Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 488 (2004) 79–83
www.elsevier.com/locate/ejphar

Short communication

Licofelone, an inhibitor of cyclooxygenase and 5-lipoxygenase, specifically inhibits cyclooxygenase-1-dependent platelet activation

Serenella Rotondo*, Katarzyna Krauze-Brzósko, Stefano Manarini, Virgilio Evangelista, Chiara Cerletti[1]

Laboratory of Vascular Biology and Pharmacology, Consorzio Mario Negri Sud, Via Nazionale, 66030 Santa Maria Imbaro, Italy

Received 10 December 2003; received in revised form 4 February 2004; accepted 6 February 2004

Abstract

5-Lipoxygenase/cyclooxygenase inhibitors, possessing anti-inflammatory action and gastric safety due to cyclooxygenase-2 and 5-lipoxygenase inhibition and antiplatelet activity due to cyclooxygenase-1 blockade, would be beneficial in the treatment of ischemic disease because they may reduce, at the same time, inflammation, underlying the atherosclerotic process, and platelet activation, responsible for acute thrombotic events. In this study, we characterized the antiplatelet effects of the new 5-lipoxygenase/cyclooxygenase inhibitor licofelone ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3,dihydro-1*H*-pyrrolizine-5-yl]-acetic acid). Licofelone completely prevented platelet aggregation induced in platelet-rich plasma by threshold aggregating concentrations of arachidonic acid (0.87 ± 0.14 mM) at threshold inhibitory concentrations of 0.75 ± 0.35 μM ($n = 5$). Platelet-rich plasma aggregation induced by threshold aggregating concentrations of collagen/adrenalin (0.3 ± 0.05 μg/ml and 0.4 ± 0.1 μM, respectively) was reduced to $3.2 \pm 2\%$ of control at licofelone 100 μM, ($P < 0.05$, $n = 6$). Washed platelet aggregation induced by threshold aggregating concentrations of thrombin (0.07 ± 0.01 U/ml) was only partially affected by licofelone at concentrations one or two order of magnitude higher than those fully preventing arachidonic acid-induced aggregation ($44 \pm 11\%$ of control at 100 μM, $P < 0.05$, $n = 7$). Failure to prevent aggregation triggered by high concentrations of collagen/adrenalin in aspirin-treated platelets supports cyclooxygenase-1 as a specific target of licofelone. In fact, licofelone inhibited thromboxane B_2 (TxB_2) production by all the agonists tested at concentrations between 0.5 and 50 μM. At this concentration, TxB_2 production was reduced at values similar to those of unstimulated platelets. These results indicate that, at clinically relevant concentrations, licofelone exerts a potent antiplatelet effect mediated by the inhibition of cyclooxygenase-1 activity.

Keywords: Licofelone; Cyclooxygenase-lipoxygenase inhibitor; Platelet aggregation, human; Thromboxane A22

1. Introduction

Thrombosis—the current most common cause of ischemic disease such as myocardial infarction and stroke—is the late complication of atherosclerosis, a progressive inflammatory disease characterized by lipid infiltration in the wall of large arteries (Ross, 1999). Platelet activation plays a crucial role in thrombosis (Cerletti et al., 2002). Antithrombotic therapy with aspirin, which at low doses acts as a selective inhibitor of platelet cyclooxygenase-1 activity, has been tested in hundreds of clinical trials and shown to reduce by 25% both secondary (Antithrombotic Trialists' Collaboration, 2002) and primary (Hayden et al., 2002) incidence of myocardial infarction and cerebrovascular disease. However, a major limitation of aspirin is given by its gastrotoxicity, which is mainly linked to suppression of cytoprotective prostaglandins (Weil et al., 1995).

Selective cyclooxygenase-2 inhibitors, which are anti-inflammatory agents with lower gastrotoxicity than aspirin, may also down-regulate inflammatory components of the atherosclerotic plaque that play a major part in atherothrombotic disease (de Gaetano et al., 2003). Nevertheless, experimental studies (de Gaetano et al., 2003) suggested that selective blockade of cyclooxygenase-2 might increase cardiovascular risk, possibly due to endothelial prostaglan-

* Corresponding author. Tel.: +39-872-570-306; fax: +39-872-570-416.
E-mail address: rotondo@negrisud.it (S. Rotondo).
[1] Present address: Center for High Technology Research and Education in Biomedical Sciences, Catholic University, 86100, Campobasso, Italy.

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.02.006

din I2 suppression with untouched platelet thromboxane generation. However, the clinical evidence of an increased risk of cardiovascular events in patients taking cyclooxygenase-2 inhibitors remain controversial (Mukherjee et al., 2001; Konstam et al., 2001).

Newly developed inhibitors of 5-lipoxygenase and cyclooxygenase share the anti-inflammatory effect and gastric safety of cyclooxygenase-2 inhibitors, but also inhibit cyclooxygenase-1-mediated platelet function and 5-lipoxygenase-mediated synthesis of inflammatory leukotrienes and might thus be beneficial in the treatment of atherothrombosis (de Gaetano et al., 2003). Licofelone ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3,dihydro-1*H*-pyrrolizine-5-yl]-acetic acid, previously ML3000) is a cyclooxygenase and 5-lipoxygenase inhibitor in bovine and human platelets and granulocytes in vitro (Laufer et al., 1994a, Rotondo et al., 2002). It was specifically developed in search for an analgesic and anti-inflammatory compound with higher gastrointestinal safety than the classical non-steroidal anti-inflammatory drugs and with advantages over selective cyclooxygenase-2 inhibitors. Licofelone has shown anti-inflammatory, analgesic, and antiasthmatic effects (Laufer et al., 1994a; Laufer et al., 1995; Abraham et al., 1997). Its anti-5-lipoxygenase activity is also responsible for reduced gastrointestinal side-effects, as compared with conventional non-steroidal antinflammatory drugs, which are mainly due to leukotriene-induced polymorphonuclear leukocytes adherence to gastric microvessels and consequent mucosal injury (Wallace et al., 1994; Laufer et al., 1994b).

The aim of this study was to investigate the effect of licofelone on in vitro human platelet activation induced by cyclooxygenase-1-dependent agonists, such as arachidonic acid, and by agonists only partially dependent on cyclooxygenase-1 activity, such as the combination collagen/adrenalin and thrombin.

The direct cyclooxygenase-1 inhibitor diclofenac (Todd and Sorkin, 1988) and {(*R*)-2-[4-(quinolin-2-yl-methoxy)-phenyl]-2-cyclopentyl acetic acid} (BAY-X1005), which indirectly blocks 5-lipoxygenase activity by inhibiting its translocation from the cytosol to the membrane (Hatzelmann et al., 1993), were used as reference compounds.

2. Materials and methods

2.1. Materials

Arachidonic acid (sodium salt), adrenalin, thrombin from human plasma (2000 U/mg NIH protein), were from Sigma (St. Louis, MO, USA); collagen was from Mascia Brunelli (Milano, Italy); anti-thromboxane B_2 (TxB_2) monoclonal antibody was kindly provided by Dr. G. Ciabattoni (University of Chieti, Italy). Licofelone, kindly provided by Merckle (Ulm, Germany), was dissolved in dimethyl sulfoxide (DMSO). Diclofenac, provided by Alfa Wassermann (Alanno, Italy), was dissolved in bidistilled water. BAY-X1005 (*R*)-2[4-(quinolin-2-yl-methoxy)phenyl]-2-cyclopentyl acetic acid, from Bayer (Wuppertal, Germany), was dissolved in ethanol. Aspirin, water-soluble lysine salt Flectadol, was from Maggioni (Milano, Italy).

2.2. Methods

Blood (anticoagulated with 3.8% of trisodium citrate, 9:1, vol/vol) was collected from the antecubital vein of healthy volunteers, free from any medication for at least 2 weeks, under informed consent.

Platelets were isolated from whole blood using standard procedures performed at room temperature (Rotondo et al., 2002). Briefly, platelet-rich plasma was prepared by centrifuging whole blood at $250 \times g$ for 25 min. Washed platelets were separated from platelet-rich plasma by centrifugation in the presence of 2 μM prostaglandin E1, and the pellet was then washed with 10 ml of HEPES-Tyrode buffer (129 mM NaCl, 9.9 mM $NaHCO_3$, 2.8 mM KCl, 0.8 mM KH_2PO_4, 5.6 mM dextrose, 10 mM HEPES, pH 7.4), containing 2 μM prostaglandin E1 and 5 mM EGTA. Platelets were resuspended in HEPES-Tyrode buffer, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

In some experiments, platelet-rich plasma was treated with 300 μM of aspirin for 30 min at room temperature, to irreversibly block platelet cyclooxygenase-1. Absence of aggregation in response to high concentrations (2–4 mM) of arachidonic acid proved complete cyclooxygenase-1 inhibition.

2.3. Platelet aggregation and thromboxane B_2 measurements

Platelet aggregation was studied in a lumiaggregometer (Platelet Ionized Calcium Aggregometer, Chronolog, Mascia Brunelli) at 37 °C, under constant stirring at 1000 revolution per minute (rpm). After 10 min of preincubation at room temperature with licofelone, the reference drugs, or their solvent (control), added in a volume not exceeding 0.5% of total sample, 500 μl platelet-rich plasma, or 500 μl of washed platelet suspension (at 2×10^8 platelets/ml) were stimulated with threshold concentrations of arachidonic acid, the combination of collagen/adrenalin, or thrombin. Aggregation was recorded as change in light transmission and quantified as peak height, in centimeters, after 3 min of stimulation. Data are reported as percent of platelet aggregation with respect to samples stimulated in the presence of the same volume of the solvent (control). Threshold aggregating concentration of agonists was defined, for each individual sample, as the lowest concentration inducing maximal, irreversible aggregation (Di Minno et al., 1979). Threshold inhibitory concentration of licofelone was defined as the minimal concentration completely preventing platelet aggregation.

TxB_2, the stable metabolite of TxA_2, was measured by radioimmunoassay (Patrono et al., 1980) in the supernatant

obtained by centrifuging platelet-rich plasma or washed platelet samples for 3 min at 14,000 rpm, and expressed as pmol/ml by comparison with a standard curve.

2.4. Statistical analysis

Data are presented as means ± S.E.M. for the indicated number of independently performed experiments. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test; a P value of 0.05 or less was considered significant.

3. Results

3.1. Effect of licofelone on platelet aggregation and thromboxane B_2 production

Licofelone completely prevented platelet aggregation induced in platelet-rich plasma by threshold aggregating concentrations of arachidonic acid (0.87 ± 0.14 mM, $n = 5$) at threshold inhibitory concentrations of 0.75 ± 0.35 μM ($n = 5$). Platelet aggregation by arachidonic acid was similarly blocked by diclofenac (threshold inhibitory concentrations 0.75 ± 0.25 μM, $n = 3$), while it was not modified by BAY-X1005.

Platelet aggregation induced in platelet-rich plasma by threshold aggregating concentrations of collagen/adrenalin (0.3 ± 0.05 μg/ml and 0.4 ± 0.1 μM, for collagen and adrenalin, respectively, $n = 6$) was inhibited by licofelone in a concentration-dependent manner. The aggregation was reduced to 59 ± 19% and 3.2 ± 2% of control at 1 and 100 μM, respectively ($P < 0.05$, $n = 6$). Diclofenac showed an inhibitory effect similar to licofelone, while BAY-X1005 was ineffective (Fig. 1, panel A).

Raising collagen/adrenalin at concentrations that induce platelet aggregation independently from TxA_2 synthesis overcame the inhibitory effect of licofelone (not shown).

Licofelone and diclofenac, up to 100 μM, did not affect aggregation stimulated by collagen/adrenalin in aspirin-treated platelet-rich plasma (not shown).

Washed platelet aggregation induced by threshold aggregating concentrations of thrombin (0.07 ± 0.01 U/ml, $n = 7$) was only partially reduced by 25 μM licofelone to 42 ± 14% of control ($P < 0.05$, $n = 7$). Higher concentrations did not enhance the inhibitory effect.

Diclofenac and BAY-X1005 partially inhibited platelet aggregation induced by thrombin only at the highest concentration tested (100 μM), to 57 ± 16% ($P < 0.05$) and to 65 ± 18% of control (ns, $n = 5$), respectively (Fig. 1, panel B).

As with collagen/adrenalin, raising thrombin at concentrations that induce platelet aggregation independently from TxA_2 synthesis overcame the inhibitory effect of licofelone (not shown).

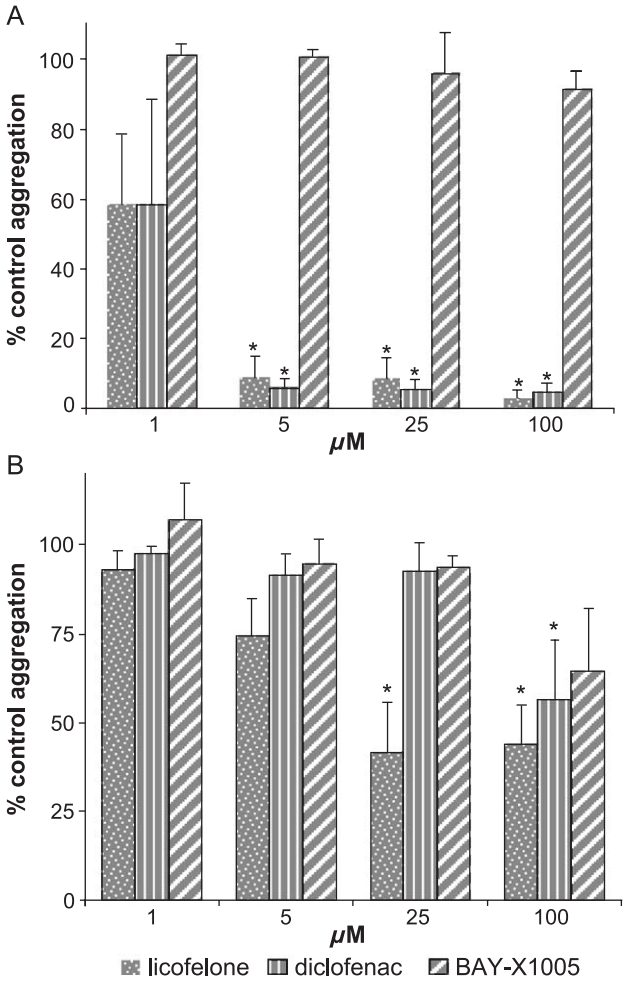

Fig. 1. Effect of licofelone, diclofenac, and BAY-X1005 on platelet aggregation induced by collagen/adrenalin combination (A) and thrombin (B). After 10 min of preincubation at room temperature with the drugs or their solvent (control), 500 μl of platelet-rich plasma (A) or of washed platelet suspension (2×10^8/ml) (B) were stimulated with threshold aggregating concentrations of the indicated stimuli. Aggregation was recorded as change in light transmission and quantified as peak height, in centimeters, after 3 min of stimulation, at 37 °C, at 1000 rpm. Data (means ± S.E.M, $n = 5–7$) are reported as percent of platelet aggregation in control samples; *$P < 0.05$, as compared to control values, at one-way ANOVA – Dunnett's test.

Licofelone inhibited TxB_2 production by all the agonists tested at concentrations between 0.5 and 50 μM. At this concentration, TxB_2 production was reduced by licofelone at values similar to those of unstimulated platelets (Table 1).

The effect of licofelone on platelet cyclooxygenase-1 activity was reversible. In fact, platelets in platelet-rich plasma pretreated with licofelone 10 and 50 μM, washed to eliminate the drug, and then stimulated with thrombin 0.5 U/ml, produced an amount of TxB_2 corresponding to 81 ± 2% and 56 ± 1% of control platelets, respectively ($P < 0.05$, $n = 3$, for both).

Table 1
Effect of licofelone on TxB_2 production by platelets stimulated with different agonists

	Platelet-rich plasma (pmol/ml)	Washed platelets (pmol/ml)	
Basal	0.5 ± 0.2	5.7 ± 1.4	
	Arachidonic acid	Collagen/adrenalin	Thrombin
DMSO 0.5% (control)	405 ± 101	21 ± 7	48 ± 1.8
Licofelone (μM)			
0.5	191 ± 90[a]	–	–
1	149 ± 50[a]	6.4 ± 1.5[a]	23 ± 10[a]
2.5	74 ± 36[a]	3.5 ± 0.7[a]	19 ± 3.5[a]
10	7.5 ± 4[a]	1.7 ± 0.3[a]	9.0 ± 5.0[a]
50	0.6 ± 0.1[a]	0.5 ± 0.1[a]	3.6 ± 1.7[a]

Platelet-rich plasma or washed platelets, preincubated with DMSO or licofelone for 10 min at room temperature, were stimulated with threshold aggregating concentrations of the indicated stimuli for 3 min at 37 °C, at 1000 rpm; TxB_2 was measured by radioimmunoassay in the supernatant obtained by centrifuging samples for 3 min at 14,000 rpm, and expressed as pmol/ml by comparison with a standard curve. Data are means ± S.E.M., $n = 3$.

[a] $P < 0.05$, with respect to control values, at one-way ANOVA – Dunnett's test.

The incomplete recovery of TxB_2 after platelet washing might be explained by the entrapment of the drug, which is highly lipophilic, in the platelet membrane.

In the same conditions, acetyl salicylic acid-pretreated platelets produced 0.7 ± 0.08% of TxB_2 in respect to control platelets ($n = 3$), which is in agreement with the known irreversible acetylation of platelet cyclooxygenase-1 by aspirin.

4. Discussion

This study shows that licofelone inhibits cyclooxygenase-1-dependent platelet activation. In fact, at very low concentrations, it completely prevents platelet aggregation induced by arachidonic acid and partially prevents activation by the combination collagen/adrenalin. The latter only in part depends on cyclooxygenase-1 activity and is restored just slightly raising agonists concentrations (Cerletti et al., 1986).

Licofelone inhibits platelet aggregation induced by thrombin at concentrations one or two order of magnitude higher than those fully preventing arachidonic acid-induced aggregation.

Licofelone does not prevent platelet aggregation induced by high concentrations of collagen/adrenalin, or of thrombin, as well as in aspirin-treated platelets. Moreover, the synthesis of thromboxane A_2 (TxA_2), the main product of cyclooxygenase-1 enzymatic action on arachidonic acid in platelets, triggered by different stimuli is strongly inhibited by licofelone in the same range of concentrations.

All together, these results support the conclusion that the platelet inhibitory effect of licofelone is mainly mediated by cyclooxygenase-1 blockade.

This indication is reinforced by the similar behaviour of the cyclooxygenase-1 inhibitor diclofenac, and by the lack of effect of the 5-lipoxygenase inhibitor BAY-X1005 in the same experimental systems.

The concentrations of licofelone active on platelets in vitro are in the range of the peak plasma levels (from 2 to 7 μM) measured in healthy volunteers after single or 7 days oral administration of 200 mg of the drug (Albrecht et al., 2002).

In conclusion, our results confirm and extend previous findings on antiplatelet effects of licofelone (Tries et al., 2002). The anti-cyclooxygenase-1 activity, combined with its anti-inflammatory action, make this type of drug of potential interest for the treatment of atherothrombosis.

Acknowledgements

We gratefully thank Dr. Giovanni de Gaetano, from the Center for High Technology Research and Education in Biomedical Sciences, Catholic University, Campobasso, Italy, for the helpful discussion, and the Gustave A. Pfeiffer Memorial Library staff for its contribution in editing the manuscript. Katarzyna Krauze-Brzósko was the recipient of a Marie Curie Host Fellowship of the European Union (Contract N. QLK4-CT-2000-60029).

References

Abraham, W.M., Laufer, S., Tries, S., 1997. The effects of ML3000 on antigen-induced responses in sheep. Pulm. Pharmacol. Ther. 10, 167–173.

Albrecht, W., Bias, P., Lammerich, A., Carré, C., Clerch, L., 2002. Pharmacokinetics, safety and tolerability of licofelone (ML3000) 200 mg b.i.d. given with food in young and elderly healthy volunteers. Ann. Rheum. Dis. 61 (S1), 422.

Antithrombotic Trialists' Collaboration, 2002. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 324, 71–86.

Cerletti, C., Carriero, M.R., de Gaetano, G., 1986. Platelet-aggregation response to single or paired aggregating stimuli after low-dose aspirin. N. Engl. J. Med. 314, 316–318.

Cerletti, C., Evangelista, V., Lorenzet, R., de Gaetano, G., 2002. Platelet-leukocyte interactions relevant to vascular damage and thrombosis. In: Gresele, P., Page, C.P., Fuster, V., Vermylen, J. (Eds.), Platelets in Thrombotic and Non-thrombotic Disorders. Pathophysiology, Pharmachology and Therapeutics. Cambridge University Press, Cambridge, UK, pp. 412–431.

de Gaetano, G., Donati, M.B., Cerletti, C., 2003. Prevention of thrombosis and vascular inflammation: benefits and limitations of selective or combined cyclooxygenase-1, cyclooxygenase-2 and 5-lipoxygenase inhibitors. Trends Pharmacol. Sci. 24, 245–252.

Di Minno, G., Silver, M.J., de Gaetano, G., 1979. Prostaglandins as inhibitors of human platelet aggregation. Br. J. Haematol. 43, 637–647.

Hatzelmann, A., Fruchtmann, R., Mohrs, K.H., Raddatz, S., Muller-Peddinghaus, R., 1993. Mode of action of the new selective leukotriene synthesis inhibitor BAY-X1005 ((*R*)-2-[4-(quinolin-2-yl-methoxy)-

phenyl]-2-cyclopentyl acetic acid) and structurally related compounds. Biochem. Pharmacol. 45, 101–111.

Hayden, M., Pignone, M., Phillips, C., Mulrow, C., 2002. Aspirin for the primary prevention of cardiovascular events: a summary of the evidence for the U.S. preventive services task force. Ann. Intern. Med. 136, 161–172.

Konstam, M.A., Weir, M.R., Reicin, A., Shapiro, D., Sperling, R.S., Barr, E., Gertz, B.J., 2001. Cardiovascular thrombotic events in controlled, clinical trials of rofecoxib. Circulation 104, 2280–2288.

Laufer, S., Tries, S., Augustin, J., Dannhardt, G., 1994a. Pharmacological profile of a new pyrrolizine derivative inhibiting the enzymes cyclooxygenase and 5-lipoxygenase. Arzneim.-Forsch. 44, 629–636.

Laufer, S., Tries, S., Augustin, J., Elsasser, R., Algate, D.R., Atterson, P.R., Munt, P.L., 1994b. Gastrointestinal tolerance of [2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1*H*-pyrrolizine-5-yl]-acetic acid in the rat. Arzneim.-Forsch. 12, 1329–1333.

Laufer, S., Tries, S., Augustin, J., Elsaßer, R., Albrecht, W., Guserle, R., Algate, D.R., Atterson, P.R., Munt, P.L., 1995. Acute and chronic anti-inflammatory properties of [2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1*H*-pyrrolizine-5-yl]-acetic acid. Arzneim.-Forsch. 45, 27–32.

Mukherjee, D., Nissen, S.E., Topol, E.J., 2001. Risk of cardiovascular events associated with selective cyclooxygenase-2 inhibitors. JAMA 286, 954–959.

Patrono, C., Ciabattoni, G., Pinca, E., Pugliese, F., Castrucci, G., De Salvo, A., Satta, M.A., Peskar, B.A., 1980. Low dose aspirin and inhibition of thromboxane B_2 production in healthy subjects. Thromb. Res. 17, 317–327.

Ross, R., 1999. Atherosclerosis: an inflammatory disease. N. Engl. J. Med. 340, 115–126.

Rotondo, S., Dell'Elba, G., Krauze-Brzosko, K., Manarini, S., Martelli, N., Pecce, R., Evangelista, V., Cerletti, C., 2002. Licofelone, a dual lipoxygenase-cyclooxygenase inhibitor, downregulates polymorphonuclear leukocyte and platelet function. Eur. J. Pharmacol. 453, 131–139.

Todd, P.A., Sorkin, E.M., 1988. Diclofenac sodium. A reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy. Drugs 35, 244–285.

Tries, S., Laufer, S., Radziwon, P., Breddin, K., 2002. Antithrombotic and platelet function inhibiting effects of ML3000, a new antinflammatory drug with cyclooxygenase/5-lipoxygenase inhibitory activity. Inflamm. Res. 51, 129–134.

Wallace, J.L., Carter, L., McKnight, W., Tries, S., Laufer, S., 1994. ML3000 reduces gastric prostaglandin synthesis without causing mucosal injury. Eur. J. Pharmacol. 271, 525–531.

Weil, J., Colin-Jones, D., Langman, M., Lawson, D., Logan, R., Murphy, M., Rawlins, M., Vessey, M., Wainwright, P., 1995. Prophylactic aspirin and risk of peptic ulcer bleeding. BMJ 310, 827–830.