Biochemical Pharmacology, Vol. 36, No. 20, pp. 3529–3531, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

SHORT COMMUNICATIONS

L-652,343, a novel dual cyclo/lipoxygenase inhibitor, inhibits LTB_4-production by stimulated human polymorphonuclear cells but not by stimulated human whole blood

(*Received* 18 *November* 1986; *accepted* 27 *March* 1987)

During the last few years more and more interest focused on leukotrienes (LTs) and on the development of new drugs aimed to block their synthesis or to antagonize their action.

The method most widely adopted for testing putative inhibitors of LT production has been that of using isolated purified preparations of LT-producing cells suspended in buffer and stimulated with appropriate inducers [1, 2]. However, this method may have some limitations and therefore we have recently developed and characterized a new system to study LTB_4 production by stimulated human whole blood and compared this with the traditional method which uses isolated polymorphonuclear leucocytes (PMNs) [3]. We have now tested the effects of a number of putative inhibitors of LT synthesis *in vitro* in the two systems. Essentially similar results were obtained except for one experimental compound, L-652-343 (3-hydroxy-5-trifluoromethyl- *n*- [2- (2-thienyl)- 2-phenyl-ethyl]- benzo[b]-thiophene-2-carboxamide), a novel dual cyclo/lipoxygenase inhibitor [4], which proved to be a potent inhibitor of A23187-induced LTB_4 production when tested with isolated cells but was completely inactive in whole blood.

Methods and results

Purified PMNs were prepared as previously described from human heparinized (10 U/ml) whole blood collected from drug-free healthy volunteers [3]. Aliquots (0.5 ml) of purified PMNs (10^7 cells/ml) were incubated for 10 min at 37° in a water bath in the presence of microliter amounts of the test drugs or their vehicles and then stimulated with A23187. The reactions were terminated after 10 min by centrifugation at 12,000 *g* for 2 min and the cell free supernatants were stored at −80° until assayed.

LTB_4 production by stimulated whole blood was measured as previously reported [3]. Briefly, 1-ml aliquots of heparinized (10 U/ml) human whole blood were incubated in a water bath at 37° for 1 min with microliter amounts of the test drugs or their vehicle. Then, A23187 in microliter amounts was added, the samples were thoroughly mixed by vortexing and left at 37° for 60 min. Incubations were terminated by centrifugation at 12,000 *g* for 2 min; the supernatant plasma was stored at −80° until further processing. We decided to use an incubation time at which LTB_4-production would surely be totally completed; this was different for whole blood and isolated PMNs because in the latter case LTB_4 levels tend to decrease rapidly after they have reached a peak at around 10 min [3].

LTB_4 was measured by radioimmunoassay (RIA), using a RIA Kit from Amersham (TRK.840, Amersham Int., Amersham, U.K.); unextracted samples were used when testing supernatants of isolated PMNs; plasma samples were extracted with 9 vol. aceton, dried under vacuum in a Speed Vac Concentrator (Savant Instruments Inc., Hicksville, NY, U.S.A.) and resuspended in Tris–HCl containing 0.1% gelatin, pH 8.6, before testing [3].

Thromboxane B_2 (TXB_2) was measured by a specific RIA in unextracted plasma samples, as previously described [5].

L-652,343 produced a dose-dependent inhibition of LTB_4 synthesis by isolated, purified human PMNs stimulated with A23187 with a concentration giving 50% inhibition (IC_{50}) of 1.4 μM. However, L-652,343 was inactive in A23187-stimulated whole blood in concentrations up to 10^{-3} M (Fig. 1). This loss of activity was confined to 5-lipoxygenase (5-LO) inhibition; indeed, TXB_2 formation was inhibited with an IC_{50} of 0.7 μM in A23187 stimulated whole blood (not shown). When using isolated PMNs, LTB_4 synthesis was inhibited more potently by L-652,343 than by another double cyclo/lipoxygenase inhibitor, BW755c [6], which showed an IC_{50} of 9 μM. However, BW755c inhibited LTB_4-production by A23187-stimulated whole blood with a similar potency as in isolated PMNs (Table 1). Other 5-LO inhibitors, including NDGA, nafazatrom and the novel phenothiazinone L-651,392 [7], were also nearly equipotent in the two systems; the cyclooxygenase inhibitor aspirin was inactive in both media (Table 1).

The activity of L-652,343 in the two systems was further characterized as follows: heparinized whole blood, collected from a healthy volunteer, was divided in four aliquots. Aliquots 1 and 2 were incubated for 10 min at 37° with 20 μM L-652,343 or its vehicle, respectively, and then stimulated with A23187. Aliquots 3 and 4 were also incubated with the drug or its vehicle respectively, but instead of being stimulated they were processed for the preparation of isolated PMNs, as described [3]. The PMNs thus obtained were stimulated with A23187. It was confirmed that L-652,343 did not affect LTB_4 production by stimulated whole blood; LTB_4 production by isolated PMNs prepared from whole blood which had been preincubated with the drug (aliquot 3) was also not inhibited (5.5 ± 0.4 ng/10^6 PMNs for aliquot 3 vs 5.8 ± 0.7 ng/10^6 PMNs for aliquot 4, mean ± SEM, N = 5); however, when 20 μM L-652,343 was added *in vitro* to the isolated cells a striking inhibition (99.1%) of LTB_4 synthesis was observed.

Finally, in order to evaluate the role of plasma proteins in the loss of activity of L-652,343, we carried out a dose-response study in isolated PMNs resuspended in buffer, or in buffer containing albumin (2 g/100 ml) (Behringwerke AG, Marburg, F.R.G.) or in a mixture of buffer and autologous plasma (50/50 v/v). Again the drug inhibited A23187-induced LTB_4-production in PMNs resuspended in buffer, but was inactive both in buffer plus albumin or in buffer plus plasma (Table 1).

Discussion

The use of a novel method to study LTB_4 production by stimulated whole blood [3] has enabled us to identify an important discrepancy in the effectivness of a new double cyclo/lipoxygenase inhibitor, L-652,343, when tested in a suspension of isolated PMNs or in whole blood. This drug is almost equally potent in inhibiting ram-seminal vesicle cyclooxygenase and RBL-1 cells 5-lipoxygenase *in vitro* [4]. In our study L-652,343 behaved as a strong inhibitor of LTB_4 synthesis in stimulated isolated PMNs but was ineffective in whole blood. The loss of activity appears to be due to the presence of proteins; indeed, when adding human plasma or simply human serum albumin to a suspension of isolated PMNs in buffer, the LTB_4-inhibitory activity was equally lost. Pharmacokinetic studies have shown an exten-

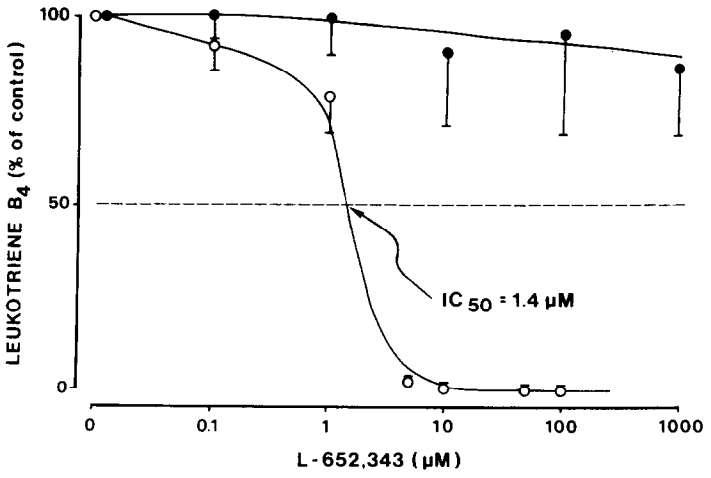

Fig. 1. Effect of L-652,343 on LTB_4 production by A23187 (10 μM)-stimulated human PMNs (○—○) or whole blood (●—●). Data represent mean ± SEM (N = 4–6). In this experiment, in the absence of the drug (control), A23187-stimulated PMNs produced 3.1 ± 0.6 ng $LTB_4/10^6$ cells (mean ± SEM, N = 4) in buffer and 4.2 ± 2.0 ng $LTB_4/10^6$ PMNs in whole blood (mean ± SEM, N = 6).

Table 1. IC_{50} (μM) of some drugs on LTB_4-production by A23187*-stimulated PMNs in different media

Compound	Medium	
	Buffer	Whole blood
L-652,343	1.4	>1000†
BW755c	9	4.7
NDGA	0.9	0.4
Nafazatrom	1.1	0.5
L-651,392	0.7‡	0.3
Aspirin	>1000	>1000

* A23187 was used at 10 μM final concentration.

† IC_{50} > 1000 μM was also obtained in buffer + plasma and in buffer + human serum albumin (see Methods).

‡ Data from Guindon *et al.* [7]

sive (>99.9%) protein binding of the drug (M. Goldenberg, personal communication).

A puzzling finding was that the loss of activity was confined almost exclusively to the 5-LO inhibitory effect: in A-23187-stimulated whole blood the drug continued to inhibit TXB_2 formation indicating the persistence of the blocking activity on platelet cyclooxygenase. Whether leucocyte cyclooxygenase was also inhibited is not known because leucocyte contribution to TXB_2 production by stimulated whole blood is probably negligible [8], and isolated purified PMNs stimulated with A23187 did not produce detectable amounts of TXB_2 in our experimental conditions (data not shown). Most of the other compounds tested, including the potent pure 5-LO inhibitor L-651-392, were almost equally potent in whole blood and in buffer.

Ex vivo, after acute or chronic dosing with L-652,343 to healthy volunteers, we obtained results similar to those described above: no significant effects on LTB_4 production by stimulated whole blood were seen despite a strong inhibition of TXB_2 synthesis and also of arachidonic acid-induced platelet aggregation [9].

The use of stimulated whole blood for testing drugs potentially active against LTB_4 synthesis may be advantageous over the more traditional system utilizing purified PMNs because it may reflect a situation closer to the physiologic one [3, 10]. It may be advisable to check the effectiveness of LT-synthesis inhibitory drugs in this system even when they have been shown to be effective in purified systems.

Whether the effectiveness of a compound in inhibiting LTB_4 production by stimulated whole blood is relevant for its pharmacological activity *in vivo* remains to be established. Animal studies seem to indicate that L-652,343 is effective in a number of models which are thought to be LT-mediated despite its lack of activity in our system [4].

In summary, L-652,343, a novel dual cyclo/lipoxygenase inhibitor, is a potent inhibitory of LTB_4-synthesis when using isolated PMNs while it is totally ineffective in whole blood. The addition of plasma or human serum albumin to PMN suspensions abolishes the LTB_4-inhibitory activity of L-652,343 indicating that extensive protein binding most probably accounts for the loss of activity of this drug in whole blood. Drugs potentially inhibiting LTB_4 synthesis should not be tested only in purified cell systems which lack the complex interactions with other cells and plasma components normally present in blood.

Acknowledgements—The technical assistance of Miss M. Bruynseels, G. Pieters and A. Hoogmartens is gratefully acknowledged. The authors thank Merck, Sharp & Dohme, and in particular Mrs A. Buntinx and Dr N. Baber, for support during these studies. We are indebted to Dr Marvin Goldenberg (Merck, Sharp & Dohme, Rahaway, NJ) for helpful discussions. The following chemicals were kindly donated: LTB_4, L-652,343 and L-651,392 by Dr J Rokach, Merk Frosst, Canada; BW 775c by Dr G. A. Higgs, Wellcome Res. Labs., Beckenham, U.K.; nafazatrom by Dr E. Philipp, Bayer AG, Wuppertal, F.R.G.

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Biochemical Pharmacology, Vol. 36, No. 20, pp. 3531–3533, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Polarography: a new tool in the elucidation of drug–albumin interactions

(*Received* 10 *November* 1986; *accepted* 13 *March* 1987)

Many benzodiazepine derivatives are widely used in the treatment of various psychic disorders, and much information is available about their clinical and pharmacological effects [1].

Human serum albumin (HSA) is known to possess several sets of binding sites for drugs. The widely used benzodiazepines generally show extensive binding to HSA, whereas other plasma proteins do not appreciably bind these drugs.

The equilibrium constants and the stoichiometries of the benzodiazepine–HSA complexes have a fundamental role in determining the free drug concentration in the plasma which, in turn, influences the pharmacological activities of these drugs. By using gel filtration, circular dichroism and microcalorimetric techniques, several authors [2–6] have calculated the equilibrium constants of various benzodiazepine–HSA complexes.

Quite recently* we proposed an original a.c. polarographic method for the study of the interaction between chlordiazepoxide and albumin. This method was based on the depression of the polarographic diffusion current of chlordiazepoxide by albumin. A similar phenomenon has been observed for some reducible substances when proteins are added and has been ascribed to complex formation between the reducible molecules and proteins [7–13]. We herein report the determination of the formation constant for the complex clorazepate–albumin using an a.c. polarographic method.

HSA (concentration 5%, mol. wt 69,000) was a product of the Sigma Chemical Co. Drugs were obtained from Lab. Chile, S.A. Santiago-Chile. The clorazepate concentration used was 0.25 mM. Sorensen buffer (pH 6–9) was used throughout these studies. The ionic strength was kept constant at 0.3 M with KCl. Polarographic measurements were performed in the TACUSSEL assembly previously described [14]. Potentials (Ep) were measured against a saturated calomel electrode.

The polarographic behaviour of clorazepate (CZP) has been well studied [15]. By a.c. polarography, we obtained an irreversible, diffusion-controlled and pH-dependent peak with a peak potential of −1.08 V at pH 7.4. The height of this peak showed a linear correlation with the CZP concentration. When increasing quantitites of HSA were added to a solution containing CZP, we observed a decrease in the height until a limiting value was reached. This effect was due to the interactions between HSA and CZP and could be used to calculate the most important binding parameters.

Ip and $(Ip)_o$ are the diffusion currents of the drug in the presence and the absence of HSA, respectively, and C, C_b and C_f are the concentrations of total, bound and free drugs, where

$$C = C_f + C_b \tag{1}$$

$$Ip/(Ip)_o = C_f + kC_b/C \tag{2}$$

the fractional coefficient, k, is the value of $Ip/(Ip)_o$ when enough excess HSA has been added to result in all the CZP being bound. This k value is obtained by an extrapolation method using the following condition:

$$\lim_{C_f \to 0} Ip/(Ip)_o = k \tag{3}$$

The experimental data were obtained by plotting $Ip/(Ip)_o$ versus HSA concentration at a fixed CZP concentration. Furthermore, decreasing CZP concentrations resulted in a decrease in the $Ip/(Ip)_o$ ratio at a constant HSA concentration. If protein adsorption to the drop, or another non-specific factor, were responsible for the diffusion current decrease, $Ip/(Ip)_o$ should, at a given HSA concentration, be independent of the concentration of CZP. Probably with a short drop time the HSA adsorption was non-competitive, with a highly favored interaction between HSA and CZP.

* J. A. Squella, E. Papic and L. J. Nunez-Vergara, presented at the Eighth International Symposium on Bioelectrochemistry and Bioenergetics, Bologna, June 1985.