci/mmol, New England Nuclear) for 24 h, as previously reported (Kim et al, 1987). At the end of the 24 h incubation, the spent media (the pretreatment sample) were collected, and the labeled cultures were washed twice with Dulbecco's phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} before chasing for 30 min in PBS containing varying concentrations of each agent (the treatment sample). PLL (average molecular weight 7,500) and ATP were prepared and administered to cultures in PBS. Glycyrrhizin, prunetin and morroniside were dissolved in dimethylsulfoxide and administered in PBS (final concentrations of dimethylsulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. PBS solution between this range and 0.5% dimethylsulfoxide did not affect mucin secretion from HTSE cells. Floating cells and cell debris were removed by centrifugation of samples at 12,000×g for 5 min. The samples were stored at −80°C until assayed for their ^{3}H-mucin contents.

Quantitation of ^{3}H-mucins

High molecular weight glycoconjugates excluded after Sepharose CL-4B gel-filtration column chromatography and resistant to hyaluronidase were defined as mucins and measured by the column chromatography as previously reported (Kim et al, 1985). Media samples were adjusted to pH 5.0 with 0.1 M citric acid and treated with 100 U/ml of testicular hyaluronidase (Type VI-S) at 37°C for 16 h. At the end of the incubation, the digestion mixture were neutralized to pH 7.4 using 0.2 M NaOH, boiled for 2 min and centrifuged. The supernatants were applied to Sepharose CL-4B columns (1×50 cm) equilibrated with PBS containing 0.1% (w/v) sodium dodecyl sulfate (SDS). Columns were eluted with the same buffer at a constant flow rate of 0.336 ml/min and fractions of 0.42 ml were collected. Void volume fractions (4 peak fractions) were mixed with 4 ml of scintillation cocktail, hydrofluor (National Diagnostic) and the radioactivity of fractions were counted using a liquid scintillation counter (LSC). The sum of radioactivity in four peak fractions was defined as the amount of mucin in the sample. The effect of agents on mucin secretion was measured as follows: the amount of mucin secreted during the treatment period was divided by the amount of mucin secreted during the pretreatment period and the ratio was expressed as a secretory index. Means of secretory indices of each group were compared and the differences were assessed using statistics.

Statistics

Means of individual group were converted to percent control and expressed as mean S.E.M. The difference between groups was assessed using student's t-test for unpaired samples. $p<0.05$ was considered as significantly different.

RESULTS

Effect of glycyrrhizin on mucin secretion

Glycyrrhizin significantly increased mucin secretion during 30 min of treatment period. The amounts of mucin in the spent media of drug-treated cultures were 100±7%, 99±3%, 98±3%, 127±4% and 430±41% for control, 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively. For comparison, both 2.0×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive controls (Fig. 1).

Effect of prunetin on mucin secretion

Prunetin did not affect mucin secretion, during 30 min of treatment period. The amounts of mucin in the spent media of drug-treated cultures were 100±2%, 90±2%, 95±3%, 108±14% and 85±6% for control, 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively. For comparison, both 2.0×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive

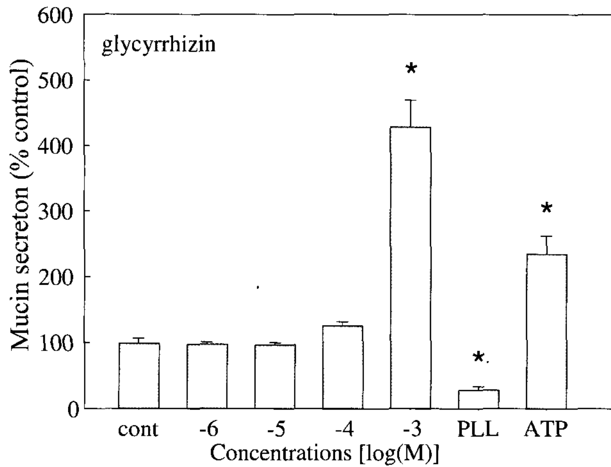

Fig. 1. Effect of glycyrrhizin on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of agent. For comparison, both 200 μM of ATP, a well-known mucin secretagogue and 10 μM of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion were used as positive controls. The amounts of ^{3}H-mucins in the spent media were measured as described in METHODS. Each bar represents a mean S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p < 0.05$). ATP, adenosine triphosphate; PLL, poly-L-lysine.

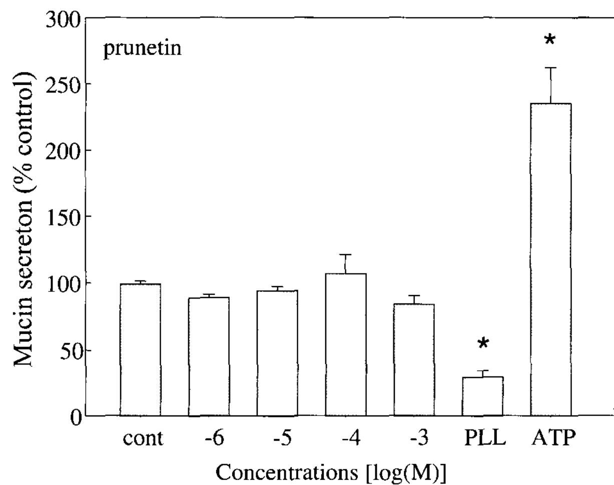

Fig. 2. Effect of prunetin on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of drug. For comparison, both 200 μM of ATP, a well-known mucin secretagogue and 10 μM of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion were used as positive controls. The amounts of ^{3}H-mucins in the spent media were measured as described in METHODS. Each bar represents a mean S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p < 0.05$). ATP, adenosine triphosphate; PLL, poly-L-lysine.

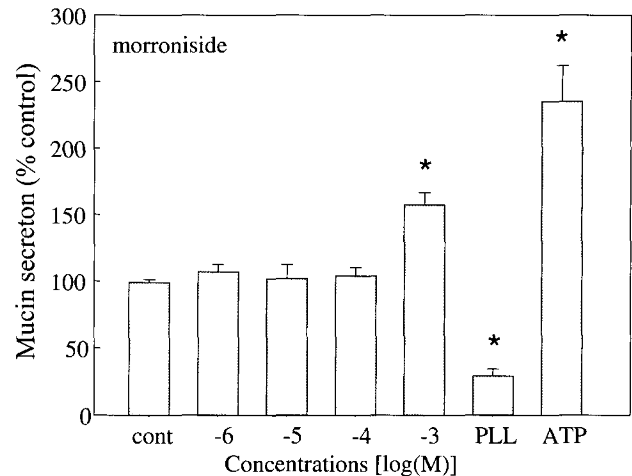

Fig. 3. Effect of morroniside on mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of morroniside. For comparison, both 200 μM of ATP, a well-known mucin secretagogue and 10 μM of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion were used as positive controls. The amounts of ^{3}H-mucins in the spent media were measured as described in METHODS. Each bar represents a mean S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p < 0.05$). ATP, adenosine triphosphate; PLL, poly-L-lysine.

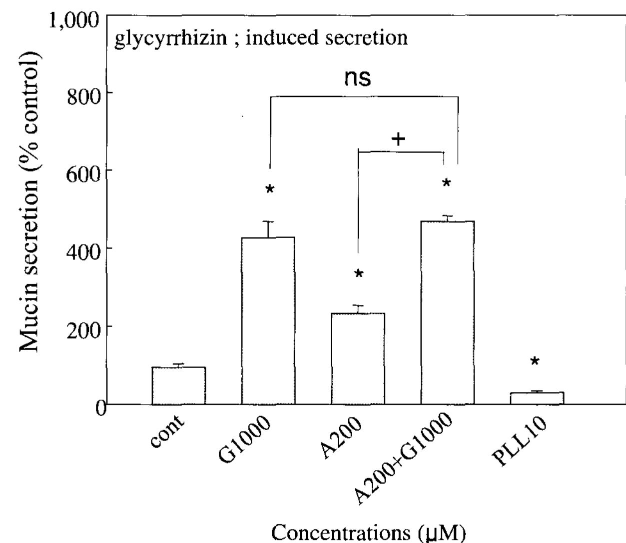

Fig. 4. Effect of glycyrrhizin on ATP-induced mucin secretion. Confluent HTSE cells were metabolically radiolabeled with ^{3}H-glucosamine for 24 h and chased for 30 min in the presence of varying concentrations of each agent. For comparison, 10 μM of PLL (MW 7,500) which is reported to be an inhibitor of mucin secretion was used as positive control. The amounts of ^{3}H-mucins in the spent media were measured as described in METHODS. Each bar represents a mean S.E.M. of four culture wells in comparison with that of control set at 100%. *significantly different from control ($p < 0.05$). $^+$significantly different from 200 μM of ATP alone ($p < 0.05$). Cont, control; A, ATP, adenosine triphosphate; G, glycyrrhizin; PLL, poly- L-lysine.

controls (Fig. 2).

Effect of morroniside on mucin secretion

Morroniside stimulated mucin secretion slightly during 30 min of treatment period. The amounts of mucin in the spent media of drug-treated cultures were 100±2%, 108±5%, 103±10%, 105±6% and 158±9% for control, 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively. For comparison, both 2.0×10^{-4} M ATP and 10^{-5} M PLL (MW 7,500) were used as positive controls (Fig. 3).

Effect of glycyrrhizin on ATP-induced mucin secretion

As shown in Fig. 4, glycyrrhizin did not inhibit ATP-induced mucin secretion and maintained the original activity on basal mucin secretion. The amounts of mucin in the spent media of glycyrrhizin-treated cultures were 100±7%, 430±41%, 236±20%, 472±13% and 30 5% for control, glycyrrhizin 10^{-3} M, 2×10^{-4} M of ATP alone, 2×10^{-4} M of ATP plus glycyrrhizin 10^{-3} M and PLL 7,500 (positive control) 10^{-5} M, respectively (Fig. 4).

DISCUSSION

Compounds derived from Glycyrrhizae Radix showed diverse biological effects (Jang, 2003). Glycyrrhizin showed anti-viral activity on corona virus, a SARS (specific acute respiratory syndrome)-associated virus through inhibiting the replication of virus (Cinatl et al, 2003; Chen et al, 2004). Yu et al. reported that glycyrrhizin reduced the severity of rapid shock by bacterial endotoxin. They suggested glycyrrhizin should be used as an agent alleviating the acute respiratory distress syndrome observed in sepsis (Yu et al, 2005). However, to the best of our knowledge, there is no report about a trial of investigating the effect of compounds derived from Glycyrrhizae Radix on hypersecretion of mucus observed in diverse respiratory diseases. Thus, we tried to investigate the possible effects of glycyrrhizin and prunetin - another compound originated from Glycyrrhizae Radix - on mucin secretion from airway. As shown in results, glycyrrhizin significantly increased basal mucin secretion (Fig. 1). However, prunetin did not show any activity (Fig. 2). On the other hand, it is expected that local extracellular ATP concentrations in the airway can reach high levels from lysed epithelial or inflammatory cells during airway inflammation, since intracellular ATP concentrations are greater than 5 mM (Gordon, 1986) and ATP has been reported to stimulate mucin secretion from airway (Kim et al, 1997). Therefore, we tried to test the effect of glycyrrhizin on induced mucin secretion by ATP. However, glycyrrhizin did not inhibit ATP-induced mucin secretion and maintained the original activity on basal mucin secretion, during 30 min of treatment period (Fig. 4). Also, there is no report about the effect of morroniside, an iridoid compound originated from Corni Fructus. Thus, we checked the activity of morroniside on mucin secretion from airway. As shown in Fig. 3, morroniside slightly increased airway mucin secretion at the highest concentration. This result explains, at least in part, the scientific background of using Glycyrrhizae Radix and Corni Fructus as herbal expectorants in oriental folk medicine. That is to say, they may help the removal of airway mucus by inducing cough due to increased secretion of mucus. The underlying mechanisms of action of glycyrrhizin and morroniside on basal and ATP-induced mucin secretion are not clear at present and should be elucidated through future research. Based on our results, we failed to find a natural compound that showed a remarkable inhibitory action on mucin secretion. However, from the viewpoint of management of airway mucus hypersecretion, it is of a great interest to find the possible inhibitory effects of novel compounds on mucin secretion. Additionally, the result from this study suggests glycyrrhizin and morroniside can be used as expectorants for acute upper respiratory tract infections, though mechanisms of action should be elucidated through further studies.

ACKNOWLEDGEMENT

This work was supported by 2005 and 2006 grant of Korea Food and Drug Administration.

REFERENCES

Chen F, Chan KH, Jiang Y, Kao RY, Lu HT, Fan KW, Cheng VC, Tsui WH, Hung IF, Lee TS, Guan Y, Peiris JS, Yuen KY. In vitro susceptibility of 10 clinical isolates of SARS coronavirus to selected antiviral compounds. *J Clin Virol* 31: 69–75, 2004

Cinatl J, Morgenstern B, Bauer G, Chandra P, Rabenau H, Doerr HW. Glycyrrhizin, an active component of liquorice roots, and replication of SARS–associated coronavirus. *Lancet* 361: 2045–2046, 2003

Ellis EF. Asthma in childhood. *J Allergy Clin Immunol* 72: 526–539, 1985

Gordon JL. Extracellular ATP: effects, sources and fate. *Biochem J* 233: 309–319, 1986

Jang IM. *Treatise on Asian Herbal Medicines.* 1st ed. Haksulpyunsu-kwan in Research Institute of Natural Products of Seoul National University, Seoul, p 2103, 2800, 3655, 2003

Kim KC, McCracken K, Lee BC, Shin CY, Jo MJ, Lee CJ, Ko KH. Airway goblet cell mucin: its structure and regulation of secretion. *Eur Respir J* 10: 2644–2649, 1997

Kim KC, Rearick JI, Nettesheim P, Jetten AM. Biochemical characterization of mucous glycoproteins synthesized and secreted by hamster tracheal epithelial cells in primary culture. *J Biol Chem* 260: 4021–4027, 1985

Kim KC, Wasano K, Niles RM, Schuster JE, Stone PJ, Brody JS. Human neutrophil elastase releases cell surface mucins from primary cultures of hamster tracheal epithelial cells. *Proc Natl Acad Sci USA* 84: 9304–9308, 1987

Ko KH, Lee CJ, Shin CY, Jo M, Kim KC. Inhibition of mucin release from airway goblet cells by polycationic peptides. *Am J Physiol* 277: L811–L815, 1999

Lee CJ, Lee JH, Seok JH, Hur GM, Park JS, Bae S, Lim JH, Park YC. Effects of betaine, coumarin and flavonoids on mucin release from cultured hamster tracheal surface epithelial cells. *Phytother Res* 18: 301–305, 2004a

Lee CJ, Lee JH, Seok JH, Hur GM, Park YC, Seol IC, Kim YH. Effects of baicalein, berberine, curcumin and hesperidin on mucin release from airway goblet cells. *Planta Med* 69: 523–526, 2003

Lee CJ, Paik SH, Ko KH, Kim KC. Effects of polycationic peptides on mucin release from airway goblet cells: relationship between polymer size and activity. *Inflamm Res* 51: 490–494, 2002

Lee CJ, Seok JH, Hur GM, Lee JH, Park JS, Seol IC, Kim YH. Effects of ursolic acid, betulin and sulfur-containing compounds on mucin release from airway goblet cells. *Planta Med* 70: 1119–1122, 2004b

Mutschler E, Derendorf H. *Drug Actions.* Boca Raton, CRC press, Florida, p 410–411, 1995

Newhouse MT, Biennenstock J. Respiratory tract defense mechanism. In: Baum GL, Wolinsky E ed, *Textbook of Pulmonary Disease*. 3rd ed. Little Brown and Company, Boston, Massachusetts,1983

Wasano K, Kim KC, Niles RM, Brody JS. Membrane differentiation markers of airway epithelial secretory cells. *J Histochem Cytochem* 36: 167−178, 1988

Yu Z, Ohtaki Y, Kai K, Sasano T, Shimauchi H, Yokochi T, Takada H, Sugawara S, Kumagai K, Endo Y. Critical roles of platelets in lipopolysaccharide−induced lethality: effects of glycyrrhizin and possible strategy for acute respiratory distress syndrome. *Int Immuno Pharmacol* 5: 571−580, 2005