Department of Gynecologic Oncology
Roswell Park Memorial Institute
Buffalo, New York 14263
U.S.A.

KANAKENDU CHOUDHURY*
SUNIL K. CHATTERJEE
MALAYA BHATTACHARYA
JOSEPH J. BARLOW

* Present address: Department of Biochemistry, Chittaranjan National Cancer Research Centre, 37, S. P. Mukherjee Road, Calcutta 700026, India.

REFERENCES

1. M. Bhattacharya, S. K. Chatterjee and J. J. Barlow, *Cancer Res.* **36**, 2096 (1976).
2. S. K. Chatterjee, M. Bhattacharya and J. J. Barlow, *Proc. Am. Ass. Cancer Res.* **19**, 165 (1978).
3. S. K. Chatterjee, M. Bhattacharya and J. J. Barlow, *Cancer Lett.* **5**, 239 (1978).
4. S. K. Chatterjee, M. Bhattacharya and J. J. Barlow, *Cancer Res.* **39**, 1943 (1979).
5. B. S. Khatra, D. G. Herries and K. Brew, *Eur. J. Biochem.* **44**, 537 (1974).
6. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934)
7. S. K. Chatterjee and U. Kim, *J. natn. Cancer Inst.* **58**, 273 (1977).

Biochemical Pharmacology, Vol. 31, No. 3, pp. 460–463, 1982.
Printed in Great Britain.

0006-2952/82/030460-04 $03.00/0

The effect of indomethacin and its ester on lysosomal enzyme release from polymorphonuclear leukocytes and intracellular levels of cAMP and cGMP after phagocytosis of urate crystals

(*Received* 28 *November* 1980; *accepted* 30 *July* 1981)

Lysosomal enzymes are implicated in the pathogenesis of articular tissue degradation in several rheumatic diseases. The main sources of these enzymes are polymorphonuclear leukocytes and mononuclear phagocytes which release lysosomal enzymes after exposure to various phagocytic stimuli. Release of lysosomal enzymes from invading polymorphonuclears and other cells could be one of the targets for the therapeutic action of antirheumatic drugs. Many studies performed so far have yielded controversial results. This confusion originated from a great number of variable conditions used in experimental systems. Various authors used cell-free systems, cultured polymorphonuclear leukocytes and macrophages, different types of phagocytic stimuli (zymosan, aggregated IgG, urate crystals etc.). Relatively good agreement has been achieved when cultured polymorphonuclear leukocytes and macrophages have been used as test system—most clinically active non-steroidal antirheumatic drugs inhibited the release of lysosomal enzymes in concentrations higher than those achieved in clinical therapy. This is well documented in the case of indomethacin which in some experimental systems has been active in inhibiting lysosomal enzyme release in concentrations of 10^{-3} M to 10^{-4} M [1]. Lower concentrations were not significantly effective [2, 3], whereas higher concentrations (above 10^{-4} M) resulted in one experiment even in cytotoxicity and stimulation of zymosan-induced enzyme release [4].

The objective of our study was to examine the effect of indomethacin and ester of indomethacin with tropic acid—Tropesin (2-phenyl-3-(1-*p*-chlorbenzoyl-2-methyl-5-methoxy-3-indolyl) (acetoxypropionic acid in racemic form) both on the release of selected lysosomal enzyme from PMN leukocytes and on the levels of cyclic nucleotides involved in regulatory mechanism of the lysosmal enzymes release into the extracellular space. Microcrystals of sodium urate were used as stimulators of phagocytosis. Indomethacin and Tropesin were prepared in Research Institute for Pharmacy and Biochemistry, Prague. Monosodium urate (MSU) microcrystals (0.5–30 μm) were a gift from Dr. Továrek, University Hospital, Brno, Czechoslovakia. Phenolphthalein-β-D-glucuronide was from Koch-Light and cyclic AMP and cyclic GMP RIA kits from Amersham.

Separation of leukocytes [5]. Leukocytes were obtained from venous blood of healthy young men. Blood (450 ml) was drawn into plastic flasks with 0.9 ml of heparin (5000 U per ml) and 90 ml of a 6% dextran solution. Sedimentation was allowed to proceed in the same flasks for 40 min at room temperature. The cell-rich supernatant was sedimented at 100 *g* for 8 min at room temperature. The erythrocytes were removed by hypotonic lysis (90 ml 0.85% NaCl for 30 sec, 270 ml distilled water added for 20 sec, 90 ml 2.6% NaCl added), and the leukocytes were washed two more times in 0.15 M NaCl and resuspended in the buffered medium to a concentration of 5×10^7 leukocytes per ml medium (1% glucose in phosphate buffered saline, pH 7.4, containing 500 U of heparin per 100 ml). Neutrophils were 60–75 per cent of total leukocytes.

Measurement of enzyme release. Portions of cell suspension (0.7 ml) were dispensed into 10×75 mm plastic test tubes. The cells were incubated at 37° with gentle shaking with indomethacin or with Tropesin in various concentrations. Autologous serum was added to a concentration of 10%. After 1 hr incubation the cells were exposed for 1 hr to particles of microcrystalline monosodium urate. The final concentration was 0.5 mg urate per ml medium. At the end of experiments, tubes were centrifuged at 755 *g* at 4°. The cell-free supernatant fractions were used for enzyme determination. Portions (0.5 ml) of 0.05 M Tris–HCl buffer (pH 7.5) containing 4 mM EDTA were added to sediments (EDTA acts as a phosphodiesterase inhibitor to prevent degradation of cyclic nucleotides by plasma enzymes). Samples were heated to 100° and centrifuged at low speed.

Supernatant fractions were stored at −20° for determination of cyclic nucleotides.

Estimation of enzymatic activities. Lactate dehydrogenase (EC 1.1.1.27) was determined by the method of Bergmeyer *et al.* [6]. One unit of activity of lactate dehydrogenase was defined as decrease of absorbancy 0.001 value per min at 340 nm at 25°. Neutral proteases (EC 3.4.4.—) activity was estimated by the procedure reported by Ignarro [7]. Beta glucuronidase (EC 3.2.1.31) activity was measured with phenolphthalein glucuronide as substrate [8].

Determinations of total enzyme activities were made after cells, either incubated with tested drugs for one hour or without incubation, were lysed by six freeze–thaw cycles. Broken cell preparations were centrifuged and enzyme activities were then determined. Incubation with added drugs did not influence the total enzyme activity in comparison with untreated cells.

The cyclic nucleotide levels were estimated by cyclic AMP and cyclic GMP assay kit [9–11].

PMN leukocytes incubated with sodium urate microcrystals released studied lysosomal enzymes. Phagocytosis of urate crystals led to an increase in cytoplasmic lactate dehydrogenase (Tables 1 and 2). The phagocytic stimulation by urate crystals led, at the same time, to an increase in cGMP levels and a decrease in intracellular cAMP (Table 3). Preincubation with indomethacin or Tropesin, depending on the concentration used in medium after stimulation by urate crystals, reduced the level of released lysosomal enzymes (Tables 1 and 2). Only the lowest concentration of Tropesin was without any effect. Both agents significantly increased levels of cAMP. At the same time, after the incubation with indomethacin and its derivative, decreased levels of cGMP were detected.

The effect of non-steroidal antirheumatic agents has been explained by an interference into a number of processes concurrent with inflammatory reaction. Significant effects are those on prostaglandin synthesis and on the process of lysosomal enzyme release from PMN leukocytes and macrophages. Indomethacin belongs to the active agents

Table 1. The effect of indomethacin (IND) on release of lysosomal enzyme after phagocytosis of urate crystals (U)

	Lactate dehydrogenase (units)	Neutral protease (μg tyrosine/18 hr)	β-Glucuronidase (μg phenolphthalein/18 hr)
Polymorphonuclear leukocytes (PMN)	107.0 ± 4.1 (7.5%)	915.6 ± 2.7 (9.3%)	227.1 ± 2.1 (12.3%)
PMN + urate (U)	252.0 ± 3.3 (17.8%)	3048.6 ± 5.6 (31.1%)	625.9 ± 3.4 (34.1%)
PMN + U + 10^{-3} M IND	172.0 ± 3.2* (12.1%)	996.6 ± 4.6* (10.1%)	276.0 ± 2.7* (15.0%)
PMN + U + 5×10^{-4} M IND	208.0 ± 3.5* (14.7%)	1812.0 ± 4.5* (18.4%)	347.6 ± 2.1* (18.9%)
PMN + U + 10^{-4} M IND	227.0 ± 3.9* (16.0%)	2221.0 ± 8.3* (22.6%)	365.0 ± 3.1* (19.8%)
PMN + U + 5×10^{-5} M IND	213.3 ± 1.6* (15.1%)	2392.6 ± 1.8* (24.3%)	408.7 ± 2.0* (22.3%)
PMN + U + 10^{-5} M IND	226.6 ± 2.1* (16.0%)	2793.2 ± 2.7* (28.3%)	619.3 ± 2.9 (33.7%)
Total activity (100%)	1410.0 ± 12.1	9861.2 ± 15.6	1836.1 ± 13.4

Each number represents the mean of five samples ± S.D. All activities were calculated for 5×10^7 cells.
* Statistically significant difference against PMN + U, $P < 0.01$.

Table 2. The effect of Tropesin (TR) on release of lysosomal enzymes after phagocytosis of urate crystals (U)

	Lactate dehydrogenase (units)	Neutral protease (μg tyrosine/18 hr)	β-Glucuronidase (μg phenolphthalein/18 hr)
Polymorphonuclear leukocytes (PMN)	61.0 ± 1.3 (4.8%)	857.6 ± 10.6 (9.1%)	198.0 ± 1.7 (11.5%)
PMN + urate (U)	208.0 ± 1.5 (16.5%)	3053.1 ± 12.4 (32.4%)	481.3 ± 3.7 (28.6%)
PMN + U + 10^{-3} M TR	168.1 ± 1.4* (13.3%)	2655.3 ± 13.7* (28.2%)	265.6 ± 3.3* (15.5%)
PMN + U + 5×10^{-4} M TR	165.0 ± 0.8* (13.1%)	3014.3 ± 17.4† (31.8%)	304.3 ± 2.9* (17.8%)
PMN + U + 10^{-4} TR	202.6 ± 1.8† (16.1%)	3024.1 ± 10.8† (31.9%)	432.6 ± 6.1† (25.3%)
PMN + U + 5×10^{-5} M TR	199.3 ± 1.6† (15.8%)	3043.3 ± 7.2† (32.1%)	471.0 ± 1.5† (27.5%)
PMN + U + 10^{-5} M TR	206.6 ± 1.7 (16.4%)	3055.5 ± 12.5 (32.3%)	485.7 ± 2.9 (28.4%)
Total activity (100%)	1255.5 ± 14.1	9459.4 ± 22.5	1709.3 ± 12.2

Each number represents the mean of five samples ± S.D. All activities were calculated for 5×10^7 cells.
* Statistically significant difference against PMN + U, $P < 0.01$.
† Statistically significant difference against PMN + U, $P < 0.02$.

Table 3. The effect of indomethacin (IND) and Tropesin (TR) on cyclic nucleotides level after phagocytosis of urate crystals (U)

	cAMP (pmoles/5 × 10^7 cells)	cGMP (pmoles/5 × 10^7 cells)
Polymorphonuclear leukocytes (PMN)	12.63 ± 0.13	7.17 ± 0.26
PMN + urate (U)	7.01 ± 0.11	12.26 ± 0.47
PMN + U + 10^{-3} M TR	15.17 ± 0.18*	6.63 ± 0.12*
PMN + U + 5 × 10^{-4} M TR	13.43 ± 0.17*	8.90 ± 0.17*
PMN + U + 10^{-4} M TR	12.30 ± 0.23*	7.77 ± 0.16*
PMN + U + 5 × 10^{-5} M TR	11.12 ± 0.16*	9.12 ± 0.31*
PMN + U + 10^{-5} M TR	10.53 ± 0.33*	9.87 ± 0.20*
PMN + U + 10^{-3} M IND	19.06 ± 0.24*	5.70 ± 0.15*
PMN + U + 5 × 10^{-4} M IND	16.13 ± 0.26*	6.56 ± 0.24*
PMN + U + 10^{-4} M IND	13.36 ± 0.29*	7.25 ± 0.18*
PMN + U + 5 × 10^{-5} M IND	10.37 ± 0.18*	7.53 ± 0.26*
PMN + U + 10^{-5} M IND	9.89 ± 0.15*	7.62 ± 0.23*

Each number represents the mean of five samples ± S.D.
* Statistically significant difference against PMN + U, $P < 0.01$.
TR: Tropesin.
IND: indomethacin.

which have an effect on both the former and latter processes. Indomethacin concentration necessary for inhibition of lysosomal enzyme release is relatively higher than that needed for inhibiting the synthesis of prostaglandins [12]. In the individual studies it ranges between 10^{-4} M and 10^{-5} M [1, 3, 4]. Incubation of mouse peritoneal macrophages for 1 day to 4 weeks resulted in an increase in intracellular content of lysosomal enzymes and, at the same time, to the inhibition of their release [13]. The mechanism of effect of indomethacin can be explained in terms of lysosomal membrane stabilization [14], inhibition of phagocytosis [3, 15] and decrease in leukocyte motility [16]. Study of the distribution of marked indomethacin in rat embryonic fibroblasts did not suggest that indomethacin directly stabilises lysosomal membranes as it was not found in connection with lysosomes [17].

Indomethacin effects the level of cAMP which is involved in the transfer mechanisms of lysosomal enzyme release. The effect of the agent depends, again, on the concentration used on the experimental system. After short incubation (5 min) indomethacin in the rat skin led to an increase in the level of cAMP; the 30 min exposure resulted, however, in a decrease in the level of this cyclic nucleotide [18].

Indomethacin inhibits proteinkinases which are dependent on cAMP and are mediators of cAMP effect on metabolic processes in the cell [19]. The administration of indomethacin to the rats led to a decrease of cAMP in leukocytes of pleural exudations [20]. The rise in cAMP is explained by inhibition of cyclic AMP phosphodiesterase [18, 21]. An increase in adenylate cyclase is not excluded either [22]. Tropesin manifested in our experiment inhibitory properties identical to those of indomethacin. The ester used does not split in short-term incubation (up to 20 hr) and remains as an intact molecule. It maintains, at the same time, the pharmacobiochemical properties of the original indomethacin. This notion is an encouraging one, as this substance manifests in animals a lower gastrotoxicity and thus can be considered a prospective agent for clinical use [23].

Conclusion. We have made an *in vitro* study of the effect of indomethacin and Tropesin (2-phenyl-3-(1-*p*-chlorbenzoyl-2-methyl-5-methoxy-3-indolyl) acetoxypropionic acid in racemic form) on the lysosomal enzymes release from PMN leukocytes after phagocytosis of urate crystals. Both agents, depending on their concentration, reduced the amount of released β-glucuronidase, neutral protease and intracellular level of cGMP. After incubation with both agents the cAMP levels, however, increased. A regulation of lysosomal enzyme release by means of indomethacin and Tropesin at the level of cyclic nucleotides is considered.

Research Institute of Rheumatic Diseases
921 01 Piešťany
Czechoslovakia

DANIELA MIKULÍKOVÁ
KAREL TRNAVSKÝ

REFERENCES

1. R. J. Perper and A. L. Oronsky, *Arthr. Rheum.* **17**, 47 (1974).
2. K. Phadke, S. Nanda and K. Lee, *Biochem. Pharmac.* **28**, 3671 (1979).
3. R. J. Smith, *J. Pharmac. exp. Ther.* **200**, 647 (1977).
4. P. S. Ringrose, M. A. Parr and M. McLaren, *Biochem. Pharmac.* **24**, 607 (1975).
5. R. B. Zurier, S. Hoffstein and G. Weissmann, *J. Cell Biol.* **58**, 27 (1973).
6. H. U. Bergmeyer, E. Bernt and B. Hees, in *Methods in Enzymatic Analysis* (Ed. H. U. Bergmeyer) p. 736. Academic Press, New York (1965).
7. L. J. Ignarro, *Arthr. Rheum.* **17**, 25 (1974).
8. A. J. Barret, in *Lysosomes—A Laboratory Handbook* (Ed. J. T. Dingle), p. 116. North-Holland, Amsterdam (1972).
9. B. L. Brown, J. D. M. Albano, R. P. Ekins and A. M. Scherzi, *Biochem. J.* **121**, 561 (1971).
10. A. G. Gilmann *Proc. natn. Acad. Sci. U.S.A.* **67**, 305 (1970).
11. R. Kobayashi and V. S. Fang, *Biochem. biophys. Res. Commun.* **67**, 493 (1975).
12. R. J. Gryglewski, in *Anti-inflammatory Drugs* (Eds. J. R. Vane and S. H. Ferreira), p. 3. Springer, Berlin (1979).
13. C. Finlay, P. Davies and A. C. Allison, *Agents Acts* **5**, 345 (1975).
14. K. Goto, M. Hisadome, Y. Maruyama and H. Imamura, *Jap. J. Pharmac.* **28**, 433 (1978).
15. I. Spilberg, A. Gallacher, B. Mandell and D. Rosenberg, *Agents Acts* **7**, 153 (1977).
16. S. Spisani, G. Vanzini and S. Traniello, *Experientia* **35**, 803 (1979).
17. J. C. Blanchard, Y. J. Schneider, P. Tulkens and A. Trouet, *Archs. int. Pharmacodyn. Ther.* **240**, 4 (1979).
18. W. J. Hall and M. G. O'Regan, *J. Physiol.* **247**, 31 (1975).
19. H. S. Kantor and M. Hampton, *Nature* **276**, 841 (1978).

20. D. A. Deporter, C. J. Dunn and D. A. Willoughby, *Br. J. Pharmac.* **65**, 163 (1979).
21. V. Stefanovich, *Res. Commun. chem. path. Pharmac.* **7**, 573 (1974).
22. E. Remold-O'Donell and H. G. Remold, *J. biol. Chem.* **249**, 3622 (1974).
23. J. Grimová, L. Fišnerová, Z. Roubal, Z. Trnavská and O. Němeček, *Abstracts of International Meeting on Future Trends in Inflammation*, Abstract No 103, London, 18–22 February 1980.

Biochemical Pharmacology, Vol. 31, No. 3, pp. 463–466, 1982.
Printed in Great Britain.

0006-2952/82/030463-04 $03.00/0

Discordant findings concerning the stimulation by chlorpromazine of erythrocyte sugar transfer

(Received 10 *April* 1981; *accepted* 15 *July* 1981)

It is commonly observed that chlorpromazine and other psychoactive phenothiazines suppress permeation or transport mechanisms in biological membranes. The obvious potential relevance to the clinical applications of these drugs has focused attention particularly on their interference with the uptake of the prominent biogenic amines in brain slices [1–4] and in synaptosomes [3–9]. But similar blocking by chlorpromazine is evident also not only in the non-neural traffic in amino compounds [1, 10–15], but in a wide assortment of membrane transports, dealing with the active turnover of Na^+ and K^+ [16–19], the accumulation of Ca^{2+} within sarcotubular vesicles and other organelles [20–23], and the uptake of glucose and other simple sugars by various mammalian cells [19, 24–27] and even by protozoa [28].

However, one series of studies indicates that the action of chlorpromazine and related agents on sugar transport can be strongly stimulatory, rather than inhibitory. Examining the effects of such drugs on the facilitated diffusion of hexoses through human red-cell membranes, Baker and Rogers [29–32] found a sharply biphasic or triphasic response, as a function of the concentrations applied. Indeed, their dose–response curves show a virtual discontinuity at a critical chlorpromazine concentration range, where the general pattern of progressive transport inhibition is interrupted by an abrupt and marked acceleration of the net transfer of glucose or sorbose. In contrast, their parallel isotopic-tracer studies revealed no significant response to chlorpromazine in the equilibrium exchange of glucose, either at the critical levels where net entry and net exit were stimulated or (as confirmed by Morais *et al.* [27]) at the higher levels which distinctly blocked net movements.

Analysis of the operation of this mechanism that mediates the equilibration of monosaccharides across human erythrocyte membranes had been a central concern in our laboratory for many years, and we were hopeful of utilizing the unusual behavior of chlorpromazine described by Baker and Rogers, as a tool for improving differential radiochemical labeling of the membrane components involved in the transport. The feature that particularly engaged our attention was the marked enhancement of net sugar transport inducible with this drug (a response not seen in this insulin-independent system with any of the several other identified classes of inhibitors). However, we have been quite unable to confirm this critical phenomenon with chlorpromazine, using either of the two principal test procedures adopted by Baker and Rogers.

The studies reported here were pursued in three episodes: first (by D. M. S., as a student pilot project) throughout the academic year 1975–76, then (by P. G. LeF.) for several weeks in late 1977 and again in early 1980. Similar results were obtained on each occasion.

Methods. Human blood, either heparinized or in standard acid–citrate–dextrose bags, was obtained from the Nassau–Suffolk (N.Y.) Inter-County Blood Services, and stored at about 3°. A few hours prior to experimental work, the plasma and additives, together with the major part of the white cells, were removed by several serial centrifugal washes, first with isotonic saline solution (sometimes lightly citrated) until clotting factors were sufficiently diluted, and then with a balanced-salt medium.

Duplication of the nominal essentials of Baker and Rogers' principal experiments required only minor procedural alterations in techniques employed extensively in our laboratory for many years. For the densitometric (Ørskov) recording of D-glucose exit, the erythrocytes were suspended at approximately 5% (v/v) in the medium, with D-glucose at 75–150 mM, and incubated at 37.5° for 50–80 min, to assure equilibration of the sugar throughout the cell water. The suspensions were then centrifugally concentrated to varying degrees as high as 64% (v/v), to allow adequate dilution of the sugar upon reduction of the cell density to the level of about 0.3% required in the cuvette for satisfactory Ørskov densitometric recording [33]. The initial volume in the cuvette (continuously stirred at 37.5°) was usually either 12 or 13 ml, and contained the chosen concentrations of chlorpromazine and glucose. Recording was begun at the instant when glucose exit was initiated by injection of a fixed volume (varying on different occasions from 60 μl up to 1 ml) of the cell suspension. "Exit times" were estimated directly from the records as described by Sen and Widdas [34]. On a few occasions (noted below), the assigned levels of chlorpromazine were added also to the glucose-loaded cells, permitting preincubation with the drug prior to the start of sugar exit.

The isotopic tracing of L-sorbose uptake requires denser cell suspensions, in which a significant fraction of the drug is removed by the cells [35], subtracting to a variable degree from the nominal concentrations applied. For this reason, aliquots of the cell preparations were taken through three initial centrifugal washes in approximately 100-fold volumes of medium containing the several assigned chlorpromazine concentrations, and each was then brought to a fixed volume at approximately normal blood hematocrit. A small volume (generally 50 μl) from one of these dense suspensions was taken for each sorbose-uptake assay (run at 37–38°). Uptake was initiated by addition of an equal