Biochemical Pharmacology, Vol. 45, No. 4, pp. 978–981, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

Criteria for the identification of non-redox inhibitors of 5-lipoxygenase

(*Received* 14 *August* 1992; *accepted* 9 *November* 1992)

Abstract—Methoxyalkyl thiazoles have been identified as a novel series of selective 5-lipoxygenase inhibitors with anti-inflammatory properties (Bird *et al.*, *J Med Chem* **34**: 2176–2186, 1991). Based on their structure, it was proposed that the potency of these compounds is not due to redox or iron-chelating properties. In the studies reported here, it was found that the model compounds 1-[3-(naphth-2-ylmethoxy)phenyl]-1-(thiazol-2-yl)propyl methyl ether (ICI 211965) and 3-[1-(4-chlorobenzyl)-4-methyl-6-(5-phenylpyridin-2-ylmethoxy)-4,5-dihydro-1*H*-thiopyrano[2,3,4-*c*,*d*]indol-2-yl]-2,2-dimethylpropanoic acid (L-689,065) (1) are inactive as reducing substrates in the 5-lipoxygenase-catalyzed decomposition of lipid hydroperoxides, (2) inhibit the 5-lipoxygenase-catalyzed reaction of reducing agents with lipid hydroperoxides, and (3) strongly inhibit the turnover-dependent inactivation of 5-lipoxygenase. These three observations with ICI 211965 and L-689,065 are in contrast to the behavior of other potent 5-lipoxygenase inhibitors from other structural classes, such as L-670,630, BW A4C, and zileuton, which all function as reducing substrates for 5-lipoxygenase. The data indicate that methoxyalkyl thiazoles and thiopyranoindoles are reversible dead-end inhibitors of 5-lipoxygenase and that the effects of inhibitors on the pseudoperoxidase activity and rate of enzyme inactivation provide simple tests to distinguish between redox and non-redox inhibitors of 5-lipoxygenase.

Recent clinical data indicate that leukotrienes play a role in asthma and inflammatory bowel disease and that inhibition of their biosynthesis by blocking 5-lipoxygenase activity may be useful in the treatment of these diseases [1, 2]. Most of the currently available inhibitors of 5-lipoxygenase contain functional groups such as phenol, hydroxamate or *N*-hydroxyurea, and act by a redox mechanism or by chelation of the active site iron. Studies with soybean lipoxygenase as a model have demonstrated that some of these inhibitors convert the ferric enzyme to an inactive ferrous form [3, 4]. Indirect evidence that benzofuranol, hydroxamate and *N*-hydroxyurea inhibitors also reduce 5-lipoxygenase has been obtained from the observations that the inhibitors (1) stimulate the 5-lipoxygenase-catalyzed degradation of hydroperoxides (pseudoperoxidase activity), and (2) are oxidized by the enzyme during the reaction [5, 6]. In addition, certain redox-based inhibitors have been shown to increase the rate of enzyme inactivation during the lipoxygenase reaction [7] and to be converted by 5-lipoxygenase to unstable species leading to protein alkylation [6].

The ability of 5-lipoxygenase to catalyze drug oxidation implies that reactive radical species could be generated during the process of inhibition. Because of the limited stability and the potential toxicity of reducing inhibitors, it is of importance to identify inhibitors acting by a different mechanism. In the present study, we describe the characteristics of inhibition of 5-lipoxygenase by methoxyalkyl thiazole and thiopyranoindole derivatives, new classes of selective and chiral inhibitors of the enzyme [8–10]. The inhibitory effects of these classes of compounds have been evaluated in previous studies using intact cells or cell-free preparations from leukocytes as a source of 5-lipoxygenase activity [8–10]. The present results with purified human 5-lipoxygenase show that, in contrast to previously described inhibitors, these compounds are inactive as reducing substrates for 5-lipoxygenase and cause a marked protection against turnover-dependent inactivation.

* Abbreviations: 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 13-HPOD, 13-hydroperoxyoctadecadienoic acid; ICI 211965, 1-[3-(naphth-2-ylmethoxy)phenyl]-1-(thiazol-2-yl)propyl methyl ether; L-689,065, 3-[1-(4-chlorobenzyl)-4-methyl-6-(5-phenylpyridin-2-ylmethoxy)-4,5-dihydro-1*H*-thiopyrano[2,3,4-*c*,*d*]indol-2-yl]2,2-dimethylpropanoic acid; PC, phosphatidylcholine; and RP-HPLC, reverse phase-high pressure liquid chromatography.

Materials and Methods

Chemicals. The various inhibitors of 5-lipoxygenase were prepared at the Department of Medicinal Chemistry, Merck Frosst Research Centre for Therapeutic Research: zileuton [11], BW A4C [12], L-670,630 [13], ICI 211965* and analog [9] and L-689,065 [10] (structures and IC_{50} values are given in Fig. 1).

5-Lipoxygenase purification and assay. Human 5-lipoxygenase was prepared from Sf9 cells infected with recombinant baculovirus rvH5L0(8-1). The soluble cell-free fraction (S100) containing high levels of 5-lipoxygenase or the enzyme purified by ATP-agarose affinity chromatography (>95%) was prepared as previously described [14]. The activity of the oxygenase reaction was measured using a spectrophotometric assay by monitoring 5-hydroperoxyeicosatetraenoic acid (5-HPETE) production (A_{234}) after incubation of enzyme (0.3 μg/mL, 20 μmol 5-HPETE/mg protein) with arachidonic acid (20 μM), ATP (0.2 mM), $CaCl_2$ (0.4 mM), phosphatidylcholine (PC) (24 μg/mL) in 0.05 M sodium phosphate, pH 7.4 [7]. Enzyme activity was determined from the optimal (initial) change in A_{234} and the apparent rate constant of enzyme inactivation was estimated assuming a first-order decay of enzyme activity [7, 15]. Inhibitors were added from 500-fold concentrated stock solutions in dimethyl sulfoxide. Inhibitor concentrations causing a 50% decrease of the initial velocity were determined from five-point titration curves by non-linear regression analysis.

Measurement of pseudoperoxidase activity. The pseudoperoxidase activity was determined from the inhibitor-dependent consumption of 13-hydroperoxyoctadecadienoic acid (13-HPOD) catalyzed by the purified 5-lipoxygenase using the variation in A_{234} as previously described [5]. The assay mixture contained 10 μM 13-HPOD, 10 μM inhibitor, 0.4 mM $CaCl_2$, 0.2 mM ATP, 24 μg/mL PC and purified 5-lipoxygenase (3 μg/mL) in 0.05 M sodium phosphate, pH 7.4.

Results and Discussion

Effects of inhibitors on the pseudoperoxidase activity of 5-lipoxygenase. We have reported previously that inhibitors containing benzofuranol, hydroxamate and *N*-hydroxyurea

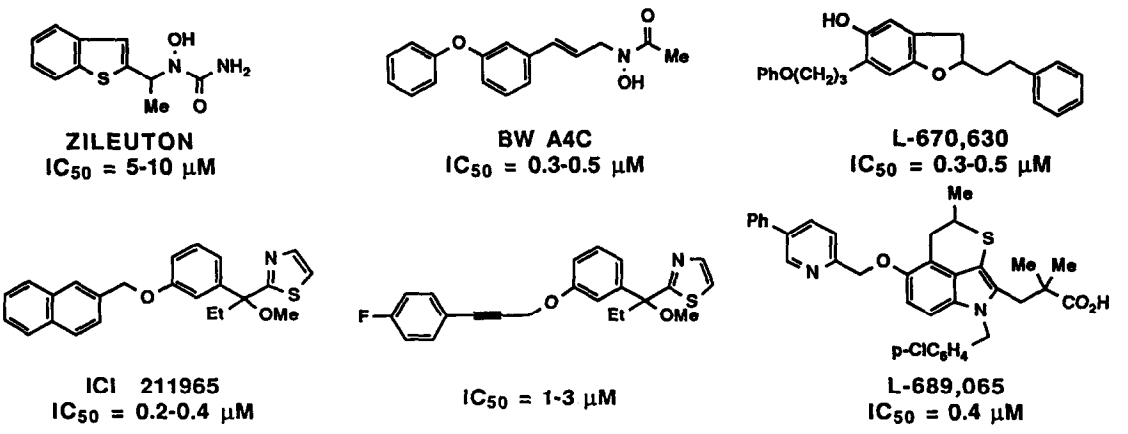

Fig. 1. Structures and potencies of 5-lipoxygenase inhibitors. The concentration of inhibitor causing a 50% decrease of the optimal rate of 5-HPETE production (IC_{50}) by purified human recombinant 5-lipoxygenase was determined from a five-point titration curve for each inhibitor (N = 2–3).

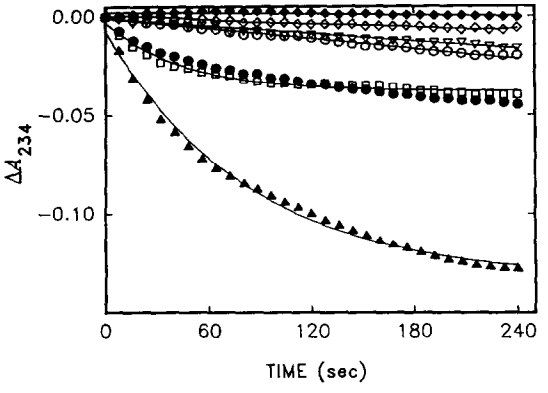

Fig. 2. Effects of ICI 211965, L-689,065 and reducing inhibitors on 5-lipoxygenase-catalyzed consumption of 13-HPOD. The conditions of incubation were as described for the pseudoperoxidase assay using 10 μM L-670,630 (▲), 10 μM zileuton (●), 10 μM BW A4C (□), 10 μM L-689,065 (◊) and 10 μM ICI 211965 in the presence (○) or absence (▽) of enzyme. The stable baseline observed with 10 μM 13-HPOD in the absence of inhibitor and enzyme is also given (♦).

moieties can function as reducing agents for the porcine leukocyte 5-lipoxygenase on the basis that they stimulate the pseudoperoxidase activity of the enzyme [5]. Figure 2 demonstrates that purified recombinant human 5-lipoxygenase also exhibited a pseudoperoxidase activity when incubated with 10 μM 13-HPOD and a 10 μM concentration of either L-670,630, BW A4C or zileuton. In contrast to these inhibitors, the methoxyalkyl thiazole derivative ICI 211965, its fluorophenyl alkynyl analog, and L-689,065 did not cause any significant stimulation of the pseudoperoxidase activity with a change in A_{234} of less than 3% of that observed with L-670,630. The slow decrease in A_{234} observed in the presence of ICI 211965 appeared to be due to changes in inhibitor solubility with time, rather than to the pseudoperoxidase reaction since it was independent of the presence of enzyme and did not correspond to a significant decrease in 13-HPOD as measured by reverse phase-high pressure liquid chromatography (RP-HPLC). It should be noted that the inhibitor concentration used in these assays (10 μM) was 5- to 30-fold higher than the IC_{50} value for the inhibition of the 5-lipoxygenase reaction. Furthermore, the potencies of ICI 211965 and L-689,065 were comparable to those of L-670,630 or BW A4C and more than 10-fold higher than that of zileuton (Fig. 1). These results indicate that the methoxyalkyl thiazole and thiopyranoindole inhibitors did not convert 5-lipoxygenase to a form which could be reoxidized by hydroperoxide, despite the fact that their inhibition potencies are within the range of those of reducing inhibitors.

Inhibition of the pseudoperoxidase and leukotriene A_4 (LTA_4) synthase activities of 5-lipoxygenase by non-redox inhibitors. The effects of the methoxyalkyl thiazole and the thiopyranoindole on the pseudoperoxidase activity driven by reducing inhibitors were examined. Figure 3 shows the time course of 13-HPOD consumption by 5-lipoxygenase (stimulated by a 10 μM concentration of the benzofuranol L-670,630) and the progressive inhibition by increasing amounts of ICI 211965 or L-689,065. This result demonstrates that the compounds can interact with 5-lipoxygenase under conditions where they did not cause any significant reduction of the enzyme. In addition, ICI 211965 was found to inhibit the synthesis of LTA_4 during arachidonic acid oxidation by 5-lipoxygenase, in parallel with the formation of 5-HPETE (data not shown). Thus, ICI 211965 can block all of the catalytic activities associated with 5-lipoxygenase.

Protection against enzyme inactivation by non-redox inhibitors. The 5-lipoxygenase shows non-linear kinetics with a rapid first-order decay in enzyme activity during arachidonic acid oxidation [14–15]. Progress curves for the oxygenase reaction of the purified enzyme in the presence of various inhibitors are given in Fig. 4. The apparent rate constant of enzyme inactivation (k_{in}) was 0.91 min^{-1} for the non-inhibited reaction and was increased slightly during inhibition by zileuton (k_{in} = 1.2 min^{-1} at 5 μM) (Fig. 4A) or BW A4C (k_{in} = 1.0 min^{-1} at 0.4 μM) (Fig. 4B). In sharp contrast, the rate of oxygenation of arachidonic acid in the presence of methoxyalkyl thiazoles was linear for the first 3 min of the reaction (Fig. 4, C and D) and was accompanied by a pronounced decrease in the rate of enzyme inactivation. The effect is such that a 30% increase in total 5-HPETE

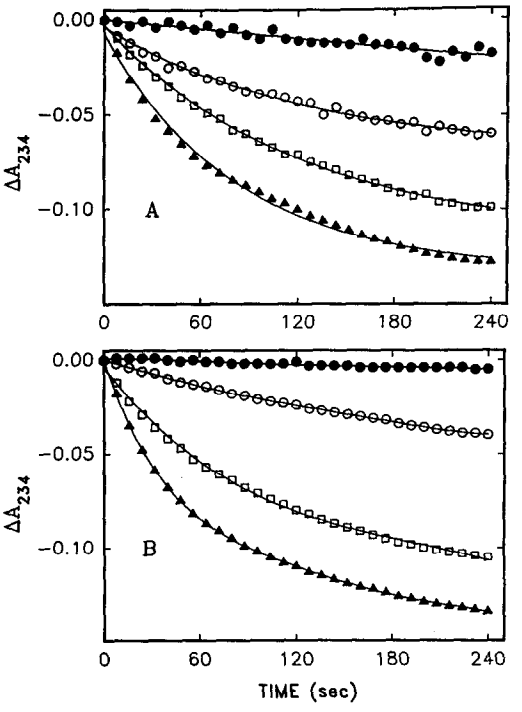

Fig. 3. Inhibition of the pseudoperoxidase activity of 5-lipoxygenase by ICI 211965 and L-689,065. The consumption of 13-HPOD (ΔA_{234}) in the pseudoperoxidase reaction stimulated by 10 μM L-670,630 was measured (A) in the absence (▲) and in the presence of 3.3 μM (□) or 10 μM (○) ICI 211965 and (B) in the absence (▲) and in the presence of 0.37 μM (□) or 3.3 μM (○) L-689,065. The variations in A_{234} for control incubation mixtures using 10 μM 13-HPOD and 10 μM inhibitor (no enzyme) are also shown (●).

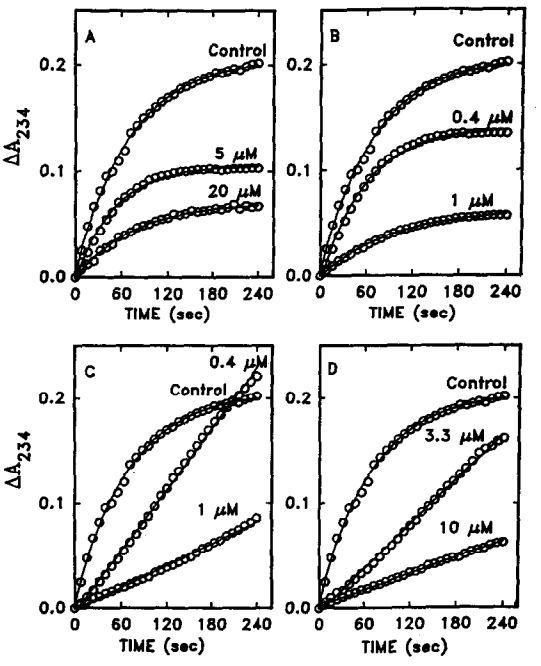

Fig. 4. Time course of 5-HPETE formation by purified 5-lipoxygenase in the presence of various types of inhibitors. The reaction was measured in the absence of inhibitors (control) and in the presence of the indicated concentrations of zileuton (A), BW A4C (B), ICI 211965 (C) and the fluorophenyl alkynyl analog of ICI 211965 (D).

accumulation could be obtained for the reaction inhibited by 0.4 μM ICI 211965 for which the initial rate was only 30% of that of the control experiment. L-689,065 also caused a significant decrease in the rate of enzyme inactivation ($k_{in} = 0.46\ min^{-1}$ at 1 μM). The simplest interpretation for the present data is that methoxyalkyl thiazoles and the thiopyranoindole inhibitor block 5-lipoxygenase activity by forming dead-end reversible complexes with the eznyme. Unfortunately, it has not been possible to determine if the inhibition is competitive in nature with the arachidonic substrate. Because of the difficulty in estimating initial rates and of the complexity of the 5-lipoxygenase reaction involving product activation, substrate inhibition (above 20 μM arachidonic acid) and rapid inactivation [16], steady-state kinetic analyses have not yet been performed successfully with this enzyme.

In conclusion, the present results show that methoxyalkyl thiazoles and thiopyranoindoles represent classes of potent 5-lipoxygenase inhibitors which do not function primarily as redox-based inhibitors, in contrast to compounds containing phenolic, hydroxamate and hydroxyurea functional groups (see Ref. 17 for a brief review). The most distinctive property of these non-redox inhibitors was the marked protection against enzyme inactivation which could be explained in part by a decrease in the self-catalyzed inactivation due to the reduced turnover of the inhibited reaction. Additional stabilizing effects of the methoxyalkyl thiazole inhibitors, such as competition with reactive species generated during the reaction, must also be envisaged since the accumulation of 5-HPETE reaction product could be increased by ICI 211965. It should be noted that no protection has been obtained for any of the compounds from other chemical series including hydroxyureas, 4- and 5-hydroxybenzofurans, hydroxamates (this study), flavonoids, quinones, phenothiazinones, BW 755C and phenidone [7]. The non-redox inhibitor should thus prove to be a useful tool to inhibit 5-lipoxygenase activity without general anti-oxidant effects and to characterize further the mechanism of lipoxygenase self-inactivation.

Acknowledgements—We would like to thank Y. Ducharme, P. Roy and D. Delorme for the synthesis of the inhibitors, and M. D. Percival and M. J. Gresser for valuable discussion during the course of this work.

*Departments of *Biochemistry and †Medicinal Chemistry*
Merck Frosst Centre for Therapeutic Research
Kirkland, Québec
Canada H9H 3L1

JEAN-PIERRE FALGUEYRET*
JOHN H. HUTCHINSON†
DENIS RIENDEAU*‡

REFERENCES

1. Lammers J-WJ, Van Daele P, Van den Elshout FMJ, Decramer M, Buntinx A, De Lepeleire I and Friedman B, Bronchodilator properties of an inhaled leukotriene

‡ Corresponding author: Dr. D. Riendeau, Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire-Dorval, Quebec, Canada H9R 4P8. Tel. (514) 428-2673); FAX (514) 695-0693.

D_4 antagonist (Verlukast—MK-0679) in asthmatic patients. *Pulmonary Pharmacol* **5**: 121–125, 1992.
2. Staerk Laursen L, Naesdal J, Bukhave K, Lauritsen K and Rask-Madsen J, Selective 5-lipoxygenase inhibition in ulcerative colitis. *Lancet* **335**: 683–685, 1990.
3. Clapp CH, Banerjee A and Rotenberg SA, Inhibition of soybean lipoxygenase 1 by *N*-alkylhydroxylamines. *Biochemistry* **24**: 1826–1830, 1985.
4. Kemal C, Louis-Flamberg P, Krupinski-Olsen R and Shorter AL, Reductive inactivation of soybean lipoxygenase 1 by catechols: A possible mechanism for regulation of lipoxygenase activity. *Biochemistry* **26**: 7064–7072, 1987.
5. Riendeau D, Falgueyret J-P, Guay J, Ueda N and Yamamoto S, Pseudoperoxidase activity of 5-lipoxygenase stimulated by potent benzofuranol and *N*-hydroxyurea inhibitors of the lipoxygenase reaction. *Biochem J* **274**: 287–292, 1991.
6. Rouzer CA, Riendeau D, Falgueyret J-P, Lau CK and Gresser MJ, Inhibition of human leukocyte 5-lipoxygenase by a 4-hydroxybenzofuran, L-656,224. Evidence for enzyme reduction and inhibitor degradation. *Biochem Pharmacol* **41**: 1365–1373, 1991.
7. Riendeau D, Falgueyret J-P, Nathaniel DJ, Rokach J, Ueda N and Yamamoto S, Sensitivity of immunoaffinity-purified porcine 5-lipoxygenase to inhibitors and activating lipid hydroperoxides. *Biochem Pharmacol* **38**: 2313–2321, 1989.
8. McMillan RM, Girodeau J-M and Foster SJ, Selective chiral inhibitors of 5-lipoxygenase with anti-inflammatory activity. *Br J Pharmacol* **101**: 501–503, 1990.
9. Bird TGC, Bruneau P, Crawley GC, Edwards MP, Foster SJ, Girodeau J-M, Kingston JF and McMillan RM, (Methoxyalkyl)thiazoles. A new series of potent, selective, and orally active 5-lipoxygenase inhibitors displaying high enantioselectivity. *J Med Chem* **34**: 2176–2186, 1991.
10. Hutchinson JH, Prasit P, Choo LY, Riendeau D, Charleson S, Evans JF, Piechuta H and Ball RG, Development of L-689,065—The prototype of a new-class of potent 5-lipoxygenase inhibitors. *BioMed Chem Lett* **2**: 1699–1702, 1992.
11. Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL, Summers JB and Brooks DW, 5-Lipoxygenase inhibitory activity of zileuton. *J Pharmacol Exp Ther* **256**: 929–937, 1991.
12. Jackson WP, Islip PJ, Kneen G, Pugh A and Wates PJ, Acetohydroxamic acids as potent, selective, orally active 5-lipoxygenase inhibitors. *J Med Chem* **31**: 499–500, 1988.
13. Lau CK, Belanger PC, Dufresne C, Scheigetz J, Therien M, Fitzsimmons B, Young RN, Ford-Hutchinson AW, Riendeau D, Denis D, Guay J, Charleson S, Piechuta H, McFarlane CS, Chiu S-HL, Eline D, Alvaro RF, Miwa G and Walsh JL, Development of 2,3-dihydro-6-(3-phenoxypropyl)-2-(2-phenylethyl)-5-benzofuranol (L-670,630) as a potent and orally active inhibitor of 5-lipoxygenase. *J Med Chem* **35**: 1299–1318, 1992.
14. Denis D, Falgueyret J-P, Riendeau D and Abramovitz M, Characterization of the activity of purified recombinant human 5-lipoxygenase in the absence and presence of leukocyte factors. *J Biol Chem* **266**: 5072–5079, 1991.
15. Rouzer CA, Thornberry NA and Bull HG, Kinetic effects of ATP and two cellular stimulatory components on human leukocyte 5-lipoxygenase. *Ann NY Acad Sci* **524**: 1–11, 1988.
16. DeWolf WE Jr, Enzymatic studies on purified 5-lipoxygenase. In: *Lipoxygenases and Their Products* (Eds. Crooke ST and Wong A), pp. 105–135. Academic Press, San Diego, 1991.
17. Riendeau D, Denis D, Falgueyret J-P, Percival MD and Gresser MJ, Catalytic properties and reaction mechanism of 5-lipoxygenase. In: *Prostaglandins, Leukotrienes, Lipoxins and PAF* (Ed. Bailey JM), pp. 31–37. Plenum Press, New York, 1991.