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Guinea pig whole blood 5-lipoxygenase assay: utility in the assessment of potential 5-lipoxygenase inhibitors

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Abstract—Guinea pigs are widely used in the study of the role of leukotrienes in airway pathophysiology. Extensive research efforts have utilized this species in the development of potential therapeutic agents associated with inhibition of leukotriene production (e.g. 5-lipoxygenase inhibitors and 5-lipoxygenase-activating protein antagonists) for the treatment of acute bronchospasm in asthma. We now report, for the first time, an *ex vivo* whole blood 5-lipoxygenase assay in guinea pigs which should prove useful in the future development of leukotriene biosynthesis inhibitors. Addition of 150 μM arachidonic acid (AA) to heparinized whole blood for 5 min prior to the stimulation with 20 μg/mL A23187 resulted in a 10-fold increase in leukotriene B_4 (LTB_4; 11.36 ± 1.55 ng/mL) above basal (0.96 ± 0.29 ng/mL) within 10 min. To further validate the utility of the assay, we utilized the 5-lipoxygenase inhibitor BW A4C. Pretreatment of guinea pig whole blood with BW A4C *in vitro* prior to stimulation resulted in a concentration-dependent inhibition of LTB_4 production (IC_{50} = 229 nM), whereas thromboxane B_2 (TXB_2) production was unaffected. Likewise, when BW A4C was administered to guinea pigs intravenously (3 mg/kg), we observed a rapid and marked (≈90%) reduction in whole blood LTB_4 production which returned to control (pre-drug values) by 5 hr. In contrast, TXB_2 production was unaffected over the same experimental time period. In summary, we have described a whole blood assay which can discriminate 5-lipoxygenase inhibitors both *in vitro* and *in vivo*. Furthermore, this assay system will be of use in determining the potency, efficacy, selectivity, and pharmacodynamic properties of 5-lipoxygenase inhibitors in guinea pigs.

Elucidation of the role of leukotrienes in asthma has been an area of intense research in recent years. Guinea pigs provide a useful animal model for studying the role of leukotrienes in airway hyperreactivity [1] and have demonstrated utility in the development of potential therapeutic 5-lipoxygenase inhibitors [2]. Validation of the therapeutic efficacy of experimental compounds continues to be of vital importance in drug development. Specifically, quantitation of eicosanoids in plasma has proven to be a simple, yet effective method for evaluating bioavailability and pharmacodynamic properties of a variety of classes of therapeutic compounds including: 5-lipoxygenase inhibitors [3], thromboxane synthase inhibitors, platelet-activating factor antagonists [4], and dual cyclooxygenase/5-lipoxygenase inhibitors [4, 5] *in vivo*. Indeed, in a recent clinical trial with the 5-lipoxygenase inhibitor A-64077, Israel *et al.* [6] utilized an *ex vivo* whole blood assay in human asthmatics to validate drug efficacy and selectivity, highlighting the importance of a simple, reproducible assay in drug development. To date, the species in which whole blood *ex vivo* assays have been developed include rabbit [4], rat [3, 7], mouse [5, 7], and human [6–9]. However, a similar assay has not been reported in guinea pigs, the animal most utilized in the study of the role of leukotrienes in asthma. To further understand the relationship between *in vivo* efficacy of 5-lipoxygenase inhibitors in models of asthma, their metabolic selectivity, and pharmacodynamic properties, we have developed an *ex vivo* 5-lipoxygenase assay in guinea pig whole blood. In the present report, we (1) describe the assay development, (2) validate its utility to discriminate 5-lipoxygenase inhibitors with the use of the 5-lipoxygenase inhibitor BW A4C [3], and the cyclooxygenase inhibitor indomethacin *in vitro*, and (3) utilize the assay to define the *in vivo* pharmacodynamic properties and selectivity of BW A4C in this species.

Materials and Methods

Whole blood eicosanoid production. Male Hartley guinea pigs (Charles Rivers, Portage, MI) were anesthetized with a combination of ketamine (35 mg/kg; Aveco Co., Fort Dodge, IA) and xylazine (10 mg/kg; Butler Co., Columbus, OH) administered subcutaneously. Heparinized blood (20 U/mL) was obtained via cardiac puncture and was stimulated within 5 min. We examined a variety of stimulation conditions to optimize leukotriene B_4 (LTB_4*) production. Duplicate 1-mL aliquots of whole blood were incubated with various concentrations of the sodium salt of arachidonic acid (AA; Nu Chek Prep, Elysian, MN) in the presence or absence of various concentrations of the ionophore A23187 (supplied by Ms. Louise Crandall, Eli Lilly & Co.) at 37°. The reaction was terminated by centrifugation at 4° at various times after stimulation, and plasma was stored at −32°. From these initial concentration–response and time–response studies, we determined optimal conditions for LTB_4 production in guinea pig whole blood. In subsequent studies, whole blood was incubated with AA (150 μM) for 5 min at 37° prior to stimulation with A23187 (20 μg/mL). The reaction was allowed to proceed for an additional 10 min and was terminated by centrifugation; direct quantitation of eicosanoids in diluted plasma was carried out utilizing enzyme immunoassay (EIA) techniques.

Quantitation of eicosanoids by enzyme immunoassay. Direct quantitation of plasma eicosanoids was achieved by EIA utilizing reagents purchased from the Cayman Chemical Co. (Ann Arbor, MI) as outlined in the instructions obtained therein. Because of studies reporting cross-reactivity of 12-hydroxyeicosatetraenoic acid (12-HETE) in the immunoassay of plasma LTB_4 [7], we included the platelet 12-lipoxygenase metabolite, 12(*S*)-hydroxyeicosatetraenoic acid (12(*S*)-HETE) [10], in the LTB_4 immunoassay to determine cross-reactivity of this metabolite in the EIA.

Chromatographic purification of LTB_4 by reversed phase high-pressure liquid chromatography (RP-HPLC). To further examine the potential interference of 12-HETE in the immunoassay of plasma LTB_4 [7], in a single series of experiments we performed chromatographic purification of LTB_4 by RP-HPLC, modified from the method of Powell [11]. Briefly, blood was collected and incubated with AA (150 μM) for 5 min at 37° prior to stimulation with A23187 (20 μg/mL) for 10 min; plasma was recovered as described. Plasma was deproteinated by addition of 60% methanol (final concentration) and stored at −32°. Following

* Abbreviations: LTB_4, leukotriene B_4; LTA_4, leukotriene A_4; AA, arachidonic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; 12(*S*)-HETE, 12(*S*)-hydroxyeicosatetraenoic acid; TXB_2, thromboxane B_2; and EIA, enzyme immunoassay.

Table 1. Effects of various stimuli on eicosanoid production

Stimulus	N	LTB_4 (ng/mL)	TXB_2 (ng/mL)
Basal	6	0.96 ± 0.29	<0.10
AA (200 μM)	5	3.16 ± 1.06*	8747 ± 678*
A23187 (20 μg/mL)	4	3.64 ± 1.14*	991 ± 279*
AA (200 μM) + A23187 (20 μg/mL)	4	2.43 ± 0.35*	ND†

Blood was incubated in the presence or absence of stimuli for 10 min. Values are means ± SEM.

* Significantly greater than basal levels, $P < 0.05$.

† ND, not determined.

centrifugation, an aliquot of methanolic plasma was applied to a C18 (7 μm, 0.32 × 1.5 cm i.d.) guard column (Brownlee Laboratories Inc., Santa Clara, CA) and AA metabolites were eluted onto a Ultrasphere C18 (5 μm, 0.46 × 25 cm i.d.) analytical column (Beckman Instruments, Fullerton, CA). The mobile phase consisted of acidified water (pH 3.3 with phosphoric acid)/methanol. Separation was achieved utilizing a discontinuous methanol gradient [60–95% (v/v) over 22 min] at a flow rate of 1.8 mL/min. The fraction co-eluting with authentic LTB_4 was collected, dried under vacuum, reconstituted in immunoassay buffer, and stored at −32° until assayed. All reagents were of the highest possible quality.

Effect of the 5-lipoxygenase inhibitor BW A4C or the cyclooxygenase inhibitor indomethacin on whole blood LTB_4 production. To further validate the assay, blood was preincubated for 10 min at 37° in the presence of various concentrations of the 5-lipoxygenase inhibitor BW A4C (synthesized by Dr. T. Clarke, Erl Wood Research Laboratories, Eli Lilly & Co.) prior to the addition of AA and A23187. Since assay conditions were optimized for LTB_4 production, we included the cyclooxygenase inhibitor indomethacin (10 μM; Sigma Chemical Co., St. Louis, MO) to determine whether this whole blood assay would identify cyclooxygenase inhibitors. Data are expressed as a percentage of control eicosanoid production.

Pharmacodynamic profile and selectivity of BW A4C in vivo: Effect of BW A4C on whole blood eicosanoid production ex vivo. Additional experiments were carried out *in vivo* to demonstrate the utility of the *ex vivo* assay in determining the pharmacodynamic profile of 5-lipoxygenase inhibitors. Eighteen hours prior to experimentation, guinea pigs were anesthetized with halothane (Butler Co., Columbus, OH) and the left carotid artery was cannulated. BW A4C (3 mg/kg, i.v.) was administered and heparinized blood collected and stimulated immediately prior to, and at various times after, drug administration. Plasma was stored at −32° prior to quantitation of eicosanoids. Due to animal to animal variability, each donor served as its own control. Data are expressed as a percentage of pre-drug control (time 0) eicosanoid production.

Statistical analysis. Statistical analysis was performed utilizing a Student's *t*-test. A difference was considered to be significant at the $P < 0.05$ level.

Results and Discussion

Whole blood eicosanoid production. Various stimulation parameters were examined to determine optimal conditions for LTB_4 production in guinea pig whole blood. Basal immunoreactive LTB_4 in plasma isolated from guinea pigs was low (Table 1). In initial studies we found that stimulation of whole blood with either AA (200 μM) or A23187 (20 μg/mL) alone resulted in a modest and highly variable, though significant, increase in the production of LTB_4 (Table 1). The degree of stimulation was far less than that observed in other species [4–9]. Likewise, simultaneous administration of AA (200 μM) and A23187 (20 μg/mL) to guinea pig blood did not result in a significant increase in LTB_4 production above basal (Table 1). The assay was, however, functional as an increase in thromboxane B_2 (TXB_2) production was observed with the different stimuli (Table 1). We therefore determined that production of LTB_4 by whole blood of this species was aided by a 5-min preincubation with AA alone prior to the addition of A23187. Addition of 150 μM AA for 5 min prior to the stimulation with 20 μg/mL A23187 resulted in a 10-fold increase in LTB_4 above basal within 10 min; LTB_4 continued to increase, but at a slower rate, over the next 20 min (Fig. 1). For consistency, production of LTB_4 was measured 10 min after the addition of 20 μg/mL A23187 in subsequent assays. Next, we altered the amount of AA added in the initial 5-min period and noted a concentration-dependent increase in LTB_4 which peaked at 150 μM (Fig. 2a). Similarly, altering the concentration of A23187 in the presence of 100 μM AA resulted in a bell-shaped curve which peaked at 20 μg/mL A23187 (Fig. 2b). From these findings, subsequent *ex vivo* assays utilized a 5-min preincubation of guinea pig blood with 150 μM AA followed by a 10-min stimulation with 20 μg/mL A23187.

Unlike other species [4–9], stimulation of whole blood with A23187 alone resulted in only a modest (2- to 3-fold) increase in plasma LTB_4, and we can offer no plausible explanation for these species differences. We have observed, however, that utilization of this stimulation protocol in a variety of other species including rat, monkey, and human resulted in a more pronounced stimulation of LTB_4 production when compared to A23187 alone (data not shown). This would seem to indicate that a combined stimulation with AA and A23187 is generally a more effective stimulus for LTB_4 production in whole blood, most likely due to the added availability of substrate for 5-lipoxygenation following enzyme activation with A23187.

Effect of 12(S)-HETE on the immunoassay of LTB_4. Recently, Carey *et al.* [7] reported cross-reactivity of 12-HETE, in the radioimmunoassay of plasma LTB_4 which results in artifically elevated levels of immunoreactive LTB_4. To exclude the possibility of plasma 12-HETE interference in the immunoassay of LTB_4, we chromatographically purified plasma LTB_4 prior to quantitation with EIA. In a single series of experiments, plasma leukotrienes were extracted and fractionated by RP-HPLC. The immunoreactivity co-eluting with authentic LTB_4 was 38.67 ± 6.59 ng/mL compared with 9.51 ± 2.07 ng/mL when assayed prior to RP-HPLC (mean ± SEM, N = 5), indicating that plasma 12-HETE does not artifactually elevate LTB_4 levels when plasma is assayed directly. Additionally, the levels of LTB_4 detected following HPLC purification were higher than the values obtained following direct quantitation of diluted plasma, indicating that a substance(s) in plasma reduces the apparent immunoreactivity of LTB_4, possibly due to a high degree of plasma protein binding of LTB_4.

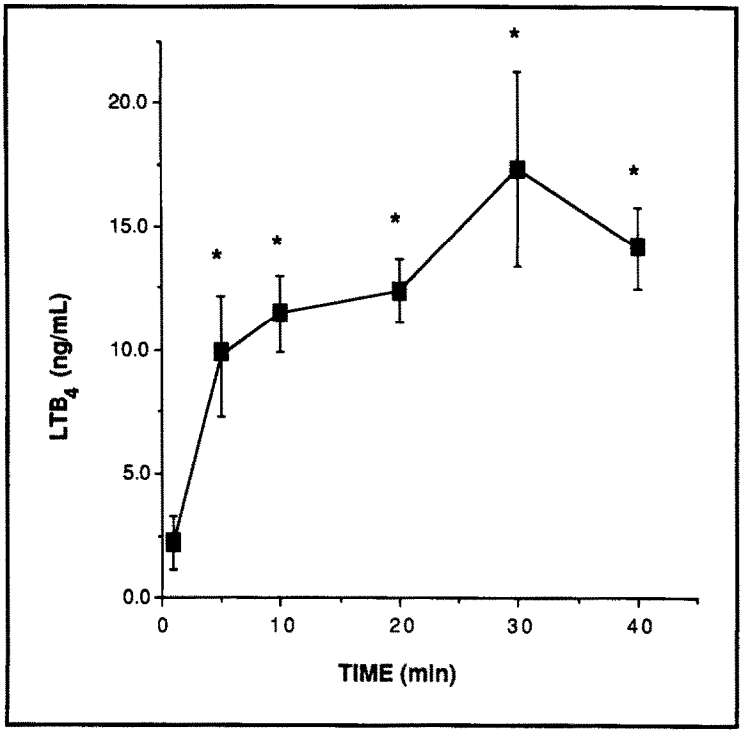

Fig. 1. Time course of LTB_4 production in guinea pig whole blood. Heparinized whole blood was incubated with 150 μM arachidonic acid for 5 min at 37° prior to stimulation with 20 μg/mL A23187. The reaction was terminated by centrifugation at 1, 5, 10, 20, 30, and 40 min post-A23187. Plasma LTB_4 concentrations were determined by EIA. Values are means ± SEM, N = 4. Key: * Significantly greater than basal levels, $P < 0.05$.

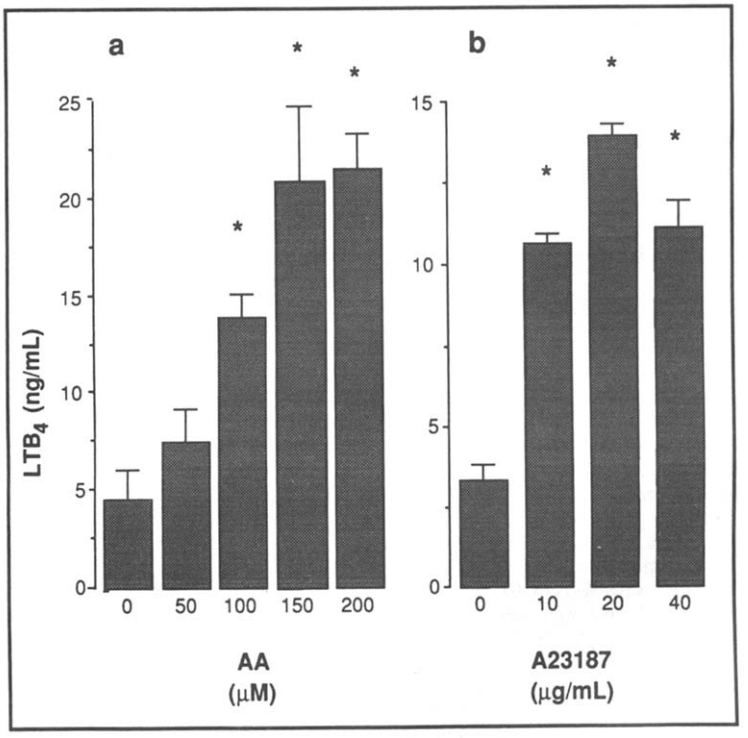

Fig. 2. Optimization of the whole blood 5-lipoxygenase assay. Heparinized whole blood was preincubated with either (a) various concentrations of AA, or (b) 100 μM AA, for 5 min at 37°. After 5 min, the blood was stimulated with (a) 20 μg/mL A23187, or (b) various concentrations of A23187. The reaction was allowed to proceed for an additional 10 min and was terminated by centrifugation. Plasma eicosanoid concentrations were determined by EIA. Values are means ± SEM, N = 3. Key: * Significantly greater than basal levels, $P < 0.05$.

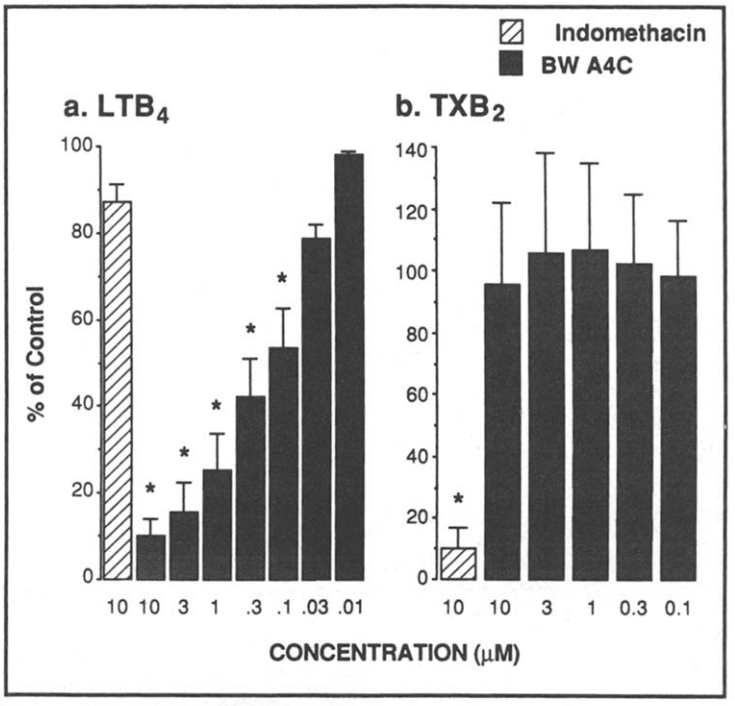

Fig. 3. Effect of various concentrations of BW A4C, or indomethacin (10 μM) on (a) LTB_4 or (b) TXB_2 production in guinea pig blood. Heparinized whole blood was preincubated with either BW A4C or indomethacin for 10 min at 37° prior to the addition of 150 μM arachidonic acid. After 5 min, the blood was stimulated with 20 μg/mL A23187. The reaction was allowed to proceed for an additional 10 min and was terminated by centrifugation. Plasma eicosanoid concentrations were determined by EIA. Control values were: LTB_4, 19.55 ± 7.18 ng/mL; TXB_2, 1190 ± 357 ng/mL. Values are means ± SEM, N = 3. Key: * Significantly less than control levels, $P < 0.05$.

To further exclude the possibility of plasma 12-HETE interference in the immunoassay of LTB_4, we evaluated the cross-reactivity of 12-HETE with the antibody used in these studies. Since the 12-lipoxygenase metabolite is likely derived from the platelet 12-lipoxygenase and therefore the 12(*S*)stereoisomer [10], we included this metabolite in parallel immunoassays. 12(*S*)-HETE at concentrations up to 50 μg/mL plasma, 25 times greater than the amount of 12-HETE produced in guinea pig blood [12] and greater than 5000-fold higher than the concentration of LTB_4, did not interfere. Collectively, these data indicate that the 12(*S*)-HETE cross-reactivity with the antibody used in these studies was minimal. In all subsequent experiments, eicosanoids were quantitated directly in plasma.

Effect of BW A4C or indomethacin on whole blood eicosanoid production in vitro. We next examined the effect of BW A4C, a 5-lipoxygenase inhibitor [3], on eicosanoid production in response to stimulation of whole blood with AA (150 μM) for 5 min followed by A23187 (20 μg/mL) for 10 min. Production of LTB_4 was inhibited in a concentration-dependent manner by preincubation of blood with BW A4C, resulting in an IC_{50} value of 229 nM (Fig. 3a). Additionally, BW A4C demonstrated specificity for 5-lipoxygenase in this assay system; there was no significant inhibition of TXB_2 production by this compound (Fig. 3b). These results are similar to those reported by Tateson *et al.* [3] in the rat. Preincubation of whole rat blood with BW A4C resulted in an IC_{50} of 100 nM for LTB_4 production. These investigators also report an IC_{50} value of 16.6 μM for TXB_2 production. We did not achieve these concentrations in our studies. However, since the assay we describe in guinea pigs was optimized for LTB_4 production, it was necessary to demonstrate that the assay would discriminate cyclooxygenase inhibitors. We therefore included indomethacin as a control. Preincubation of whole blood with indomethacin (10 μM) resulted in marked (≈90%) suppression of TXB_2 production without any significant effect on LTB_4 formation (Fig. 3).

The selectivity of BW A4C for 5-lipoxygenase *in vitro* has been reported [3]. In contrast to the 30- to >100-fold selectivity for 5-lipoxygenase compared to cyclooxygenase in a variety of test systems, BW A4C was reported to inhibit leukotriene A_4 (LTA_4) synthase. This activity could aid in its efficacy for LTB_4 inhibition observed in our studies in guinea pig whole blood. However, the studies on LTA_4 synthase reported by Tateson *et al.* were conducted in a partially purified enzyme preparation and it is uncertain whether this activity would translate to LTA_4 synthase inhibition in whole blood. Modest inhibition of 12- and 15-HETE formation was also noted. We did not confirm these results in the present guinea pig whole blood studies.

Pharmacodynamic profile and selectivity of BW A4C in vivo. As a final demonstration of the utility of this assay in documenting the efficacy, selectivity, and pharmacodynamic profile of 5-lipoxygenase inhibitors, we monitored LTB_4 and TXB_2 production in whole blood *ex vivo* immediately prior to, and at various times after, administration of BW A4C. Intravenous administration of BW A4C (3 mg/kg) resulted in a rapid suppression (≈90%) of LTB_4 production *ex vivo* within 10 min which was maintained for 1 hr and returned to pre-drug values by 5 hr (Fig. 4a). In marked contrast, TXB_2 production was

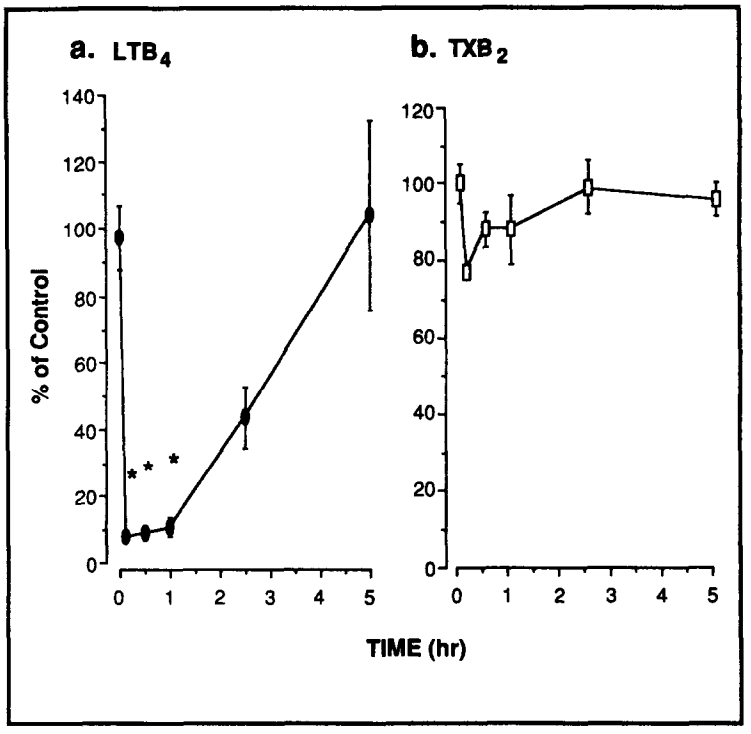

Fig. 4. Pharmacodynamic profile of BW A4C: Effect of BW A4C (3 mg/kg, i.v.) *in vivo* on (a) LTB_4 or (b) TXB_2 production in guinea pig blood *ex vivo*. Heparinized whole blood was collected immediately prior to (time 0), and at various times after, administration of BW A4C and incubated with 150 μM arachidonic acid for 5 min at 37°. Blood was stimulated with 20 μg/mL A23187, the reaction was allowed to proceed for an additional 10 min, and was terminated by centrifugation. Plasma LTB_4 and TXB_2 concentrations were determined by EIA. Control values were: LTB_4, 28.97 ± 4.20 ng/mL; TXB_2, 372 ± 125 ng/mL. Values are mean ± SEM, N = 3. Key: * Significantly less than pre-drug control levels, $P < 0.05$.

virtually intact over the same time period (Fig. 4b). The selectivity of BW A4C for inhibition of LTB_4 production is similar to the results reported by Tateson *et al.* [3] in rats following a dose of 50 mg/kg, p.o., a dose 17 times higher than that used in our studies. Marked (>80%) inhibition of LTB_4 production *ex vivo* was maintained over the 6-hr experimental time period while TXB_2 production was not affected, further documenting the selectivity and *in vivo* utility of this compound in the study of the role of leukotrienes in animal models of disease.

Thus, we have described an assay system to measure LTB_4 production *ex vivo* from guinea pig whole blood. This assay system can be used to discriminate 5-lipoxygenase inhibitors both *in vitro* and *in vivo* and will be of use in determining the efficacy, potency, selectivity, and pharmacodynamic properties of 5-lipoxygenase/leukotriene biosynthesis inhibitors in guinea pigs.

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In vitro inhibition studies of the glucuronidation of 3′-azido-3′-deoxythymidine catalysed by human liver UDP-glucuronosyl transferase

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3′-Azido-3′-deoxythymidine (zidovudine*), formerly known as azidothymidine (AZT), is at present the drug of choice for the treatment of AIDS and ARC although newly developed drugs such as ddI and 2′,3′-dideoxycytidine are currently being evaluated in clinical trials. Zidovudine is phosphorylated by cellular enzymes to the respective 5′-triphosphate which inhibits HIV, the aetiological agent of AIDS, replication by preferential inhibition of HIV reverse transcriptase and termination of viral DNA chain elongation [1]. In man, zidovudine has a half-life of 1 hr [2] and is metabolized extensively to an ether glucuronide (GAZT; [3]), thereby making the metabolite more polar and hence more suitable for renal excretion. To date, the UDPGT responsible for the metabolism of zidovudine remains unidentified although the $UDPGT_2$ form has been implicated recently [4]. In addition, there is the possibility of extrahepatic metabolism [5].

Because of the nature of the disease, patients with AIDS and ARC will be receiving, in addition to their AZT, a spectrum of other drugs. Whenever there is a multi-drug use there is the potential for drug interaction [6]. Interference with the conjugation of AZT could lead to the enhancement of AZT effect and potentially increased toxicity of the drug. Alternatively, the deliberate administration of a competing drug could have an economic benefit by enabling a reduction in AZT dosage regimens. In this regard, De Miranda *et al.* [7] and Kornhauser *et al.* [8] have advocated the use of probenecid, which inhibits AZT glucuronidation and the renal excretion of GAZT, to reduce the daily requirement of AZT. De Simone *et al.* [9] have suggested the use of inosine pranobex to inhibit the hepatic metabolism of AZT.

From a toxicological perspective it is essential to pinpoint drugs which are capable of interfering with the hepatic metabolism of AZT. Since an important group of patients presenting with AIDS or ARC are drug abusers we have examined the effects of a number of drugs commonly used by such patients (morphine, codeine, methadone and cocaine) on the glucuronidation of AZT by human liver microsomes. Previous work has demonstrated that both morphine and codeine are glucuronidated by human liver microsomes [10]. In addition, a range of other drugs known to be conjugated have been studied.

* Abbreviations: zidovudine, 3′-azido-3′-deoxythymidine; AZT, azidothymidine; AIDS, acquired immune deficiency syndrome; ARC, AIDS-related complex; GAZT, 3′-azido-3′-deoxy-5′-β-D-glucopyranosylthymidine; UDPGT, glucuronosyltransferase; UDPGA, UDP-glucuronic acid; ddI, 2′-3′-dideoxyinosine.

Materials and Methods

Chemicals. AZT and GAZT were gifts from the Wellcome Research Laboratories (Beckenham, U.K.). 2′,3′-Dideoxyinosine was a gift from Bristol-Myers Squibb (Wallingford, USA) and naproxen from Syntex Pharmaceuticals (Maidenhead, U.K.). Morphine sulphate, methadone hydrochloride, codeine, cocaine, diazepam, temazepam, flunitrazepam, probenecid, sulphamethoxazole, estrone, salicylic acid and UDPGA (ammonium salt) were purchased from the Sigma Chemical Co. (Poole, U.K.). HPLC grade acetonitrile was purchased from Fisons plc (Loughborough, U.K.). Orthophosphoric acid (Aristar grade) was purchased from BDH (Poole, U.K.). All other chemicals used were of the highest grade available.

Human liver samples. Samples of histologically normal livers were obtained from kidney transplant donors. Ethical approval for the studies was granted and consent to removal of the liver was obtained from the donors' relatives. Liver samples were transferred on ice to the laboratory within 30 min where they were sectioned into 10–20 g portions, placed in plastic vials and frozen in liquid nitrogen at −196°, immediately. Liver samples were stored at −80° until required.

Preparation of microsomes. Washed microsomes were prepared by the classical differential centrifugation technique [11]. Microsomal protein yield was determined by the method of Lowry *et al.* [12].

Glucuronidation assay. The glucuronidation of AZT was assayed in 1.5 mL microcentrifuge tubes that typically contained the following: 5–7 mg/mL microsomal protein, 5 mM $MgCl_2$, 50 mM Tris–HCl pH 7.5, 5 mM UDPGA and 0.5–10 mM AZT in a final volume of 0.2 mL. The detergent Brij 58 was used in preliminary experiments to determine optimal activation conditions. For optimal activation, microsomes were preincubated with 0.25 mg Brij 58/mg