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Effect of benzydamine on exocytosis and respiratory burst in human neutrophils and mononuclear phagocytes

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Abstract

The effect of benzydamine on stimulus-dependent respiratory burst activity and enzyme release was tested in human neutrophils, monocytes and monocyte-derived macrophages. Established anti-inflammatory compounds, indomethacin, phenylbutazone and bufexamac, were tested for comparison. Care was taken to avoid cytotoxic or cytolytic concentrations of the test compounds, and their effect on release of lactate dehydrogenase was also tested.

Release of specific and azurophil granules contents were induced in human neutrophils by A23187, PMA and fMLP with and without cytochalasin B pretreatment. Benzydamine inhibited stimulus-dependent release of vitamin B_{12}-binding proteins, a marker for the specific granules, in a concentration-dependent fashion. By contrast, phenylbutazone and bufexamac were practically inactive. The effect of benzydamine on exocytosis of azurophil granules was tested in cytochalasin B-pretreated neutrophils. Benzydamine, again in contrast to the two reference anti-inflammatory compounds, inhibited release concentration-dependently also under these conditions. The concentration of the compound which inhibited exocytosis by 50% was 30–100 μM in normal and 3–10 μM in cytochalasin B-treated neutrophils.

The effect of benzydamine and reference compounds on the respiratory burst was tested by assaying for superoxide formation in neutrophils and H_2O_2 formation in mononuclear phagocytes. Benzydamine was inactive on neutrophils and inhibited slightly the burst response of monocytes and macrophages. Two reference compounds, bufexamac and phenylbutazone, were generally more active. The strongest inhibitory effect was that of phenylbutazone on fMLP-stimulated cells. Benzydamine lacked activity under these conditions, indicating that it does not bind to the receptor of formylated chemotactic peptides.

The profile of activity of benzydamine shown in these experiments on human phagocytes suggest that this compound may act therapeutically by decreasing the release of enzymes and other granule constituents from stimulated neutrophils.

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Introduction

Benzydamine is a non-steroidal compound with anti-inflammatory properties, which differs structurally from the classical, aspirin-like acidic drugs which strongly inhibit cyclo-oxygenase. Benzydamine is a weak cyclo-oxygenase inhibitor. It affects prostaglandin synthesis at concentrations which are 20 to 80-fold higher than the plasma levels attained under therapy [1–5]. Other activities of benzydamine, e.g. the inhibition of platelet aggregation and a 'membrane-stabilizing' effect are, however, observed at concentrations of 2–8 μg/ml which correspond to the levels observed in body fluids upon systemic administration of the compound [1, 6, 7]. The 'membrane-stabilizing' activity of benzydamine, which was demonstrated using isolated rat liver lysosomes [7], suggested that this compound might act by inhibiting the release of enzymes and other mediators from inflammatory phagocytes. We have therefore studied the effects of benzydamine and some reference anti-inflammatory drugs on two most characteristic responses of human neutrophils and mononuclear phagocytes, the respiratory burst and the release of lytic enzymes. Respiratory burst refers to a dramatic severalfold increase in oxygen consumption induced in phagocytes by phagocytosis and other stimulii. The burst results from the activation of an oxidase located in the phagocyte membrane, which oxidizes cytoplasmic NADPH and reduces extracellular molecular oxygen to superoxide. Superoxide dismutates to hydrogen peroxide, and either one of these products may be determined as a measure of the cellular response [8]. Lytic enzymes, acid and neutral proteases in particular,

with a variety of pro-inflammatory properties, are released in large amounts from neutrophils. They are stored within two types of granules (specific and azurophil granules) which are discharged from stimulated cells according to different rules [9]. The discharge of the two types of granules is monitored by assessing the appearance of their characteristic constituents in the extracellular fluid. In the present study we used vitamin B_{12}-binding proteins as a marker for the specific and β-glucuronidase as a marker for the azurophil granules [10].

Methods

Cells

Buffy coats of human donor blood, obtained from the Central Laboratory of the Swiss Red Cross (Bern) were used. Neutrophils were prepared as described previously [10] and monocytes were preapred by Ficoll-Hypaque centrifugation followed by centrifugal elutriation, essentially, as described by BONT et al. [11].

Superoxide formation by human neutrophils

2×10^6 neutrophils were preincubated for 20 min at 37°C in the presence or absence of cytochalasin B in 0.6 ml of a medium consisting of 138 m*M* NaCl, 6 m*M* KCl, 1.2 m*M* Pi, 1.2 m*M* $MgSO_4$, 1.0 m*M* $CaCl_2$, 5.6 m*M* glucose, 5.0 m*M* $NaHCO_3$ and 20 m*M* Hepes, pH 7.4 and containing 85 μM cytochrome *c* and the test compounds at the concentration indicated. The stimuli, 0.1 μM fMLP or 20 ng/ml PMA, or incubation medium (controls) were then added, and the reaction was stopped after 10 min by adding 1.2 ml of ice-cold incubation medium and transferring the tubes to an ice bath. The tubes were then centrifuged and superoxide was measured in the supernatant according to CURNUTTE et al. [12].

Release of granule enzymes by human neutrophils

10^7 neutrophils were preincubated for 20 min at 37°C in the presence or absence of cytochalasin B in 1.0 ml of medium containing the test compounds at the concentration indicated. The medium was identical to that described above but did not contain cytochrome *c*. The stimuli, 1.0 μM A23187, 0.1 μM fMLP or 20 ng/ml PMA, or incubation medium (controls) were then added and the reaction was stopped after 10 min by cooling in ice followed by centrifugation. Vitamin B_{12}-binding proteins (specific granules), β-glucuronidase (azurophil granules) and lactate dehydrogenase (LDH, cytosol) were determined in the cell free supernatant and in a sample of the original cell suspension to which 0.1% Triton X-100 had been added to induce total lysis (100% value). Further details are given by DEWALD et al. [13].

Hydrogen peroxide formation by human monocytes and macrophages

Monocytes obtained in 90% purity by the method indicated were seeded in multi-well culture plates (0.1×10^6 cells with 0.2 ml of medium per well in a 96-well plate, or 10^6 cells with 1 ml of medium per well in a 24-well plate). The medium was DMEM containing 25% acid-treated [14] human serum. The cultures were either used immediately after the adherence period of 1 h at 37°C to test the effects of the compounds on monocytes, or after 5–7 days to test the effects of the compounds on macrophages. Under the conditions adopted, the differentiation of monocytes into macrophages as judged by the disappearance of peroxidase-positive granules (electron microscopy, data not shown) was complete in about 3 days. The test compounds were added to the culture media 10 min or, in some cases, 24 h before stimulation. The stimuli, either non-opsonized zymosan (1 mg/10^6 cells) or PMA (0.1 μM), were then added and the hydrogen peroxide formed during 2 h (at 37°C) was determined as described by RUCH et al. [15]. Under the conditions of the assay, benzydamine is fluorescent. This introduces a marked background fluorescence at the 100 μM level, which makes the determination of hydrogen peroxide levels below 0.5 nmoles per assay less reliable. With all test compounds and in all single experiments blank and standard controls were run, and the values obtained were appropriately deduced from the corresponding test-assay values. H_2O_2 rather than superoxide production was used to measure the respiratory burst of mononuclear phagocytes to avoid the disturbance of the latter assay through cytochrome *c* reoxidation. Comparison of O_2^- and H_2O_2 yield by neutrophils (which do not reoxidize cytochrome *c*) indicated that the test compounds do not influence dismutation (not shown).

Test compounds

For the experiments with neutrophils, benzydamine HCl was dissolved in phosphate-buffered saline at the concentration of 1 m*M* and diluted further with the incubation medium. Bufexamac and phenylbutazone were dissolved in a small volume of 0.1 *M* NaOH and brought to a concentration of 1 m*M* with phosphate-buffered saline. Further dilutions were made with the incubation medium. For the experiments with mononuclear phagocytes, benzydamine HCl was dissolved at the concentration of 10 m*M* in Hank's balanced salt solution from which appropriate dilutions were made. Bufexamac, indomethacin and phenylbutazone were dissolved in DMSO at the concentration of 60 m*M* and then appropriately diluted with medium.

Materials

Flat-bottom 96- or 24-well Nunc culture plates and Dulbecco's MEM were obtained from Gibco AG, Basel, Switzerland. Human AB serum (Seromed) was obtained from Fakola AG, Basel, Switzerland. All chemicals were reagent grade and their origin is described in the original publications [10–13].

Results

Release of granule enzymes from human neutrophils

The results obtained following stimulation with the ionophore A23187, PMA and fMLP in the presence and absence of cytochalasin B are shown in Figs 1–3. Stimulation with ionophore and PMA induced, as expected, a selective exocytosis of specific granules [9]. In both cases, benzydamine strongly inhibited this process in a concentration-dependent fashion. The maximum

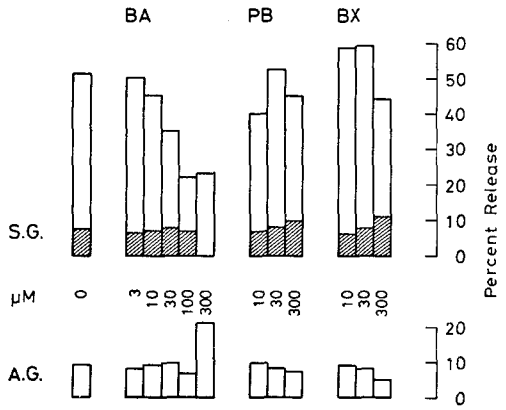

Figure 1
Effect of benzydamine (BA), phenylbutazone (PB) and bufexamac (BX) on the release of vitamin B_{12}-binding proteins from the specific granules (SG) and β-glucuronidase from the azurophil granules (AG) of human neutrophils stimulated with 1 μM A23187. The test substances were present in the assays at concentrations varying between 3 and 300 μM (indicated). The open bars express release in pecent of the initial cellular content during an incubation period of 10 min. The hatched areas within the upper bars represent release of LDH also expressed in percent of the initial cellular content. At 300 μM, benzydamine was cytolytic and LDH release was 55.7% (not indicated). Mean values from three separate experiments run in duplicate. Statistical evaluation (signed test of Dixon and Mood) of inhibition of release from specific granules: significant inhibition ($p < 0.05$) by benzydamine at 30 μM and above. No significant effect of other compounds.

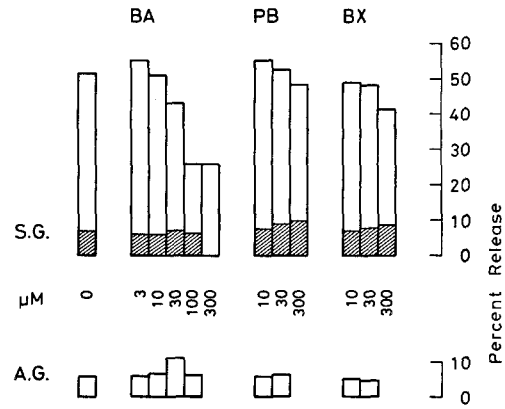

Figure 2
Experiment analogue to that described in Fig. 1 with 20 ng/ml PMA as the stimulus. The LDH release induced by 300 μM benzydamine was 57.4% (not indicated). See legend to Fig. 1 for details. Statistical evaluation (signed test of Dixon and Mood) of inhibition of release from specific granules: significant inhibition ($p < 0.05$) by benzydamine at 30 μM and above. No significant effect of other compounds.

effect was obtained at a drug concentration of 100 μM. By contrast, the two reference compounds, phenylbutazone and bufexamac, were virtually inactive. A slight inhibition of ionophore-induced release of vitamin B_{12}-binding proteins was observed with bufexamac at 300 μM only. Release of the contents of the azurophil granules, which is minimal under these experimental conditions, was not affected by the compounds, except for benzydamine at the highest concentration tested. At this level, however, benzydamine is cytolytic, as indicated by the release of the cytosolic enzyme, LDH. No release of LDH in excess of the control values was observed under all other conditions, indicating that the inhibitory effect of benzydamine was selective.

In order to probe the action of the test compounds on azurophil granule discharge as well, experiments were carried out with fMLP as the stimulus on neutrophils which had been pretreated with cytochalasin B. The results are shown in Fig. 3. In contrast to both stimuli used

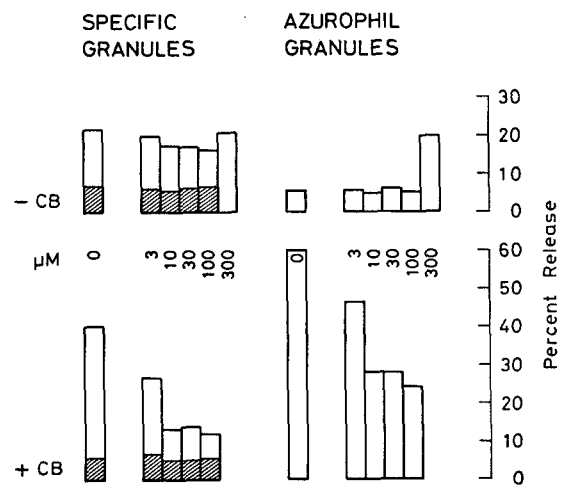

Figure 3
Effect of benzydamine on fMLP-induced release of vitamin B_{12}-binding proteins from the specific granules and β-glucuronidase from the azurophil granules of human neutrophils. Normal (−CB) and cytochalasin B-treated (+CB) neutrophils were used. Benzydamine was present in the assays at increasing concentrations between 3 and 300 μM (indicated). The results are expressed as in Fig. 1. LDH release is indicated by hatched areas in the bars showing release from the specific granules. At 300 μM, benzydamine induced the release of 46.7% LDH (not indicated). Statistical evaluation (signed test of Dixon and Mood) of inhibition of release from specific and azurophil granules of cytochalasin B-treated cells: significant inhibition ($p < 0.05$) by benzydamine at all concentrations tested.

in the former experiments, fMLP induces only a minor release of specific granule contents by normal neutrophils [13]. The data shown in the upper part of Fig. 3 indicate that benzydamine has little effect on release induced by this chemotactic factor in the absence of cytochalasin B. As in the former figures, the lytic action at the highest compound concentration is apparent from the release of LDH and β-glucuronidase. In cytochalasin B-treated neutrophils (lower graphs of Fig. 3), the inhibitory effect of benzydamine is clearly evident. A strong inhibition of the release of vitamin B_{12}-binding proteins and β-glucuronidase was obtained at all concentrations tested, i.e. already at 3 μM. Under these conditions, no increase in the release of LDH was observed. These experiments were also performed with phenylbutazone and bufexamac, but no effect of significance was obtained (data not shown).

Superoxide formation by human neutrophils

A respiratory burst was induced in human neutrophils by stimulation with PMA or fMLP. The latter stimulus was applied following pretreatment of the cells with cytochalasin B since fMLP alone induces only little production of superoxide [13]. The results are summarized in Table 1. Superoxide production induced by PMA was not influenced by any of the three compounds tested. The effect of fMLP, however, was influenced in different ways. Phenylbutazone, which is known to act as a receptor antagonist [16] inhibited the superoxide response in dependence of its concentration. Inhibition was nearly complete at 30 μM. Bufexamac inhibited this cellular response by more than a half at all concentrations tested. Benzydamine, by contrast, was much less active. It reduced superoxide production by some 25% at the highest concentration of 30 μM.

H_2O_2-production by mononuclear phagocytes

The three compounds used in the above experiments as well as indomethacin were tested on cultured human monocytes and monocyte-derived macrophages which were stimulated with either zymosan or PMA. The results are summarized in Tables 2 to 5. The respiratory burst of monocytes (Table 2) was influenced only to a minor extent by the test compounds. At the highest concentration (100 μM), benzydamine inhibited by about 50% the H_2O_2 production induced by zymosan, and bufexamac inhibited the response to both stimuli by about 60%. As indicated in Table 4, these were the strongest effects observed with monocytes. At 10 μM, benzydamine significantly lowered the response to zymosan and bufexamac had a similar effect on the response to PMA. Statistical evaluation with a sign test (see Table 2) further revealed an inhibitory effect for phenylbutazone at all concentrations tested. Indomethacin, however, was inactive. The results obtained with monocyte-derived macrophages (Table 3) were similar, except that phenylbutazone, which was only

Table 1
Effect of benzydamine and reference compounds on superoxide production by stimulated human neutrophils.[a]

Test compounds	μM	*n* moles of O_2^- formed by normal neutrophils[b]				Percent effect of drug	*n* moles of O_2^- formed by CB-treated neutrophils[b]				Percent of control
		No stimulus		PMA			No stimulus		fMLP		
None	—	6.7	7.8	64.5	64.7		0.6	0	66.5	69.1	
Benzydamine	3			63.6	63.5	96			67.9	69.1	101
	10			63.9	61.6	95			67.9	67.4	100
	30	2.0	4.1	65.9	65.9	100	0.6	0.6	51.7	51.6	76
Phenylbutazone	3			63.9	61.8	95			49.9	51.7	75
	10			63.6	64.2	97			15.4	15.4	22
	30	6.1	7.3	66.2	66.2	101	0.9	0.6	2.0	2.3	2
Bufexamac	3			65.9	65.9	100			23.5	24.1	35
	10			69.7	70.0	106			23.2	24.4	35
	30	3.8	3.8	70.8	72.3	109	0.9	0.9	20.9	23.8	32

[a] Stimulus concentration were 20 ng/ml for PMA and 0.1 μM for fMLP. Cytochalasin B (CB) was added 5 min before the stimulus at the concentration of 5 μg/ml.
[b] Mean values of two experiments run in duplicate.

Table 2
Effect of benzydamine and reference compounds on H_2O_2 production by stimulated human monocytes in culture.

Test compound	μM	H_2O_2 formation in percent of control following stimulation with					
		Zymosan		p	PMA		p
		Nr.	Median (range)		Nr.	Median (range)	
Benzydamine	1	15	95 (122–65)		12	97 (127–82)	
	10	15	91 (117–45)	0.01	12	103 (110–70)	
	100	9	54 (71–14)	0.01	6	94 (109–85)	
Bufexamac	1	9	92 (110–70)		6	88 (100–65)	
	10	9	95 (102–67)		6	82 (97–61)	0.01
	100	9	37 (49–29)	0.01	6	37 (52–31)	0.01
Indomethacin	1	9	95 (101–79)		6	86 (109–72)	
	10	9	102 (114–83)		6	98 (110–80)	
	100	6	114 (131–96)		6	108 (125–100)	
Phenylbutazone	1	9	85 (93–55)	0.01	8	91 (113–81)	
	10	9	91 (98–76)	0.01	9	98 (129–86)	
	100	9	80 (94–74)	0.01	9	89 (140–82)	

Cultures of 10^5 cells were stimulated in the presence or absence (control) of the test compounds (see Methods). Number of assays, median values and range are given. Significant differences from control at the 99% level are indicated. The signed text of Dixon and Mood was used.

Table 3
Effect of benzydamine and reference compounds on H_2O_2 production by stimulated human monocyte-derived macrophages in culture.

Test compound	μM	H_2O_2 formation in percent of control following stimulation with					
		Zymosan		p	PMA		p
		Nr.	Median (range)		Nr.	Median (range)	
Benzydamine	1	9	97 (155–75)		9	82 (103–63)	
	10	9	78 (119–38)		9	101 (109–36)	
	100	6	56 (68–36)	0.01	9	88 (105–55)	
Bufexamac	1	9	87 (155–69)		9	86 (101–74)	
	10	9	88 (113–69)		9	101 (111–57)	
	100	9	44 (116–17)	0.01	9	54 (62–13)	0.01
Indomethacin	1	6	120 (191–79)		6	85 (97–84)	
	10	6	90 (172–78)		6	107 (117–104)	
	100	6	80 (128–75)		6	110 (124–107)	
Phenylbutazone	1	6	98 (156–73)		6	65 (54–83)	0.01
	10	5	56 (103–38)		6	75 (53–92)	0.01
	100	6	39 (56–27)	0.01	6	36 (63–13)	0.01

See Table 2.

moderately active on monocytes, showed the highest inhibitory activity toward both stimuli (see also Table 4). Since mononuclear phagocytes are long-lived and may accumulate the active compounds intracellularly, experiments were also performed in which macrophages were challenged with stimuli after having been exposed to the test compounds for a 24-h period (Table 5). Lower concentrations of the drugs were used, which were both found to be cytotoxic beyond 10 μM. As shown in Table 5, benzydamine and bufexamac showed only a slight inhibition of H_2O_2 production induced by zymosan under these experimental conditions. In addition benzydamine appeared to enhance the respiratory burst response at low concentrations. The latter effect has not been investigated further. Table 5 shows a necessary control. Benzydamine, bufexamac and indomethacin did not exert apparent cytotoxic effects, as indicated by the fact that they did not

Table 4
Inhibition of H_2O_2 production by mononuclear phagocytes, Comparison of benzydamine and reference compounds.

Cell	Stimulus	Concn. of compounds (μM)	Inhibitory effect of benzydamine (+, superior, =, equal, −, inferior) compared to		
			Bufexamac	Indomethacin	Phenylbutazone
Monocytes	Zymosan	10	=	+ (0.05)	=
		100	=	+ (0.01)	+ (0.01)
	PMA	10	− (0.01)	=	=
		100	− (0.01)	=	=
Macrophages	Zymosan	100	=	+ (0.01)	=
	PMA	1	=	=	− (0.01)
		10	=	=	− (0.01)
		100	− (0.01)	=	− (0.01)

Statistical comparison (signed test of Dixon and Mood) of percent inhibition by benzydamine and reference compounds. p Values are given when benzydamine effect is statistically different (+ or −) from that of references.

Table 5
Effect of a 24-h pretreatment with benzydamine and bufexamac on stimulus-dependent H_2O_2 production by human monocyte-derived macrophages.

Test compound	μM	H_2O_2 formation in percent of control following stimulation with	
		Zymosan	PMA
Benzydamine	0.1	184, 150, 144	94, 93, 92
	1.0	141, 134, 131	94, 88, 85
	10.0	88, 78, 50	93, 92, 87
Bufexamac	0.1	113, 103, 69	102, 101, 100
	1.0	100, 91, 78	101, 97, 95
	10.0	91, 69, 53	90, 85, 78

Cultures of 10^5 macrophages were exposed for 24 h to the test compounds or control medium, and then stimulated. Data from 3 experiments (see Methods).

Table 6
Release of lactate dehydrogenase (LDH) by human monocytes exposed to benzydamine, bufexamac and indomethacin.

Test compound	μM	LDH distribution		LDH release in percent of total
		Cells	Medium	
None	—	31.62 ± 0.79	3.23 ± 0.18	9
Benzydamine	1	33.19 ± 0.41	3.62 ± 0.09	10
	10	34.22 ± 0.46	3.82 ± 0.24	10
	100	32.82 ± 1.00	3.64 ± 0.08	10
Bufexamac	1	31.55 ± 1.12	3.72 ± 0.08	11
	10	30.52 ± 0.47	3.26 ± 0.15	10
	100	34.32 ± 0.88	3.47 ± 0.20	9
Indomethacin	1	33.59 ± 0.83	3.44 ± 0.09	9
	10	32.26 ± 1.08	3.52 ± 0.15	10
	100	31.38 ± 0.41	3.00 ± 0.11	9
Overall mean		32.55 ± 1.25	3.47 ± 0.25	

Cultures of 10^6 monocytes were exposed for 2 h to the test compounds or control medium. The media were then collected and the cells were lysed in saline containing 0.1% digitonin. Mean ± SD (mU/10^6 cells) from 3 separate cultures.

influence LDH levels and LDH release by human monocytes at the concentrations used in these experiments. Phenylbutazone was shown in experiments performed on another study (data not shown) to lack cytotoxic activity in this concentration range.

Discussion

The experiments reported were designed to assess whether benzydamine influences product formation and/or release from stimulated phagocytes. Human neutrophils and mononuclear phagocytes were stimulated in various ways in the presence of increasing concentrations of the compound in question and of known non-steroidal anti-inflammatory drugs which were taken as standards.

The stimulus-dependent release of enzymes and other storage proteins was tested with human neutrophils because of the possibility to differentiate between exocytosis of azurophil and specific granules, two subcellular export compartments subject to different regulatory mechanisms [9]. Benzydamine markedly lowered exocytosis from both granules. Its effect was generally concentration-dependent and occurred under conditions of full cell viability. By comparison,

phenylbutazone and bufexamac were virtually inactive. Taken together these observations show that benzydamine has the ability to diminish stimulus-dependent exocytosis of neutrophil enzymes. Its activity was not restricted to the response to a given stimulus, suggesting that the compound is not affecting stimulus-receptor interaction. A step in the signal transduction sequence may be inhibited by the drug. Inhibition of the process of fusion of the granules with the plasma membrane is an alternative possibility.

The effect of benzydamine and the reference compounds on the respiratory burst was tested on neutrophils, monocytes and monocyte-derived macrophages. Benzydamine did not affect the response of neutrophils and inhibited zymosan-induced H_2O_2 release by monocytes and macrophages. The latter action was reproducible, although clearly apparent at the highest concentration level only, and was not related to cytotoxicity. In contrast to the inhibition of exocytosis, however, the action of benzydamine on the respiratory burst response did not stand out in comparison with that of the reference compounds. Unlike phenylbutazone, benzydamine did not exhibit antagonism toward ligands for the formyl peptide receptor.

The inhibition of neutrophil exocytosis under a variety of conditions shown in this study suggests that benzydamine may act *in vivo* by reducing the release of inflammatory and lytic components by stimulated phagocytes. Release inhibition in cytochalasin B-treated cells was observed at concentrations which are easily obtained *in vivo* upon oral administration of the compound [17]. The effect of benzydamine on the respiratory burst was less consistent and less pronounced. In this respect, benzydamine was generally less active than bufexamac and phenylbutazone. The different influence of the drugs on the two main expressions of phagocyte activation is a further indication that the underlying mechanisms of these responses are distinct.

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