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REV 2871* (CHBZ): A POTENT ANTIALLERGIC AGENT WITH A NOVEL MECHANISM OF ACTION

I. ACTIVITY PROFILE AS AN INHIBITOR OF MEDIATOR RELEASE†

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Abstract—REV 2871 (CHBZ) and its putative metabolite REV 3579-Z (also designated in the literature as RHC 3579-Z) were shown to be potent and orally effective inhibitors of passive cutaneous anaphylaxis (PCA) in the rat (ED_{50} = 12 mg/kg). The activity profiles of CHBZ, REV 3579-Z and disodium cromoglycate (DSCG) were compared as inhibitors of histamine release (HR) *in vitro* from rat mast cells, human basophils, and guinea pig lung slices. CHBZ was a potent inhibitor of both immunologic and non-immunologic HR (I_{50} 2–20 μM from rat mast cells). The activity profile of CHBZ as an inhibitor of HR from rat mast cells differed from that of DSCG and REV 3579-Z in the following respects: (a) increasing inhibition of HR with increasing preincubation time; (b) irreversibility of the inhibition; (c) lack of tachyphylaxis and cross-tachyphylaxis to DSCG; (d) potentiation of the inhibition of antigen-induced release of histamine (AIR) by DSCG; and (e) inhibition of HR induced by dextran + phosphatidyl serine, compound 48/80, ionophore A23187 and platelet activating factor (PAF). In the human basophil model, CHBZ was: (a) a potent inhibitor (I_{50} = 25 μM) of anti-IgE-induced release (AbIR), whereas DSCG and REV 3579-Z had no effect on AbIR; (b) more potent as an inhibitor of AbIR than ionophore-induced release, whereas the reverse was true for proxicromil; (c) an inhibitor of PAF-induced release, whereas proxicromil stimulated it; and (d) potentiative with proxicromil for inhibition of AbIR. In the guinea pig lung slice model, CHBZ inhibited AIR (I_{50} = 800 μM) whereas DSCG and REV 3579-Z did not (I_{50} > 300 μM). We conclude that CHBZ is an orally effective antiallergic agent whose mechanism of action as an inhibitor of mediator release is different from DSCG and proxicromil.

Disodium cromoglycate (DSCG¶) is an antiallergic drug, used prophylactically in the treatment of allergic diseases such as asthma, rhinitis, conjunctivitis, atopic eczema and allergic reactions to foods [1–6]. DSCG inhibits, both *in vitro* and *in vivo*, IgE-mediated secretion of vasoactive mediators from mast cells [7–9]. However, the therapeutic utility of DSCG has been limited because DSCG is ineffective given orally due to its poor absorption from the gastrointestinal tract [2]. As a result, many laboratories over the past 15 years have discovered orally effective inhibitors of mediator release which are in various stages of preclinical or clinical development [10–20]. However, with one exception, all of these newer antiallergic agents appear to inhibit IgE-mediated secretion of histamine from mast cells by a mechanism similar if not identical to that of DSCG as evidenced by their identical activity profiles *in vitro* and cross-tachyphylaxis to DSCG [14, 20]. The only exception is tiaramide, whose *in vitro* activity profile is different from DSCG and which does not exhibit cross-tachyphylaxis to DSCG as an inhibitor of AIR from rat mast cells [21].

2-Ethoxyethyl 5-chloro-benzoxazole-2-carboxylate (CHBZ, REV 2871) (Fig. 1) is a new orally effective antiallergic agent. In this paper we describe the novel *in vitro* activity profile of CHBZ and its putative metabolite, REV 3579-Z, as inhibitors of immunologic and non-immunologic HR from rat mast cells, human basophils and guinea pig lung slices. The accompanying paper [22] discusses the novel mechanism of action of CHBZ as an inhibitor of mediator release.

MATERIALS AND METHODS

Immunologic release of histamine from rat mast cells

Inhibition of antigen-induced release. Peritoneal cells (8–12% mast cells) from four to six rats were passively sensitized with rat antiovalbumin serum (diluted 1:8) *in vitro* at 37° for 2.5 hr. Spontaneous HR (SR, in the absence of antigen) or AIR from sensitized rat mast cells was measured. The cells were incubated with test compound for the indicated

* Also designated in the literature as RHC 2871.

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¶ Abbreviations: DSCG, disodium cromoglycate; CHBZ, REV 2871; HR, histamine release; SR, spontaneous release of histamine; AIR, antigen-induced release of histamine; AbIR, anti-IgE-induced release of histamine; DMSO, dimethyl sulfoxide; PAF, platelet-activating factor; PS, phosphatidyl serine; and PCA, passive cutaneous anaphylaxis.

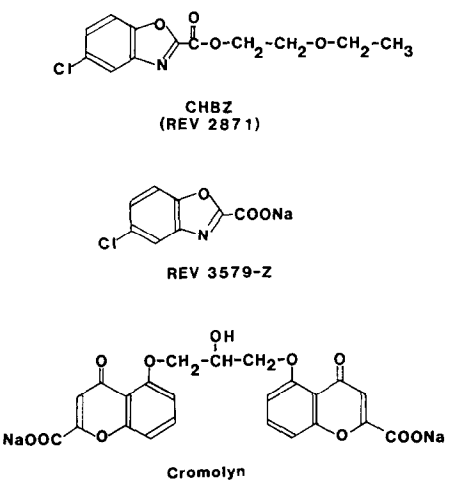

Fig. 1. Structures of CHBZ and REV 3579-Z. CHBZ: 2-ethoxyethyl 5-chloro-benzoxazole-2-carboxylate; and REV 3579-Z: 5-chlorobenzoxazole-2-carboxylic acid (sodium salt).

period of time at 37° prior to the addition of ovalbumin (final concentration 50 μg/ml) to initiate HR. Both the histamine released into the supernatant fraction and the residual histamine extracted from the rat mast cells were quantitated by an automated fluorometric method with a Technicon AutoAnalyzer. Both the SR and the AIR are expressed as percent of total cellular histamine. The compound activity is expressed as percent inhibition of control AIR or its I_{50} value (concentration required for 50% inhibition of AIR). This procedure has been described in detail previously [14, 21].

Inhibition of anti-IgE-induced release of histamine. To compare the effects of CHBZ on AbIR from purified and unpurified mast cells, rat mast cells were purified by sedimentation through 22.5% Metrizamide as described previously [23]. CHBZ was preincubated with cells for 5 min at 37° prior to the addition of rabbit anti-rat IgE (diluted 1:100) to initiate the release of histamine.

Reversibility of the inhibition of AIR by CHBZ. In these experiments, the inhibition of AIR by CHBZ was determined with a 5-min preincubation using untreated rat mast cells, as well as rat mast cells preincubated for 5 min with CHBZ, washed three times, and then challenged with antigen.

Cross-tachyphylactic properties of CHBZ and REV 3579-Z. In these experiments the inhibition of AIR by DSCG and REV 3579-Z (added simultaneously with antigen) and by CHBZ (5-min preincubation) was determined with untreated rat mast cells and with rat mast cells preincubated with DSCG for 20 min. The procedure has been described in detail previously [14].

Potentiation of the activity of DSCG by CHBZ. In these experiments the effect of pretreatment of sensitized rat mast cells with CHBZ for 5 min on the inhibition of AIR by DSCG was determined. The concentration–response curves for DSCG were determined with untreated sensitized rat mast cells and with sensitized rat mast cells preincubated with 0.1 μM CHBZ.

Non-immunologic release of histamine from rat mast cells

The HR was initiated by the addition of dextran (6 mg/ml) + PS (10 μg/ml), compound 48/80 (0.1 μg/ml), Ca^{2+} ionophore A23187 (0.01 to 0.3 μg/ml), or the platelet-activating factor (PAF, 10 μM) to washed rat mast cells. The activity of test compound was determined with either 5-min preincubation of the test compound with rat mast cells prior to the addition of the secretagogue or by the addition of the test compound simultaneously with the secretagogue. The procedure has been described in detail previously [14, 21].

Immunologic release of histamine from human basophils

Anti-IgE-induced release. The effect of test compounds on AbIR from human basophils was determined as described in detail previously [21]. Washed basophils from approximately 1 ml of blood were suspended in 2.9 ml of buffer. The cells were incubated with the test compound for 5 min, prior to the addition of 100 μl of goat anti-human IgE (100 μg/ml) to initiate HR. The incubation was continued for 60 min following which the histamine released into the supernatant fraction as well as residual cellular histamine were determined with a Technicon AutoAnalyzer II equipped with a special histamine manifold (Cat. No. 116-0746-01).

Reversibility of the inhibition of AbIR by CHBZ and proxicromil. Washed basophils were incubated with various concentrations of CHBZ or proxicromil for 10 min at 37°. The cells were then washed three times, suspended in fresh buffer, and challenged with anti-IgE to initiate HR.

Potentiation of the activity of proxicromil by CHBZ. In this experiment, the concentration–response curve for proxicromil was determined in the presence and absence of 10 μM CHBZ. Again, a 5-min preincubation for all compounds or mixtures was employed prior to immunological challenge with anti-IgE.

Non-immunologic release of histamine from human basophils

Washed basophils prepared as above were challenged with 0.5 μg of Ca^{2+} ionophore A23187 to initiate HR. CHBZ and proxicromil were incubated with basophils for 5 min prior to the addition of the secretagogue. The HR was allowed to proceed for 60 min following which residual and released histamine were quantitated by the automated fluorometric method.

Guinea pig lung slices

Briefly, lung slices from unsensitized guinea pigs were passively sensitized with guinea pig anti-ovalbumin serum (diluted 1:8) *in vitro* at 37° for 2.5 hr, washed, and challenged with ovalbumin (10 μg/ml) to initiate release of histamine. Test compounds were preincubated with sensitized lung slices for 5 min prior to the addition of antigen. This procedure has been described in detail previously [21, 24].

Data analysis for in vitro *experiments*

Calculation of percent inhibition of AIR involves

errors associated with four parameters, i.e. SR and AIR in the presence and the absence of the test compound. Therefore, the maximum percentage error of the measured quantities was calculated by substituting standard deviation of replicate values (9 for control and 3 with the test compound) into differentials of equations used in calculating results according to Daniels *et al.* [25]. A lack of overlap between maximum errors of two points, therefore, indicates a difference in the means of greater than two standard deviations, i.e. the difference is significant at the 5% level.

Materials

The synthesis, structure determination, and chemical purity of CHBZ and REV 3579-Z were carried out in the Medicinal Chemistry Department of the Revlon Health Care Group.* Proxicromil and DSCG were gifts from the Fisons Corp., Bedford, MA. Theophylline, Ca^{2+} ionophore A23187, compound 48/80, dextran, phosphatidyl serine, PAF, histamine, serotonin, ethylenediamine tetra acetic acid (EDTA), and zwitterionic buffers were purchased from the Sigma Chemical Co., St. Louis, MO. Rabbit anti-rat IgE and goat anti-human IgE were purchased from Meloy Laboratories, Springfield, VA. All other chemicals were reagent grade or better and were purchased commercially.

RESULTS

Immunologic release of histamine from rat mast cells

Antigen-induced release. The average SR and AIR values (±SE) for all experiments were 2.5 ± 1.6 and 26.6 ± 2.1% (N = 142) respectively. In the initial experiments (data not shown), comparative concentration–response curves for the inhibition of AIR by CHBZ, DSCG and REV 3579-Z were determined using a common pool of sensitized rat mast cells. These data indicated that: (a) none of the compounds significantly affected SR at concentrations of up to 300 μM; (b) added simultaneously with antigen, CHBZ and REV 3579-Z, with I_{50} values of 5 and 18 μM, respectively, were approximately 3 and 10 times less potent than DSCG as inhibitors of AIR, and (c) when preincubated with sensitized rat mast cells for 5 min prior to the addition of antigen, CHBZ inhibited AIR, whereas REV 3579-Z and DSCG had no effect. The data from several experiments show that, when added simultaneously with antigen, both CHBZ and REV 3579-Z were potent inhibitors of AIR with average I_{50} values of 5.6 and 21.0 μM respectively (Table 1). However, when these compounds were preincubated with rat mast cells for 5 min prior to the addition of antigen, REV 3579-Z did not affect AIR significantly, whereas CHBZ inhibited AIR in a concentration-dependent manner with an average I_{50} value of 1.6 ± 0.2 μM (N = 93, Table 1).

Anti-IgE-induced release. Since the peritoneal cells used to assess the effects of compounds on AIR contained only 8–12% mast cells, the effects of CHBZ on HR from purified mast cells and unpurified peritoneal cells were compared. Purification of peritoneal cells yielded a cell preparation consisting of >90% mast cells (as judged by metachromatic staining with toluidine blue) and a cell viability index of >90% (as judged by trypan blue exclusion).

CHBZ was preincubated with cells for 5 min and HR was elicited by anti-IgE (reversed anaphylaxis), since it proved difficult to passively sensitize purified rat mast cells in a consistent and reproducible manner. Both unpurified and purified rat mast cells released the same net amount of histamine (21%) upon challenge with goat anti-rat IgE, and CHBZ inhibited AbIR from both unpurified and purified rat mast cells with similar I_{50} values of 0.8 and 1.0 μM respectively. The average I_{50} value for CHBZ as an inhibitor of anti-IgE-induced release of histamine from unpurified rat mast cells was 2.0 μM (Table 1).

Effect of preincubation time on the inhibition of AIR. The results (Fig. 2) show that the inhibition of AIR by REV 3579-Z (10 μM) and DSCG (3 μM) decreased rapidly with increasing preincubation time. Both REV 3579-Z and DSCG inhibited AIR maximally when they were added simultaneously with antigen. Preincubation of REV 3579-Z or DSCG with sensitized rat mast cells for 5 min prior

* F. Huang *et al.*, manuscript submitted for publication. Revlon Health Care Group R&D was merged into Rorer Central Research in 1986.

Table 1. Effects of REV 2871 (CHBZ), 3579-Z and DSCG on histamine release from rat mast cells

	I_{50} (μM)					
	CHBZ		REV 3579-Z		DSCG	
Secretagogue	0 Min*	5 Min†	0 Min	5 Min	0 Min	5 Min
Antigen	5.6 ± 1.8 (14)‡	1.6 ± 0.2 (93)	21.0 ± 10 (9)	>100 (4)	4.5 ± 0.5 (96)	>300 (4)
Anti-IgE		2.0 ± 0.9 (4)			9.8 ± 0.8 (9)	
Compound 48/80	25.0 ± 6.2 (4)	3.1 ± 0.8 (10)	>100 (1)	>100 (1)	>00 (17)	>500 (1)
Ionophore A23187	38.3 ± 7.2 (3)	5.6 ± 0.3 (9)	>100 (1)	>100 (1)	>1000 (7)	>1000 (1)
Dextran + phosphatidyl serine	2.6 (1)	1.1 ± 1.0 (10)	>100 (2)	>100 (2)	>1000 (3)	>1000 (1)
Platelet-activating factor		20 ± 10 (2)	>100 (2)		>100 (2)	

* Compound added simultaneously with secretagogue.
† Compound preincubated with cells for 5 min prior to the addition of secretagogue.
‡ Average I_{50} values ± SE of (N) experiments carried out in triplicate.

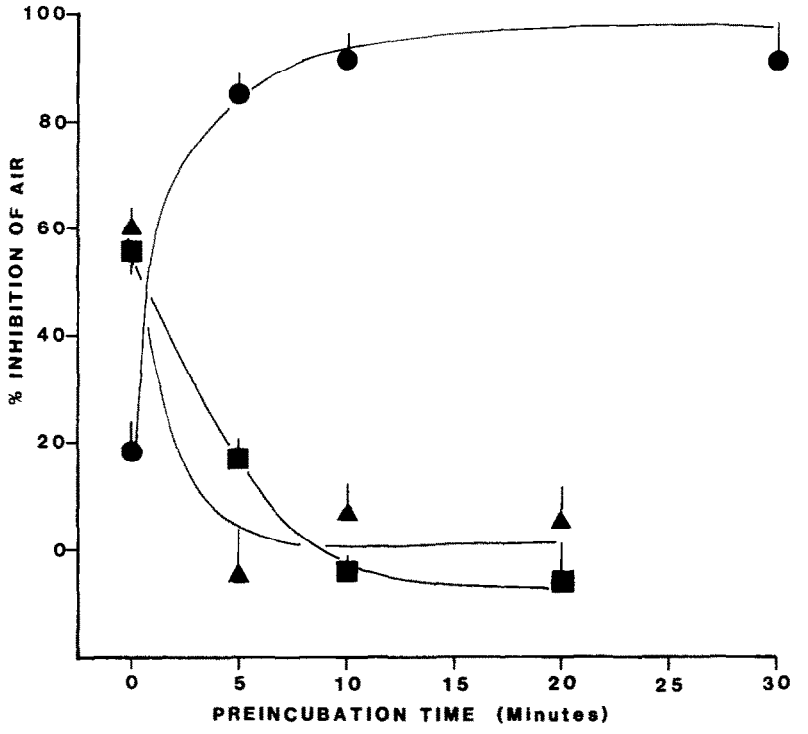

Fig. 2. Effect of preincubation time on the inhibition of antigen-induced release of histamine (AIR) from rat mast cells by CHBZ, 3579-Z and DSCG. Compounds were incubated with rat mast cells for the indicated period of time prior to the addition of antigen. Key: (●) CHBZ (3 μM, N = 3); (▲) REV 3579-Z (10 μM, N = 1); and (■) DSCG (3 μM, N = 7). Each point represents the average ± SE of (N) experiments carried out in triplicate, except for REV 3579-Z for which each point represents the average ± maximum error of triplicates.

to the addition of antigen resulted in 100 and 70% loss of their inhibitory activity respectively. In contrast, the inhibition of AIR by CHBZ increased with increasing preincubation time, with the optimal inhibition of AIR being achieved with 5–10 min preincubation of CHBZ with sensitized cells prior to the addition of antigen. Thus, with respect to the relationship between the preincubation time and inhibition of AIR, REV 3579-Z was similar to DSCG, whereas CHBZ differed from DSCG.

Reversibility of the inhibition of AIR by CHBZ. It has been reported that the inhibition of AIR by

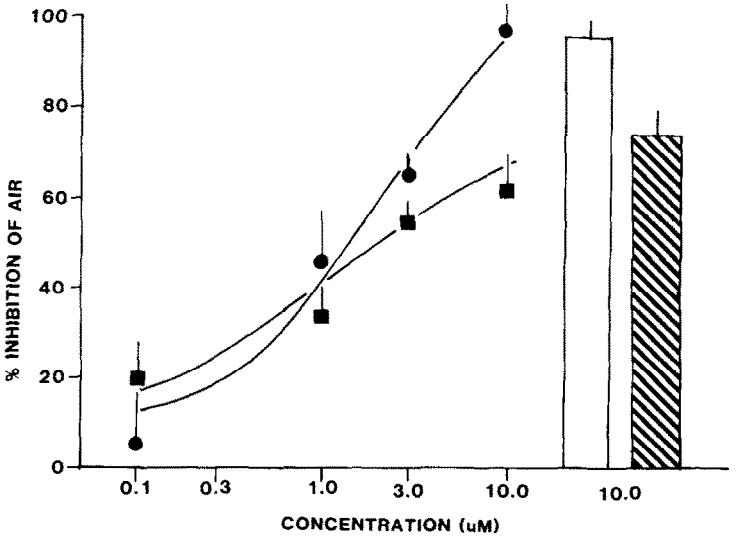

Fig. 3. Irreversibility of the inhibition of AIR from rat mast cells by CHBZ. Key: (●, □) CHBZ incubated with cells for 5 min prior to the addition of antigen (each point represents the average ± maximum errors of triplicates); and (■, ▨) cells incubated with CHBZ for 5 min, washed three times, and then challenged with antigen; each value is the average ± SE of three experiments carried out in triplicate.

DSCG is reversible [14]. In a similar experiment, the concentration–response curves for CHBZ were determined with untreated cells and with cells incubated with CHBZ for 5 min, washed three times, and then challenged with antigen. The results (Fig. 3) show that the inhibition of AIR by 0.1, 1 and 3 μM CHBZ was not reversible. Since there was a suggestion that the inhibition of AIR by 10 μM CHBZ may be reversible, the reversibility of the inhibition of AIR by 10 μM CHBZ was determined in two additional experiments. The results, depicted in Fig. 3 in the form of a bar graph, show that CHBZ (10 μM) inhibited AIR by 96.1 ± 4.8%, whereas the cells incubated with 10 μM CHBZ for 5 min, washed three times, and then challenged with antigen inhibited the AIR by 74.6 ± 13.5%. This amounts to a statistically significant ($P < 0.05$) but a small loss in the inhibition of AIR by the washing procedure. These results taken as a whole indicate that the effect of CHBZ on AIR from mast cells is largely irreversible.

Tachyphylactic properties of CHBZ, REV 3579-Z and DSCG. DSCG and other "DSCG-like" antiallergic agents have been reported to exhibit tachyphylaxis or self-inhibitory properties [8, 14, 20]. Preliminary experiments confirmed the tachyphylactic property of DSCG and indicated that REV 3579-Z also exhibited tachyphylactic properties (data not shown).

Since the preincubation-time profile of CHBZ was different from that of DSCG or REV 3579-Z (Fig. 2), it was not possible to carry out "typical" tachyphylaxis experiments with CHBZ. Instead, the concentration–response curves for CHBZ were determined with the untreated cells and cells preincubated with the indicated concentration of CHBZ for 5 min. The results (data not shown) indicated that preincubation of CHBZ with cells for 5 min did not have any effect on the inhibition of AIR by a second addition of CHBZ, indicating that CHBZ does not exhibit tachyphylactic properties.

Cross-tachyphylactic properties of CHBZ, REV 3579-Z and DSCG. The effect of pretreatment of rat mast cells with DSCG on the inhibition of AIR by CHBZ and REV 3579-Z was determined to assess their cross-tachyphylactic properties. The results (Fig. 4A and B) show that: (a) DSCG inhibited AIR and exhibited tachyphylaxis, (b) 30 and 100 μM REV 3579-Z added with antigen to untreated rat mast cells inhibited AIR by 52 and 83%, respectively, and (c) REV 3579-Z added with antigen to cells preincubated with DSCG for 20 min did not inhibit AIR significantly. This desensitization of rat mast cells to the inhibition of AIR by REV 3579-Z upon prior exposure of the cells to DSCG is a clear demonstration that REV 3579-Z is cross-tachyphylactic to DSCG. The results (Fig. 4C and D) of a similar experiment with CHBZ and DSCG showed that CHBZ inhibited AIR from untreated cells and cells preincubated with DSCG by 75 and 71% respectively. Thus, CHBZ was not cross-tachyphylactic to DSCG.

Potentiation of the activity of DSCG by CHBZ. Since CHBZ was not cross-tachyphylactic to DSCG, the effect of pretreatment of CHBZ on the inhibition of AIR by DSCG was determined. First the concentration–response curves for DSCG were determined with untreated rat mast cells as well as rat mast cells preincubated for 5 min with 0.1 μM CHBZ. The results (Fig. 5) show that DSCG inhibited AIR from untreated rat mast cells with an I_{50} value of 6 μM. Preincubation of rat mast cells with 0.1 μM CHBZ for 5 min made the cells more sensitive to the inhibitory effect of DSCG as judged by the shift in the concentration–response curve of DSCG to the left (Fig. 5), and to an I_{50} value of 1.4 μM. These results clearly demonstrate the potentiative effect of CHBZ on the inhibition of AIR by DSCG.

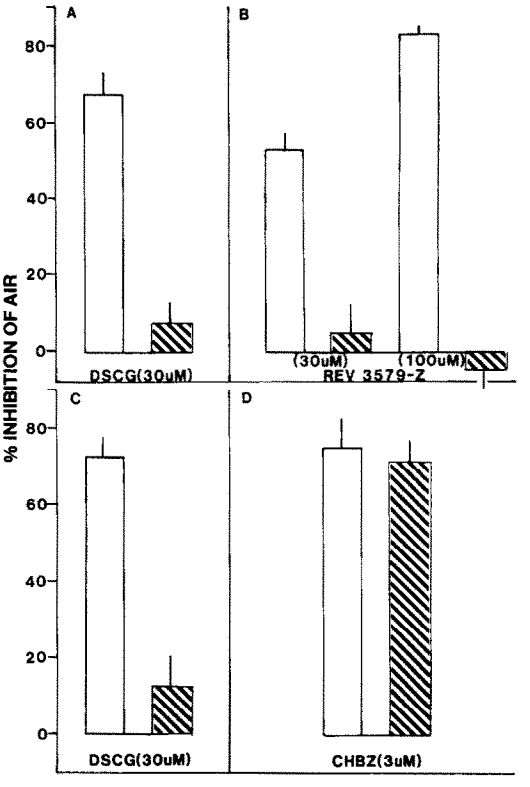

Fig. 4. Cross-tachyphylaxis of CHBZ and REV 3579-Z to DSCG as inhibitors of AIR from rat mast cells. (A, B, and C): (□) test compound added simultaneously with antigen to rat mast cells; (▨) test compound added simultaneously with antigen to rat mast cells preincubated with 30 μM DSCG for 20 min. Values are the average ± maximum error of triplicates. (D): CHBZ preincubated with rat mast cells for 5 min prior to the addition of antigen; (□) untreated cells; and (▨) cells preincubated with 30 μM DSCG for 20 min (in the absence of antigen). Values are the average ± maximum error of triplicates.

Non-immunologic release of histamine from rat mast cells

Dextran + phosphatidyl serine-induced release. A non-immunologic secretagogue which induces the release of histamine from rat mast cells and is similar to but not identical in characteristics to antigen as a secretagogue is dextran. The release of histamine from rat mast cells by dextran is extremely small but is potentiated by PS, an acidic phospholipid [26]. The average (±SE) net HR induced by dextran and PS was 46.4 ± 4.8%. The average I_{50} values for

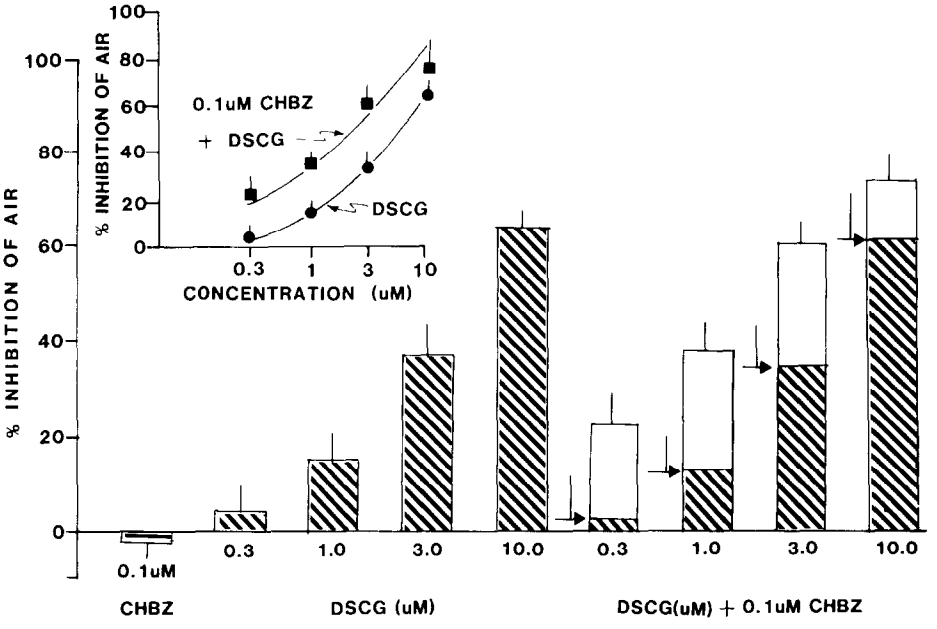

Fig. 5. Potentiation of the activity of DSCG as an inhibitor of AIR from rat mast cells by CHBZ. Key: (▤) CHBZ preincubated with rat mast cells for 5 min prior to the addition of antigen, (□) DSCG added simultaneously with antigen, and (▨) DSCG added simultaneously with antigen to rat mast cells incubated for 5 min with CHBZ in the absence of antigen. Hashed portion of DSCG + CHBZ bars represents calculated additive response of DSCG + CHBZ (also indicated by arrows ± maximum error). Each bar represents the average ± maximum error of triplicates. Arrows in the bar graph indicate calculated additive response by DSCG ± 0.1 μM CHBZ ± maximum error. (Inset) Replot of bar graph data. Each point represents the average ± maximum error of triplicates.

CHBZ as an inhibitor of dextran + PS-induced HR from rat mast cells were 2.6 and 1.1 μM, with 0-min and 5-min preincubation respectively (Table 1). In contrast, both DSCG and REV 3579-Z did not inhibit dextran + PS-induced HR (Table 1).

Compound 48/80-induced release. Compound 48/80, a polyamine, is a potent releaser of histamine from both rat mast cells and guinea pig lung slices [27, 28]. The average I_{50} values (Table 1) show that CHBZ was approximately 8 times more potent as an inhibitor of compound 48/80-induced HR from rat mast cells with 5-min preincubation (I_{50} = 3 μM) than with 0-min preincubation (I_{50} = 25 μM). In contrast, DSCG or REV 3579 (1–100 μM, added simultaneously with the secretagogue) had no effect on compound 48/80-induced HR from rat mast cells (Table 1). Even at a concentration of 500 μM, DSCG inhibited 0.1 μg/ml compound 48/80-induced HR by only 28 ± 9% (average ± SE, N = 17).

Ca^{2+} ionophore-A23187-induced release. Another non-immunologic secretagogue is a divalent cation specific ionophore, A23187. The ionophore stimulates HR from rat mast cells by facilitating influx of Ca^{2+} into the cells [29]. It is clear from Fig. 6 and Table 1 that CHBZ was approximately 7 times more potent as an inhibitor of ionophore-A23187-induced HR with 5-min preincubation (I_{50} = 5.6 μM) than with 0-min preincubation (I_{50} = 38 μM). In contrast, neither REV 3579-Z nor DSCG had a significant effect on 0.05 μg/ml ionophore-induced HR from rat mast cells (Table 1).

Next, the effect of various concentrations of ionophore on the inhibition of HR by CHBZ was investigated. The results (Fig. 6, inset) show that the net HR induced by the ionophore increased from 11.2 to 81.8% as the concentration of ionophore was increased from 0.01 to 0.3 μg/ml. The calculated I_{50} values of 5 and 3 μM for CHBZ as an inhibitor of 0.01 and 0.03 μg/ml ionophore-induced HR were similar, even though the net HR by 0.03 μg/ml ionophore (70 ± 1.3%) was 6 times greater than that promoted by 0.01 μg/ml ionophore (11.2 ± 1.5%). In contrast, when the ionophore concentration was further increased from 0.03 to 0.3 μg/ml, there was only a 16% increase in the net HR (from 70 ± 1.3 to 81.8 ± 1.2%) but an 8-fold increase in the I_{50} value of CHBZ (from 3 to 25 μM) as an inhibitor of ionophore-induced HR. Furthermore, the maximum inhibition by 30 μM CHBZ also decreased from 84% for 0.03 μM ionophore-induced HR to 56.6% for 0.3 μg/ml. These results, together with the observation [30] that ionophore at concentrations greater than 0.2 μg/ml is cytolytic for rat mast cells, suggest that as long as the HR from rat mast cells was non-cytolytic, the effectiveness of CHBZ as an inhibitor of HR was not dependent upon the net amount of HR.

Platelet-activating factor-induced release. PAF has been reported to be released from IgE-sensitized basophils and is also a secretagogue for the release of histamine from mast cells, basophils and platelets [31]. The average 10 μM PAF-induced HR (±SD) was 70.8 ± 11.7% (N = 2). CHBZ, when pre-incubated for 5 min with rat mast cells prior to the

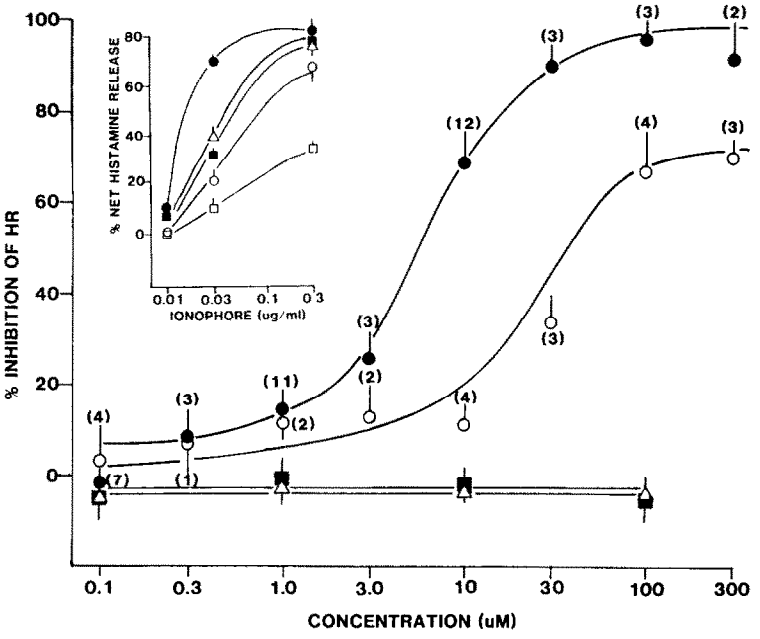

Fig. 6. Effects of CHBZ and REV 3579-Z on Ca^{2+} ionophore-induced histamine release (HR) from rat mast cells. The concentration of the ionophore was 0.05 μg/ml. The net HR induced by 0.05 μg/ml of ionophore in these experiments was 67.4 ± 5.2% (average ± SE). Key: (○) CHBZ and (△) REV 3579-Z added simultaneously with ionophore; and (●) CHBZ and (□) REV 3579-Z preincubated with rat mast cells for 5 min prior to the addition of ionophore. Each point represents the average ± SE of (N) experiments carried out in triplicate. REV 3579-Z data are the average ± SE maximum error of triplicates. (Inset) Effect of CHBZ on the HR from rat mast cells by various concentrations of the ionophore. Key: (●) control cells; (■) 1 μM, (△) 3 μM; (○) 10 μM, and (□) 30 μM CHBZ preincubated with rat mast cells for 5 min prior to the addition of ionophore. Each point represents the average ± SE of (N) experiments carried out in triplicate. REV 3579-Z data are the average ± SE maximum error of triplicates.

addition of PAF, inhibited HR in a concentration-dependent manner with an I_{50} value of 20 μM (Table 1). In contrast, neither DSCG (1–100 μM) nor REV 3579-Z (3–100 μM) had a significant effect on PAF-induced HR from rat mast cells when they were added to rat mast cells simultaneously with the PAF.

Immunologic release of histamine from human basophils

Anti-IgE-induced release. Preliminary experiments indicated that maximum net AbIR from human basophils was obtained with an anti-IgE concentration of 3.3 μg/ml. Therefore, the results described below were obtained with 3.3 μg/ml anti-IgE as the immunologic stimulus. The average SR (±SE) and net AbIR (±SE) from human basophils were 6.2 ± 0.3 and 41.4 ± 1.5% respectively (N = 90).

Neither CHBZ (1–300 μM) nor proxicromil (1–300 μM) had a significant effect on SR from human basophils. The average I_{50} values (Table 2) show that CHBZ was approximately twice as potent as proxicromil as an inhibitor of AbIR from human basophils. In contrast, REV 3579-Z (1.0 to 100 μM) did not inhibit AbIR significantly, and the inhibition of AbIR by 300 μM REV 3579-Z and 100 μM DSCG was a modest 33 ± 10 and 21 ± 8% respectively.

Effect of preincubation time on the inhibition of AbIR. The preincubation time profiles for CHBZ and proxicromil showed that the maximum inhibition of AbIR was obtained when CHBZ (40 μM) was preincubated for 5–10 min with human basophils

Table 2. Effects of REV 2871 (CHBZ) and proxicromil on histamine release from human basophils

	I_{50}* (μM)	
Secretagogue	CHBZ	Proxicromil
Anti-IgE (3.3 μg/ml)	25.0 ± 10.6 (23)	60.5 ± 15.2 (43)
Ionophore A23187 (0.5 μg/ml)	83.3 (1)	19.0 ± 1.0 (2)
PAF (10 μM)	60.0 (1)	†

* Values are average ± SE of (N) experiments.
† Proxicromil did not inhibit but stimulated PAF-induced release of histamine.

prior to the addition of anti-IgE. A further increase in the time of preincubation of CHBZ with human basophils from 10 to 60 min resulted in a gradual loss of the inhibitory activity of CHBZ. In contrast, the inhibition of AbIR by proxicromil (60 μM) was virtually independent of the time of preincubation (data not shown).

Reversibility of the inhibition of AbIR. In an initial experiment, human basophils preincubated with CHBZ for 5 min, followed by three washes with buffer, were compared with cells simply preincubated with CHBZ before challenge with anti-IgE. It was found that the washing procedure increased the I_{50} value for CHBZ about 3-fold, from 38 to 100 μM. In a second experiment, CHBZ and proxicromil (100 μM), which inhibited AbIR by 100 and 62%, respectively, prior to washing, inhibited AbIR by $\leq$10% following the washing procedure, thus confirming that the inhibition of AbIR by CHBZ and proxicromil was largely reversible.

Potentiation of the activity of proxicromil by CHBZ. In this experiment, the effect of CHBZ (10 μM) on the concentration–response curve of proxicromil as an inhibitor of AbIr was determined. CHBZ by itself did not inhibit AbIR significantly. However, 10 μM CHBZ caused a 3-fold decrease in the I_{50} value for proxicromil (from 31 to 11 μM) (data not shown). This result demonstrates that minimally effective concentrations of CHBZ potentiated that inhibition of AbIR by proxicromil.

Non-immunologic release of histamine from human basophils

Ca^{2+} *ionophore.* Ca^{2+} ionophore A23187 caused degranulation of human basophils in a concentration-dependent manner, with 0.5 μg/ml of ionophore being equivalent to 3.3 μg/ml of anti-IgE as a secretagogue. Relative potencies of CHBZ and proxicromil were determined using a common pool of cells challenged by calcium ionophore. The results (Table 2) show that CHBZ was more potent than proxicromil as an inhibitor of HR when the secretagogue was anti-IgE, whereas the reverse was true when HR was induced by ionophore.

Platelet-activating factor. PAF caused degranulation of human basophils in a concentration-related manner with 10 μM PAF being equivalent to 3.3 μg/ml of anti-IgE as a secretagogue.

CHBZ inhibited PAF-induced HR in a concentration-dependent manner with an I_{50} value of 60 μM. Thus, as an inhibitor of HR from human basophils CHBZ was approximately one-half as potent when the secretagogue was PAF rather than anti-IgE (Table 2). In contrast, proxicromil did not inhibit but rather stimulated PAF-induced HR in a concentration-related manner (data not shown), with a combination of 100 μM proxicromil and 10 μM PAF causing 87% of the cellular histamine to be released into the supernatant fraction.

Guinea pig lung slices

Antigen-induced HR from passively sensitized guinea pig lung slices is an *in vitro* model of anaphylaxis in which HR is mediated through the IgG_1 class of antibodies [20, 24, 32]. Therefore, the effects of CHBZ and REV 3579-Z on AIR from guinea pig lung slices were investigated. The results were: (a) isoproterenol (used as a positive control) inhibited AIR; (b) at concentrations of up to 300 μM, REV 3579-Z and DSCG did not significantly inhibit AIR from guinea pig lung slices; (c) CHBZ concentrations ($\leq$30 μM), which inhibited immunologic HR from rat mast cells and human basophils by more than 50%, did not have a significant effect on AIR; and (d) at higher concentrations CHBZ inhibited AIR in a concentration-dependent manner with an I_{50} value of 800 $\pm$ 100 μM.

DISCUSSION

Immunologic and/or non-immunologic HR from rat mast cells, human basophils and guinea pig lung slices are three *in vitro* models of anaphylaxis that are used to compare and contrast the antiallergic activity profile of a new agent with that of DSCG [20, 33]. The data presented here describe and compare the antiallergic activity profile of CHBZ and its putative metabolite REV 3579-Z with that of DSCG in the above-mentioned *in vitro* models of anaphylaxis.

Rat mast cells

Immunologic release. The experiment with purified mast cells and unpurified rat peritoneal cells confirmed earlier observations [23] that the histamine release into the supernatant fraction upon immunologic challenge comes from only the mast cells. Furthermore, the I_{50} values of 0.8 and 1.0 μM from unpurified peritoneal cells and purified mast cells, respectively, indicate that the presence of macrophages, monocytes and lymphocytes in the unpurified cells obtained by peritoneal lavage has no effect on the activity of CHBZ as an inhibitor of AIR from rat mast cells.

When added simultaneously with antigen, DSCG inhibited AIR from rat mast cells with an I_{50} value of 4.5 μM which is in agreement with previously reported I_{50} values of 3–10 μM [7, 8, 14, 20, 21]. Under the same conditions, CHBZ (I_{50} = 5.6 μM) and REV 3579-Z (I_{50} = 21 μM) were 0.8 and 0.2 times as potent as DSCG as inhibitors of AIR from rat mast cells. However, when test compounds were preincubated with rat mast cells for 5 min prior to the addition of antigen, DSCG, as has been reported earlier [14, 20], and REV 3579-Z had no effect on AIR, whereas CHBZ was a potent inhibitor of AIR with an average I_{50} value of 1.6 μM. CHBZ was thus approximately 4 times more potent as an inhibitor of AIR with 5-min preincubation than with 0-min preincubation. This observation is further supported by the results of preincubation time profiles which clearly demonstrate that the inhibition of AIR by CHBZ increased with increasing preincubation time, whereas that by both REV 3579-Z and DSCG decreased. Thus, with respect to preincubation time profile, CHBZ is clearly different from DSCG, indicating that CHBZ and DSCG may have a different mechanism of action. The results reported here indicate that, as an inhibitor of AIR from rat mast cells, the putative metabolite of CHBZ, REV 3579-Z, has an activity profile identical to that of DSCG in the following respects: (a) loss of inhibitory activity with

increasing preincubation time; (b) tachyphylactic properties; and (c) cross-tachyphylaxis to DSCG. In contrast, the activity profile of CHBZ as an inhibitor of AIR from rat mast cells was completely different from that of DSCG and REV 3579-Z in the following respects: (a) increase in inhibitory activity with increasing preincubation time; (b) largely irreversible inhibition of AIR; (c) lack of tachyphylaxis and, indeed, additive response of two additions of the drug 5 min apart; (d) lack of cross-tachyphylaxis to DSCG; and (e) potentiation of the activity of DSCG. The lack of cross-tachyphylaxis of CHBZ to DSCG and its potentiation of the activity of DSCG strongly support the conclusion that the mechanism of action of CHBZ as an inhibitor of AIR from rat mast cells is clearly different from DSCG and other "DSCG-like" antiallergic agents.

Non-immunologic release. In addition to immunologic stimulus, HR from rat mast cells can be elicited by a variety of non-immunologic secretagogues [33]. The most widely used among these are dextran + PS, compound 48/80, and the calcium ionophore A23187. It is reported that the dextran + PS-induced HR is similar in character to that induced by antigen [24, 34]. Compound 48/80 is reported to stimulate the HR by interaction with a specific receptor on the mast cell [27, 35]. The divalent cation specific ionophore A23187 stimulates the release process by facilitating the influx of Ca^{2+} into the mast cells [36]. The effect of DSCG on non-immunologic HR from rat mast cells is hard to assess because of conflicting results reported in the literature which have been discussed in detail previously [14]. In results reported here neither DSCG nor a "DSCG-like" agent, REV 3579-Z, at concentrations 10–200 times greater than their respective I_{50} values as an inhibitor of AIR from rat mast cells, significantly inhibited non-immunologic HR from rat mast cells. In contrast, CHBZ was a potent inhibitor of HR from rat mast cells stimulated by all of the non-immunologic secretagogues investigated.

As in the case of AIR, CHBZ was a more potent inhibitor of HR when it was preincubated with rat mast cells for 5 min prior to the addition of the secretagogue than when it was added with the secretagogue. In contrast, DSCG and "DSCG-like" agents, when reported as inhibiting non-immunologic HR, lose their inhibitory activity with increasing preincubation time [18, 20, 37]. In addition, it has been reported that the ability of DSCG to inhibit compound 48/80- and ionophore-induced HR decreases greatly as the concentration of secretagogue (and the net percent of cellular HR into the media) increases [38–41]. Our findings that increasing concentration of compound 48/80 results in an increase in the percent of cellular HR is in agreement with previously reported results [40, 41]. The ability of 10 and 30 μM CHBZ to completely inhibit HR induced by 0.1, 0.3 or 3.0 μg/ml of compound 48/80 coupled with observations of Orr *et al.* [41] that 250 μM DSCG inhibited by 59% HR induced by 0.1 μg/ml of compound 48/80 but that 500 μM DSCG had no effect on 0.2, 0.4 or 1.0 μg/ml of compound 48/80-induced HR, support our conclusion that CHBZ is a much more potent inhibitor of compound 48/80-induced HR than DSCG, regardless of the concentration of the secretagogue used. Similarly, as long as the concentration of the ionophore was non-cytolytic (<0.2 μg/ml), CHBZ was a potent inhibitor of ionophore-induced HR (data not shown).

Thus, the aggregate data in the rat mast cell model indicate that: (1) CHBZ is a potent inhibitor of HR induced by immunological or non-immunologic stimuli; (2) the efficacy of CHBZ is independent of secretagogue concentration or the percent of total cellular HR in the media; and (3) CHBZ does not exhibit cross-tachyphylaxis to DSCG but rather potentiates its activity as an inhibitor of AIR. These observations lead us to conclude that CHBZ inhibits the HR from rat mast cells: (1) at a site distal to the IgE receptor; (2) by affecting a biochemical step in the release process which is common for all secretagogues, and (3) by a mechanism different from that for DSCG.

Human basophils

Histamine release from human basophils is an *in vitro* model of anaphylaxis which allows one to assess the effect of a new agent on human cells [32]. DSCG does not inhibit IgE-mediated HR from human basophils, whereas proxicromil, an antiallergic chromone, has been reported to be a potent inhibitor of HR from human basophils [14, 42]. The observation that HR from human basophils can be elicited by cross-linking receptors on its surface with anti-IgE (Table 2), but not anti-IgG, is in agreement with results reported previously [43, 44]. As expected, neither DSCG nor REV 3579-Z inhibited AbIR from human basophils, and proxicromil inhibited AbIR with an I_{50} value of 60 μM which is in agreement with values previously reported [42]. CHBZ with an average I_{50} value of 25 μM was twice as potent as proxicromil as an inhibitor of AbIR. Comparison of the activity profiles of CHBZ and proxicromil as inhibitors of HR from human basophils showed that: (a) the preincubation time profile of CHBZ was different from that of proxicromil; (b) inhibition of AbIR by both the compounds was largely reversible; (c) CHBZ potentiated the activity of proxicromil as an inhibitor of AbIR; (d) CHBZ was a more potent inhibitor of AbIR than ionophore-induced HR; (e) proxicromil was a more potent inhibitor of ionophore-induced HR compared to AbIR; and (f) CHBZ was a potent inhibitor of PAF-induced HR, whereas proxicromil was not. The evidence presented above suggests that CHBZ and proxicromil inhibit HR from human basophils by different mechanisms.

Guinea pig lung slices

Histamine release in this model is mediated mostly through the IgG_1-class of antibodies [24]. β-Adrenergic agonists are potent inhibitors of AIR from guinea pig lung slices, whereas DSCG has no effect [20, 21]. In agreement with these previously reported results, 0.3 μM isoproterenol inhibited AIR by 60%, whereas DSCG and REV 3579-Z did not inhibit AIR significantly. In contrast, CHBZ inhibited IgE-mediated AIR from guinea pig lung slices in a concentration-dependent manner. However, based on its I_{50} values, CHBZ was 500 and 32 times more

potent as an inhibitor of IgE-mediated HR from rat mast cells and human basophils than IgG_1-mediated HR from guinea pig lung slices respectively. Thus, again in contrast to DSCG, CHBZ is capable of inhibiting IgG_1-mediated HR, thus supporting the conclusion that CHBZ and DSCG inhibit the release process by different mechanisms.

REV 2871 and 3579-Z have also been demonstrated to inhibit the PCA reaction in the rat when administered orally (ED_{50} = 12 mg/kg, personal communication, Dr. P. Sonnino, Revlon Health Care R & D).

In conclusion, CHBZ and its putative metabolite, REV 3579-Z, are structurally novel and orally effective antiallergic agents. The antiallergic activity of both CHBZ and REV 3579-Z can be attributed to their abilities to inhibit immunological HR *in vitro* from rat mast cells. The data presented here suggest that, as an inhibitor of mediator release, the mechanism of action of CHBZ is different from that of the putative metabolite REV 3579-Z and from DSCG. The results of additional experiments designed to elucidate the mechanism of action of CHBZ, and the differences in the mechanism by which CHBZ, REV 3579-Z and DSCG inhibit the release process, are the subjects of the accompanying manuscript [22].

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