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# CARDIOVASCULAR EFFECT AND STIMULUS-DEPENDENT INHIBITION OF SUPEROXIDE GENERATION FROM HUMAN NEUTROPHILS BY TIBENELAST, 5,6-DIETHOXYBENZO(*b*)THIOPHENE-2-CARBOXYLIC ACID, SODIUM SALT (LY186655)*

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**Abstract**—Tibenelast (LY186655), 5,6,-diethoxybenzo(*b*)thiophene-2-carboxylic acid, sodium salt, is an orally active anti-anaphylactic compound in guinea pigs, and has been shown to prevent bronchospasm in moderately severe asthmatic patients. Pharmacological studies with tibenelast demonstrated that it is a selective phosphodiesterase (PDE) inhibitor in that it is moderately active against the lung and stomach enzyme while being a very weak inhibitor of the heart enzyme. The compound was without cardiovascular effects at anti-anaphylatic doses. In contrast to theophylline, tibenelast did not have a direct inotropic effect in the cat papillary muscle system. The concentration that inhibited 50% of the enzymatic activity ($IC_{50}$) for tibenelast was 20- to 30-fold lower for neutrophil PDE than for PDE of other tissues. It was 100 times more potent than aminophylline in inhibiting superoxide generation from platelet-activating factor (PAF)-primed polymorphonuclear leukocytes (PMNL) challenged with chemotactic factor, N-formyl-methionyl-leucyl-phenylalanine. However, tibenelast was less effective in the tumor necrosis factor-primed system, and did not inhibit superoxide generation during phagocytosis or when other soluble stimuli, such as phorbo-12-myristate-13-acetate or the calcium ionophore A23187, were used. Furthermore, tibenelast did not inhibit enzymes involved in arachidonic acid metabolism. These results suggest that tibenelast probably inhibits superoxide release from PMNL via a selective inhibition on PDE.

Two major components of asthma are contraction of airway smooth muscle and bronchial wall edema which are the result of the inflammatory process associated with asthma [1]. Bronchoconstriction is reversible by the administration of bronchodilators, whereas the obstruction caused by edema does not respond rapidly to therapy [2]. Among the bronchodilators, $\beta_2$-adrenergic agents produce bronchodilatation by stimulating production of intracellular adenosine 3′,5′-cyclic monophosphate (cAMP), whereas the mechanism of action of theophylline has yet to be definitively proven [3]. In the past, theophylline was generally believed to cause bronchodilatation by inhibition of the enzyme phosphodiesterase (PDE), thereby increasing intracellular cAMP concentrations. However, evidence against PDE inhibition as the mode of action of theophylline has appeared [4]. Because $\beta_2$-adrenergic agents and PDE inhibitors increase intracellular cAMP concentrations through different enzymatic reactions, one would suppose that synergy should occur between $\beta_2$-adrenergic agents and PDE inhibitors, such as theophylline. Failure to demonstrate such synergy after intravenous administration of salbutamol and aminophylline to asthmatics argues against PDE inhibition as the mode of action of theophylline [5].

Recently, increasing evidence has suggested that edema-related airway obstruction and antigen-induced late asthmatic response are dependent on the presence of granulocytes at the time of exposure to antigen [6, 7]. In an animal model, depletion of granulocytes eliminated the late phase reaction and partial repletion with neutrophils restored at least part of the pathological response [8]. One interpretation is that these cells which infiltrate the lung may produce mediators that affect airway functions. Additionally, tissue injuries such as epithelial damage and increases in permeability have been reported to be mediated by oxygen radicals generated from the infiltrating neutrophils [9]. Thus, anti-asthma drugs, such as corticosteroids and disodium cromoglycate, have been proposed to exert their actions through inhibition of leukocyte functions, thereby preventing tissue damage induced by inflammatory cells [10, 11].

We have shown recently that tibenelast (LY186655), 5,6,-diethoxybenzo(*b*)thiophene-2-carboxylic acid, sodium salt, is an orally active compound against anaphylactic shock and bronchoconstriction in guinea pigs [12]. Evidence was also obtained that the compound was therapeutically

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synergistic with epinephrine in inhibiting pulmonary function changes upon antigen challenge of an actively sensitized guinea pig. In the present study, we describe additional pharmacological properties of tibenelast.

## MATERIALS AND METHODS

*cAMP phosphodiesterases (PDE).* PDE enzymes were prepared from human polymorphonuclear leukocytes (PMNL), guinea pig peritoneal PMNL, and other guinea pig tissues. The human PMNL or guinea pig PMNL cells ($2 \times 10^7$ cells/mL) were disrupted by sonication in a Branson Sonifier, model 350, equipped with a microtip. PDE was recovered in the supernatant after low speed centrifugation at 15,000 *g* for 15 min. For guinea pig tissues, a 30% homogenate (w/v) was prepared, and the particulate matter was removed by centrifugation at 15,000 *g* for 15 min.

The assay was conducted by a modification of the method described by Thompson *et al.* [13]. The reaction mixture (0.2 mL) contained 1.6 mM Tris · HCl buffer, pH 7.5, 0.32 mM magnesium chloride, 0.8 mM dithiothreitol, 0.8 μM cAMP, 0.6 mM adenosine, 0.15 μCi/mL [2,8-$^3$H]cAMP (sp. act. 38 Ci/mmol; Amersham, Arlington Heights, IL), and PDE. Snake venom (King Cobra, Sigma, St. Louis, MO) at a concentration of 0.08 mg/mL was added to the reaction mixture immediately before the reaction started. After 1 hr of incubation at room temperature the reaction was stopped by heating. Two milliliters of a 30% Dowex · Cl (AG1 × 2) slurry containing 2 mg/mL of adenosine was added to remove the unreacted cAMP. The hydrolyzed product remained in the solution, and radioactivity in the solution was quantified in a liquid scintillation counter after the addition of 10 mL of PCS counting solution (Amersham).

*Enzymes of arachidonate metabolism.* Partially purified fatty acid cyclooxygenase and thromboxane synthetase were prepared and assayed according to previously published methods [14, 15].

5-Lipoxygenase was prepared from guinea pig peritoneal PMNL. Ten milliliters of a 2% casein solution was injected intraperitoneally into guinea pigs weighing 250–300 g. After 16–18 hr, the guinea pigs were killed by suffocation in a carbon dioxide chamber. The peritoneal cavity was infused with 70 mL of saline, and 40–50 mL of the fluid was recovered from the cavity. After centrifugation, cell pellets were washed twice in Hanks' balanced salt solution (HBSS) without calcium ion. The cells were then suspended in 5 mL of sodium phosphate buffer, pH 7.1, containing 1 mM ethylenediamine tetraacetate, and 0.1% gelatin. About $2–3 \times 10^8$ cells were obtained from one guinea pig. Analysis for cell composition indicated that more than 95% of the cells were PMNL. The PMNL suspension was disrupted by five 0.5-sec sonic pulses at a setting of 3 in a Branson Sonifier, model 350, equipped with a microtip. The sonicates were combined and centrifuged at 30,000 *g* for 10 min. The supernatant was kept frozen at −70° until used.

*5-Lipoxygenase assay.* Enzyme activity was determined by assaying for 5-hydroxyeicosatetraenoic acid (5-HETE) formation by incubating 0.2 mL of the supernatant obtained from the PMNL sonicate with 5 μM [1-$^{14}$C]arachidonic acid (sp. act. 58.4 mCi/mmol; Amersham), 1 mM calcium chloride, 2 mM adenosine triphosphate, and 1 mM glutathione for 15 min at 37°. The enzyme reaction was then stopped by the addition of 10 μL of 1 M citric acid and 10 μL of an alcohol solution containing 20 mg/mL each of indomethacin and butylated hydroxyanisole (BHA). The reaction mixture was spotted (50 μL) on a silica gel plate (Baker TLC plate S1250-PA-19C) and subjected to TLC in a solvent system that consisted of the organic phase of a mixture of ethyl acetate: 2,2,4-trimethylpentane: glacial acetic acid: water (90:50:20:100).

The radioactivity of arachidonic acid and its metabolites (5-HETE and leukotriene $B_4$) can be visualized from a developed X-ray film that has been exposed to the TLC plate for 1–2 days. The amount of 5-HETE formed can be quantitated by scraping the silica gel area which corresponds to the spot on the X-ray film, and the radioactivity determined in a Beckman LS 5800 liquid scintillation counter (Beckman Inc.).

*Anesthetized guinea pigs.* Male Hartley guinea pigs (350–900 g) were anesthetized with pentobarbital (40 mg/kg, i.p.). The jugular vein was cannulated for drug administration, and a tracheal tube was inserted for artificial respiration. A 21 gauge needle attached to a pressure transducer was inserted through the chest wall into the left ventricle. This provided continuous monitoring of heart rate, cardiac contractility (dp/dt), and peak left ventricular systolic blood pressure (PLVP) [16]. Either drug or vehicle was administered at 3- to 5-min intervals.

*Pentobarbital-anesthetized dogs.* Adult beagle dogs (7.5 to 11.3 kg) were anesthetized with sodium pentobarbital (35 mg/kg, i.v.) and ventilated with room air. The left femoral artery was cannulated and the cannula attached to a Statham (P23Db) pressure transducer to measure mean arterial blood pressure (MAP). Lead II electrodes and a cardiotachometer were used to monitor heart rate. Contractility was determined with a Walton Brodie strain gauge arch sutured onto the right ventricle. Compounds were administered by a femoral venous catheter. Cumulative dose–response profiles were obtained for either theophylline or tibenelast by administering increasing doses of drug (0.125 or 8 mg/kg, i.v.) at 5-min intervals. Peak responses (usually less than 1 min) and 5-min responses were recorded.

*Cat papillary muscle system.* Isolated cat papillary muscles were placed in individual muscle baths in Krebs–Heinseleit buffer (2.5 mM calcium); muscles were equilibrated with 1 to 1.5 g base tension and stimulated twelve times/min with a Grass S9 Stimulator. An increase or decrease in developed tension after drug administration was taken as a change in contractility and expressed as a percent of control. Increasing concentrations of drugs were added to muscle baths at 30-min intervals.

*Human polymorphonuclear leukocytes (PMNL).* Fresh blood was drawn in sterile syringes containing

0.38% of sodium citrate/mL of blood. After centrifugation at 200 *g* for 15 min at room temperature, the platelet-rich plasma was removed and cells were resuspended in HBSS containing 0.1% human serum albumin (HSA). Isolation of PMNL was accomplished by using 15 mL of isolymph layered on top with 15–20 mL of defibrinated blood. This system was centrifuged at 200 *g* for 40 min at room temperature. Layers above the granulocytes and erythrocytes were removed, and 11 mL of 0.2% methocel was added to the remaining suspension of cells. The mixture was then placed in a 37° bath for 15 min. The upper opaque layer containing PMNL was aspirated and centrifuged at 200 *g* for 5 min. Five milliliters of a 0.2% sodium chloride solution was added to the red pellet of cells to lyse the contaminating erythrocytes. Immediately after mixing for 30 sec, 5 mL of a 1.5% sodium chloride solution was added to prevent PMNL from being lysed. The cell suspension was centrifuged again at 200 *g* for 5 min, and PMNL were suspended in HBSS containing 0.1% HSA.

*Immobilized IgG complex.* Human immunoglobulin G (IgG, Sigma) was dissolved in HBSS at 2 mg/mL and was heat-inactivated at 56° for 30 min to form an IgG complex according to the method of Henson [17]. Millipore SMWP 13 mm filters (Millipore Corp.) were immersed individually in the heat-inactivated IgG solution, and incubated at 37° for 1 hr. These immune complex (IgG complex) coated membranes were stored at 4° under sterile conditions until used.

*Assay for superoxide generation.* To 0.5 mL of PMNL suspension, $1 \times 10^{-7}$ M platelet-activating factor (PAF, Calbiochem, La Jolla, CA) or 100 units of recombinant human tumor necrosis factor (TNF, Genzyme, Cambridge, MA) in HBSS containing 0.1% HSA was added, and the mixture was incubated at 37° for 5 min. Superoxide generation was then initiated by addition of 0.5 mL of cytochrome *c* solution (2 mg/mL, Type VI, Sigma) containing $2 \times 10^{-8}$ M N-formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma) to the cell suspension. In experiments where cells were not preincubated with priming agents (PAF or TNF), either $2 \times 10^{-7}$ M FMLP or $2 \times 10^{-7}$ M recombinant human complement C5a (rhC5a, Calbiochem), was used as the stimulus. For phagocytosis-induced superoxide generation, opsonized zymosan or immobilized IgG complex was used as the stimulus. Fifteen milligrams of superoxide dismutase (SOD, Sigma) was used as a standard for the calculation of SOD inhibitable superoxide generation. Reactions were conducted in duplicate. After incubation for 5 min at 37°, samples were placed in ice water to stop the reaction and centrifuged at 200 *g* for 15 min at 10°. Supernatants were then transferred to glass tubes for spectrophotometric reading at 550 nm to measure the amount of reduced cytochrome *c* in μmol/L generated by the superoxide. For assays of inhibitor, the PMNL suspension was preincubated with test compounds for 5 min at 37° before addition of priming agents or stimuli. Results are expressed as percent of inhibition as calculated by the following equation.

$$\% \text{ Inhibition} = \left[1 - \frac{\text{Sample} - \text{blank control} - \text{control}}{\text{Stimuli control} - \text{blank control} - \text{SOD control}}\right] \times 100.$$

*Statistics.* All data are expressed as mean values ± SE. The statistical significance for inhibition was analyzed by comparing control and drug-treated groups. Values were considered significant at $P < 0.05$, using Student's *t*-test.

## RESULTS

*Inhibition of phosphodiesterase and arachidonate metabolizing enzymes by tibenelast.* Because tibenelast had been shown to inhibit antigen-induced bronchoconstriction similar to theophylline [12], we compared tibenelast with aminophylline and isobutyl methylxanthine (IBMX) in their effects on PDE from various guinea pig tissues and peritoneal PMNL. The range of concentration that inhibited 50% of the enzymatic activity ($IC_{50}$) was 200–300 μM for aminophylline for all tissues except kidney (95 μM) and liver (>500 μM), and 6–20 μM for IBMX for all tissues except liver (122 μM) (Table 1). In contrast, three groups of $IC_{50}$ values for PDE were observed for tibenelast. Tibenelast showed little or no inhibition (>380 μM) in heart, liver and kidney, whereas intermediate $IC_{50}$ values (200 μM) were obtained for lung, brain and stomach. Surprisingly, tibenelast preferentially inhibited PDE from PMNL ($IC_{50} = 14.9$ μM). Similar to aminophylline and IBMX, tibenelast was an apparent competitive inhibitor against the substrate cAMP (Fig. 1). $K_i$ values of 9.4, 284 and 16.1 μM were obtained for tibenelast, aminophylline and IBMX, respectively, for guinea pig peritoneal PMNL PDE. Similar $IC_{50}$ values were also obtained with these compounds in assays with PDE obtained from human PMNL (data not shown).

At a concentration of 350 μM, tibenelast did not inhibit fatty acid cyclooxygenase and thromboxane synthetase partially purified from human platelets, or 5-lipoxygenase isolated from guinea pig peritoneal PMNL.

*Cardiovascular profile of tibenelast in guinea pigs.* Because theophylline was believed to exert cardiac stimulation through its PDE inhibition, we set forth to determine whether tibenelast also possessed such cardiovascular activities. As shown in Fig. 2, intravenous administration of either 3 mg/kg theophylline or 3 mg/kg tibenelast resulted in comparable increases in dp/dt; however, theophylline caused the well-known and anticipated decrease in PLVP and increase in heart rate, whereas tibenelast had relatively little effect on either parameter. In fact, the effects of tibenelast on PLVP were not apparent until a dose of 30 mg/kg, i.v., was obtained. Although both agents caused dose-dependent increases in heart rate, the stimulation due to theophylline was statistically greater than that resulting from tibenelast ($P < 0.05$).

*Cardiovascular profile of tibenelast in dogs.* A comparison of the cardiovascular effects of theophylline and tibenelast in the anesthetized dog

Table 1. $IC_{50}$ Values for phosphodiesterase inhibition in guinea pig tissues

| | $IC_{50}$ (μM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Peritoneal PMN | Lung | Brain | Heart | Liver | Stomach | Spleen | Kidney | Fat |
| Aminophylline | 243 ± 81 | 333 ± 34 | 233 ± 53 | 242 ± 100 | >500 | 234 ± 19 | 226 ± 69 | 95.2 ± 42 | 316 ± 35 |
| IBMX | 13.5 ± 1.4 | 20.2 ± 5.4 | 18 ± 2.4 | 19.4 ± 10 | 122 ± 63 | 14.9 ± 4 | 8.6 ± 0.5 | 6.3 ± 1.8 | 15.8 ± 3.6 |
| Tibenelast | 14.9 ± 2.3 | 202 ± 28 | 267 ± 64 | 597 ± 97 | >600 | 219 ± 20 | 324 ± 87 | 388 ± 132 | >600 |

Values are the means ± SE of six separate experiments. Abbreviations: PMNL, polymorphonuclear leukocytes; and IBMX, isobutyl methylxanthine.

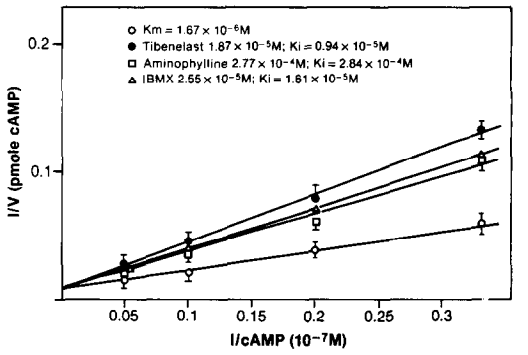


Fig. 1. Michaelis constant determination for guinea pig peritoneal PMNL phosphodiesterase. Values are the means ± SE of three separate experiments.

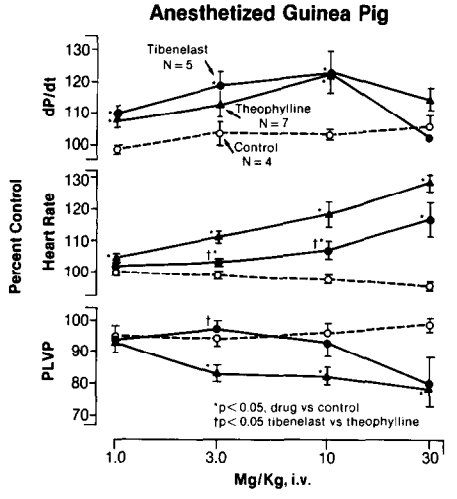


Fig. 2. Cardiovascular effects of tibenelast and theophylline in anesthetized guinea pigs. Values are the means ± SE of either four (control), seven (theophylline), or five (tibenelast) experiments. The control produced a dp/dt of 1620 ± 140 mm Hg/sec, a heart rate of 200 ± 8 beats/min, and a peak LVP of 47 ± 2 mm Hg. Statistical significance was determined by unpaired, two-tailed, *t*-test analysis.

is shown in Fig. 3. Tibenelast did not alter heart rat or contractility, but did decrease MAP in a dose-related manner. At 8 mg/kg the peak and 5-min MAP responses were 60 and 74% of control. By contrast, 8 mg/kg theophylline increased the peak and 5-min responses of heart rate by 58 and 51% and contractility by 188 and 119% respectively. Although theophylline also decreased MAP, this effect was relatively transient.

*Papillary muscle contraction by tibenelast.* In the cat papillary muscle, tibenelast caused only a slight (15%) increase in contractility at concentrations as high as 1.0 mM (Table 2). By comparison, theophylline increased contractility by 23 and 59% at 0.1 and 1.0 mM respectively. The maximum response was evident at 5 min.

*Inhibition of FMLP-induced superoxide release*

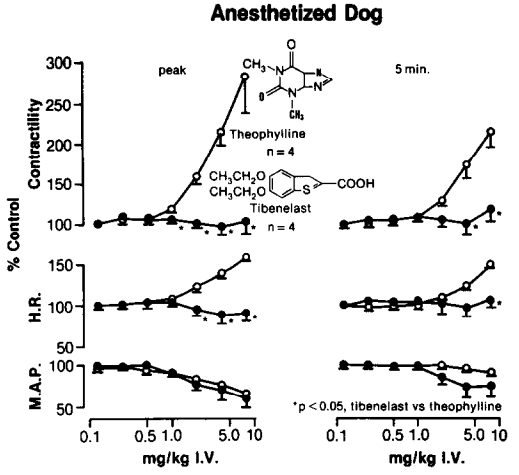


Fig. 3. Effects of tibenelast and theophylline on cardiac function in pentobarbital-anesthetized dogs. Each point is the mean ± SE of four experiments. The control produced a cardiac contractility of 91 ± 2 g, a heart rate of 130 ± 6 beats/min, and a MAP of 116 ± 5 mm Hg. Statistical significance was determined by unpaired, two-tailed, *t*-test analysis.

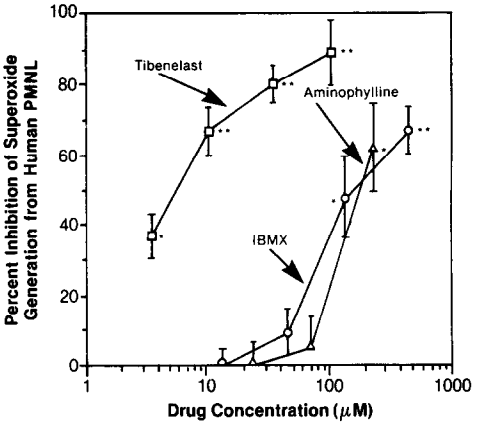


Fig. 4. Inhibition of FMLP-induced superoxide generation from human PMNL. FMLP ($2 \times 10^{-7}$ M) was added to $2 \times 10^6$ cells/mL in the presence of 5 μg/mL of cytochalasin B, and the cell suspension was incubated in the presence of cytochrome *c* (1 mg/mL) for 5 min at 37°. The control produced 18.9 ± 1.5 μmol/L of superoxide determined by the SOD-inhibitable reduction of cytochrome *c* as described under Materials and Methods. Values are means ± SE of three separate experiments performed in duplicate. Key: *P < 0.05, and **P < 0.01.

Table 2. Inotropic effects of theophylline and tibenelast in cat papillary muscles

| Increase in tension | | | |
|---|---|---|---|
| Theophylline | | Tibenelast | |
| $10^{-4}$ M | $10^{-3}$ M | $10^{-4}$ M | $10^{-3}$ M |
| 123 ± 10 (N = 10) | 159 ± 19 (N = 8) | 114 ± 8 (N = 7) | 115 ± 6* (N = 7) |

Values (mean ± SE) are expressed as a percent of control (control = 100%). The control produced an increase in tension of 0.76 ± 0.11 g after electrostimulation.
* P < 0.05, theophylline vs tibenelast.

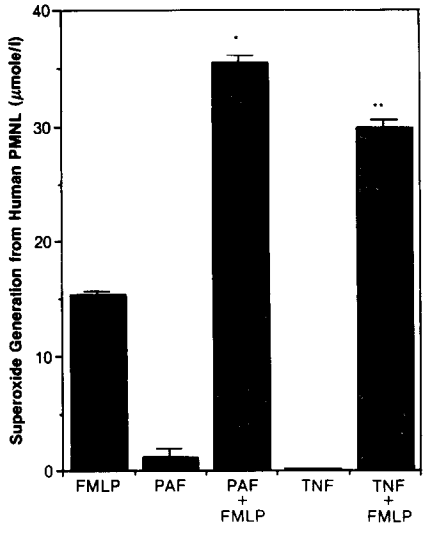


Fig. 5. Priming effects of PAF and TNF on human PMNL superoxide production by FMLP. PMNL ($2 \times 10^6$ cells/mL) were preincubated with HBSS, $1 \times 10^{-7}$ M PAF, or 100 units of TNF at 37° for 5 min before the addition of $2 \times 10^{-8}$ M FMLP. The reactions were continued for another 5 min. The results are the means of three separate experiments performed in duplicate and represent μmol/L of superoxide produced by $2 \times 10^6$ cells/mL determined by the SOD-inhibitable reduction of cytochrome *c* as described under Materials and Methods. Key: P < 0.05, PAF + FMLP vs FMLP; and **P < 0.05, TNF + FMLP vs FMLP.

*from PMNL by tibenelast.* Compounds that increase intracellular concentration of cAMP in leukocytes, such as β-adrenergic agents and prostaglandins, have been shown previously to inhibit the release of superoxide from PMNL [18]. Thus, tibenelast was examined for its ability to inhibit the chemotactic factor FMLP-induced superoxide release from human PMNL in the presence of cytochalasin B. Similar to the results reported for prostaglandin $E_1$ ($PGE_1$), no preincubation of PMNL with tibenelast was required for inhibition (Fig. 4). Tibenelast was at least 30 times more potent than aminophylline in inhibiting FMLP-induced superoxide release from human PMNL, with an $IC_{50}$ of approximately 10 μM.

*Inhibition of primed and soluble stimulus-induced and phagocytosis-induced superoxide release by tibenelast.* PMNL produce substantial amounts of superoxide during phagocytosis or upon treatment with a variety of soluble or quasi-soluble agents. Many inflammatory mediators, including PAF and TNF, can enhance or prime the neutrophil oxidative burst and the release of superoxide in response to a second stimulus and in the absence of cytochalasin B [19–22]. We first examined the effects of PAF and TNF preincubation on PMNL superoxide production in response to the chemotactic factor FMLP. Figure 5 shows that preincubation of PMNL with low

Table 3. Effect of tibenelast on human PMNL superoxide production by different stimulating agents

| Stimulating agents | PMNL superoxide production (% inhibition) |
|---|---|
| FMLP ($2 \times 10^{-7}$ M) | 89 |
| FMLP ($2 \times 10^{-8}$ M) + PAF ($1 \times 10^{-7}$ M) | 100 |
| FMLP ($2 \times 10^{-8}$ M) + TNF (100 units) | 50 |
| A23187 ($2 \times 10^{-6}$ M) | 0 |
| PMA ($3 \times 10^{-7}$ M) | 0 |
| Opsonized zymosan (1 mg/mL) | 9 |
| Immobilized immune complex | 0 |

Tibenelast ($1.04 \times 10^{-4}$ M) was added to the human PMNL ($2 \times 10^6$ cells/mL) and incubated at 37° for 5 min before the addition of various stimulating agents. The control production of superoxide was 21.5 ± 2 μmol/L for FMLP stimulation; 37.5 ± 3.3 μmol/L for PAF–FMLP stimulation; 30 ± 2 μmol/L for TNF-FMLP stimulation; 34.6 ± 3.5 μmol/L for PMA stimulation; 25.4 ± 3 μmol/L for A23187 stimulation; 38 ± 5 μmol/L for opsonized zymosan stimulation; and 37 ± 4 μmol/L for immobilized immune complex stimulation.

concentrations of PAF ($1 \times 10^{-7}$ M) or TNF (100 units) at 37° for 5 min enhanced the production of superoxide production upon the addition of $2 \times 10^{-8}$ M FMLP. Tibenelast was found to inhibit human PMNL superoxide production in these primed systems, whereas it did not inhibit superoxide release from human PMNL stimulated with opsonized zymosan, immobilized IgG, phorbo-12-myristate-13-acetate (PMA), or the calcium ionophore A23187 even at the high concentration of 100 μM (Table 3). Figure 6 shows a concentration-dependent inhibition of superoxide generation by tibenelast in these primed systems. The $IC_{50}$ was approximately 3 μM for PAF-primed and FMLP-induced superoxide generation, whereas it was less effective in the TNF-primed system ($IC_{50} = 30$ μM). Under our assay conditions, rhC5a maximally stimulated superoxide generation at a concentration of $2 \times 10^{-7}$ M, and cytochalasin B was not required for such activity. Tibenelast also inhibited rhC5a-induced superoxide generation with an $IC_{50}$ of approximately 10 μM (Fig. 6).

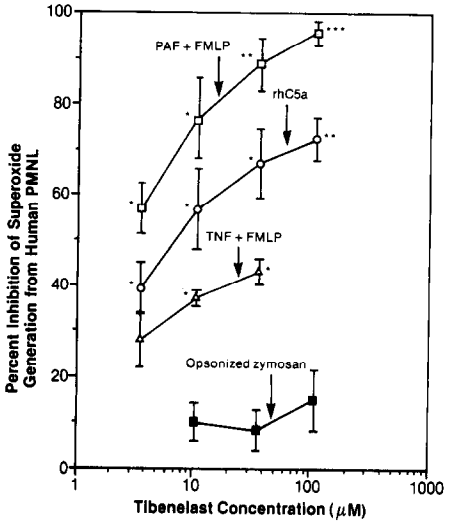


Fig. 6. Effect of tibenelast on stimulus-dependent human PMNL superoxide production. The experimental procedures were described under Materials and Methods. The control values of superoxide produced were in the range of 35 ± 5 μmol/L for all the stimuli, except for the value of rhC5a which was 20.3 ± 3.8 μmol/L. Values are means of three separate experiments performed in duplicate. Key: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$.

## DISCUSSION

Tibenelast (LY186655) is an orally active anti-anaphylactic compound in guinea pigs [12], and has been shown to prevent bronchospasm in moderately severe asthmatic patients [23]. In guinea pigs, the compound was ten times more potent than aminophylline by i.v. administration, and normalization of pulmonary function was achieved at 1 mg/kg. A therapeutic synergism between tibenelast and epinephrine was also reported and tibenelast was suggested to act at a site different from that of β-adrenergic agents [12].

Because of its immediate effect on the antigen-induced bronchoconstriction, tibenelast is by definition a bronchodilator. We have thus attempted to find the potential site of action for tibenelast as a bronchodilator. Because thromboxane and leukotrienes are major mediators released by guinea pig lung during antigen-induced bronchoconstriction [24], it is reasonable to study the effect of tibenelast on the biosynthesis of these mediators. In the cell-free enzymatic systems, tibenelast did not inhibit the synthesis of prostaglandins, thromboxanes, and leukotrienes.

Current drugs of choice for bronchodilation are theophylline and β-adrenergic agents. Although the mechanism of action for theophylline is unclear,

theophylline has been shown to be a PDE inhibitor. Interestingly, tibenelast also inhibited PDE, but it differed from theophylline in its tissue selectivity. Tibenelast was a poor inhibitor of heart PDE, and was moderately active against the enzymes from lung and stomach. Although the significance of this finding is unclear, animal studies and preliminary clinical results suggest that tibenelast is a bronchodilator associated with incidences of nausea [23]. These results suggest that the *in vivo* biological activity of tibenelast may actually reflect its *in vitro* selective inhibition of PDE.

Because PDE inhibition has been postulated to be the principal cause of the cardiac effect observed with theophylline and $\beta$-adrenergic agents [25–27], we studied the cardiovascular properties of tibenelast. In the anesthetized guinea pig, tibenelast functioned as a bronchodilator at an i.v. dose of 1.0 mg/kg but did not alter heart rate or cardiac contractility at this dose. By contrast, at its effective bronchodilatory dose of 10 mg/kg, theophylline significantly increased heart rate, cardiac contractility and PLVP.

Similar results were also obtained in the pentobarbital-anesthetized dog. The cardiovascular actions of theophylline in the dog were comparable to those observed in humans, i.e. increased contractility and heart rate. Tibenelast appeared to have little effect on these two parameters in the dog, but did cause a modest decrease in MAP. However, this effect was not apparent until a cumulative dose of 4.1 mg/kg was obtained. These results would suggest that tibenelast may relax bronchial smooth muscle without eliciting cardiovascular responses at its therapeutic doses. Further support for this suggestion is found in our study with cat papillary muscle. Compared to theophylline, tibenelast did not have a direct inotropic effect on myocardium. Thus, a possible explanation for this selectivity could reside in the relative specificities of tibenelast as a PDE inhibitor.

Of interest was the observation that the $IC_{50}$ of tibenelast for neutrophil PDE was 20- to 30-fold less than the $IC_{50}$ observed for other tissues examined. This biochemical effect was substantiated in the study of neutrophil activation. Because release of superoxide and lysosomal enzymes are two related events in neutrophil activation, we have utilized superoxide release from neutrophils as a measurement of cellular activation. At a concentration (approximately 10 $\mu$M) that inhibited neutrophil PDE, tibenelast also prevented superoxide production from human PMNL stimulated with FMLP, a synthetic chemotactic factor, or C5a, the circulating complement-derived chemotactic factor. Recently, various inflammatory mediators and cytokines have been shown to "prime" neutrophil superoxide production in response to soluble stimuli [19–22]. In our studies, we selected PAF and TNF as priming agents because of their alleged roles in the pathogenesis of asthma and shock [28–30]. Whereas both PAF and TNF did not elicit significant superoxide production at the concentrations used, combined treatment of PMNL with PAF or TNF and a soluble stimulus, such as FMLP, resulted in a 2- to 5-fold greater increase in superoxide production than that obtained using the stimulus alone. Tibenelast was equally effective in inhibiting superoxide release from PAF-primed PMNL stimulated with FMLP as from PMNL exposed to the stimulus alone. Surprisingly, inhibition of the TNF-primed system by tibenelast was decreased significantly. A possible reason for this observation may be due to a different priming mechanism between PAF and TNF.

Previous studies by Fantone *et al.* [31] have indicated that there are at least two mechanisms by which PMNL can be stimulated to produce superoxide; one is inhibited by $PGE_1$, and a second is independent of prostaglandin modulation. Since prostaglandin modulation of neutrophil activation has been correlated with the ability of $PGE_1$ to increase intracellular cAMP, tibenelast being a neutrophil PDE inhibitor may decrease superoxide release via a similar mechanism. Thus, we examined the tibenelast-mediated inhibition of neutrophil activation by two particulate stimuli, opsonized zymosan and the immobilized IgG complex, and four soluble stimuli, the chemotactic formyl peptide FMLP, rhC5a, PMA, and the calcium ionophore A23187. Consistent with the results obtained for $PGE_1$, tibenelast inhibited FMLP- and rhC5a-mediated activation but not that induced by PMA or A23187. An interesting observation in the present study is the lack of inhibition by tibenelast of particulate stimuli-mediated superoxide release from human PMNL. Under our experimental conditions, $PGE_1$ was active in inhibiting FMLP- or rhC5a-induced superoxide release ($IC_{50} = 0.1\ \mu M$) from human PMNL. However, using opsonized zymosan as the stimulus, only slight inhibition (30–40%) was observed even at a high concentration of 100 $\mu$M $PGE_1$ (unpublished results). In canine neutrophils, $PGE_1$ was reported to inhibit only 40% of the opsonized zymosan-induced superoxide production at 100 $\mu$M [32]. These findings are therefore consistent with the hypothesis that inhibition of neutrophil activation by tibenelast is a result of PDE inhibition and the resulting increase in intracellular cAMP concentration similar to those in response to $PGE_1$. More recently, selective cAMP peak IV PDE inhibitors have been shown to reduce the PMNL respiratory burst [33]. Whether tibenelast is also a selective inhibitor of peak IV PDE inhibitor remains to be determined.

In summary, tibenelast is a selective PDE inhibitor in that it is very effective against PMNL enzyme while being a weak inhibitor of the heart enzyme. The demonstration that the compound was without cardiovascular effects at the bronchodilatory doses is consistent with this biochemical result. Tibenelast inhibited stimulus-dependent human PMNL activation, and did not affect the superoxide production in the phagocytic process. Further studies with tibenelast in the late phase reaction may provide insight on the role of superoxide and neutrophil activation in asthma.

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