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SHORT COMMUNICATION

Beditine, A New Benzodioxane Derivative, As a Suppressor of Human Polymorphonuclear Leukocyte and Platelet Activation

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ABSTRACT. Beditine, 2-(2-amino-4-thiazolyl)-1,4-benzodioxane hydrochloride is a new substance which reduced platelet activation and degranulation, prevented aggregation and superoxide generation by activated human polymorphonuclear leukocytes (PMNL) and inhibited the activation of arachidonate 5-lipoxygenase. Beditine may, therefore, be a useful agent in the treatment of cardiovascular disease. BIOCHEM PHARMACOL 53;11:1753–1755, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. leukocytes; platelets; aggregation; inhibitor of 5-lipoxygenase; beditin

Beditine, 2-(2-amino-4-thiazolyl)-1,4-benzodioxane hydrochloride is a new benzodioxane derivative synthesized in the Yerevan Institute of Fine Organic Chemistry. It was indicated as an α_2-adrenoblocking and antihypoxyic agent in circulatory diseases [1]. Ischemia-induced tissue injury is associated with activation of polymorphonuclear leukocytes (PMNL) and platelets. Leukocyte-derived mediators including, arachidonic acid (AA) metabolites and oxygen-derived free radicals, are involved in this process and play an important role in cardiovascular disorders [2–5]. The aim of this work is to determine whether beditine is able to reduce PMNL and platelet activation. In this communication, the inhibitory effects of beditine on arachidonate 5-lipoxygenase, superoxide anion generation and aggregation of human PMNL are demonstrated. Beditine is also shown to inhibit platelet aggregation and degranulation.

MATERIALS AND METHODS

Preparation of PMNL and Platelets

Platelets and neutrophils were separated from the venous blood of healthy donors who had not taken any drugs for ten days prior to donation. Platelet rich plasma (PRP) was obtained according to the method of Schmidt and Rasmussen [6] and PMNL by the method of Boyum [7]. The cells were washed twice in the presence of human serum albumin (0.5%) and then resuspended in Dulbecco's phosphate buffer (pH 7.45) at a final concentration of $40 \cdot 10^6$ cells/mL (platelet and mononuclear cell contamination lower than 2%).

Blood Cell Investigations

Platelet aggregation was studied by the method of Born, using a dual-channel Payton aggregometer (Payton Co., Palo Alto, CA, U.S.A.). The aggregation was induced with ADP (10^{-5} M) or adrenaline (10^{-5} M). ADP-induced ATP release was studied using the luciferin-luciferase method [8]. Aggregation of PMNL was also studied using the standard Payton platelet aggregometer [9]. Superoxide anion ($O_2^{-\cdot}$) generation by activated PMNL ($5 \cdot 10^6$ cells/mL) was measured as superoxide dismutase-inhibitable luminol chemiluminescence [10]. Chemiluminescence was recorded by Chrono Log ATP (Chronolog Ltd., Haverton, PA, U.S.A.) spectrophotometer. PMNL aggregation and $O_2^{-\cdot}$ generation were stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a final concentration of 10^{-7} M.

Biosynthesis

Ten μL aqueous solutions of beditine or idazoxane were added to a 1 mL suspension of PMNL ($40 \cdot 10^6$ cells/mL), in buffer containing 1 mM ATP, 1.6 mM $CaCl_2$ and the solution warmed to 37°C. At the 5 min ethanolic solutions of arachidonic acid and ionophore A-23187 (CalBiochem Behring, Mannheim, FRG) were added to the incubation mixture at final concentrations of 33 and 5 μM respec-

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Abbreviations: PMNL, polymorphonuclear leukocytes; AA, arachidonic acid; PRP, platelet rich plasma; fMLF, *N*-formyl-methionyl-leucyl-phenylalanine; 5-HETE, 5S-hydroxy-6,8,11,14(E,Z,Z,Z)-eicosatetraenoic acid; 12-HETE, 12S-hydroxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid; 15-HETE, 15S-hydroxy-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid; LTB_4, leukotriene B_4; HHT, 12S-hydroxy-5,8,10(Z,Z,E)-heptadecatrienoic acid

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TABLE 1. Effects (in IC_{50}, M) of beditine, nifedipine, aspirin and idazoxan on the activation of the platelets and PMNL

Effect	Stimulus	Nifedipine	Aspirin	Idazoxan	Beditine
Inhibition of platelet aggregation	ADP	$2.3 \pm 0.3\ 10^{-5}$	$7.7 \pm 0.5\ 10^{-5}$	NS	$7.0 \pm 0.6\ 10^{-5}$
Inhibition of platelet aggregation	adrenaline	$9.4 \pm 0.8\ 10^{-7}$	$1.1 \pm 0.9\ 10^{-4}$	$1.6 \pm 0.7\ 10^{-5}$	$9.1 \pm 1.2\ 10^{-5}$
Inhibition of ATP release	ADP	$3.7 \pm 0.5\ 10^{-6}$	—	—	$5.8 \pm 0.9\ 10^{-6}$
Suppression of PMNL aggregation	fMLP	$1.1 \pm 0.2\ 10^{-5}$	NS	NS	$1.9 \pm 0.1\ 10^{-5}$
Suppression of $O_2^{-\cdot}$ generation	fMLP	$4.4 \pm 0.7\ 10^{-5}$	NS	NS	$2.5 \pm 0.7\ 10^{-5}$

NS—not statistically significant effect, —effect not studied. Results show mean ± SEM for 6 tests.

tively. The incubations (5 min) were stopped by the addition of 1.5 mL methanol containing prostaglandin B_2 (PGB_2, 25 ng/10^6 cells). The mixture was centrifuged (10 min, 600 g), the supernatant diluted with water to 10 mL, acidified to pH 3 and subjected to solid phase extraction by a C18 Sep-Pak cartridge (Waters Associates Inc., Milford, MA, U.S.A.). Elution was performed stepwise with 10 mL water, 10 mL hexane, 5 mL water, 10 mL methanol-water (15:85, v/v) and 10 mL of methanol. The methanol fraction was evaporated to dryness and redissolved in HPLC eluting solvent.

HPLC Analysis

HPLC was performed using a Nucleosil C18 (5 μm) column (Machery-Nagel GmbH & Co. KG, Duren, FRG) using as a mobile phase methanol-water-acetic acid, 70:30:0,01 (v/v), pH 5,5 (by addition of aqueous ammonia, 35% solution) at a flow rate of 1 mL/min. Detectors used were the LKB-Bromma 2151 variable wavelength monitor (LKB, Bromma, Sweden) at 280 nm (0–18 min) and 235 nm (18–60 min), and a Polychrom-9060 diode array detector (Varian, Sunnyvale, CA, U.S.A.) at the wavelength range of 190–367 nm. Ultraviolet spectra were obtained on HPLC peaks using the scanning facility of the Polychrom-9060 detector. Identification of eicosanoids was performed on the basis of the UV and HPLC data by comparison with authentic standards of 5S-hydroxy-6,8,11,14(E,Z,Z,Z)-eicosatetraenoic acid (5-HETE), 12S-hydroxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid (12-HETE), 15S-hydroxy-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid (15-HETE) and leukotriene B_4 (LTB_4), 12S-hydroxy-5,8,10(Z,Z,E)-heptadecatrienoic acid (HHT) (Sigma Chemical Co.).

Statistical Analysis

The values were expressed as mean ± SE mean. Differences between means were analyzed by Mann–Whitney's U-test. A value of $P < 0.05$ was considered statistically significant. EC_{50} was calculated by regression analysis [11].

RESULTS AND DISCUSSION

As shown in Table 1, beditine reduces platelet aggregation and ATP release, with these effects being comparable to those of aspirin and nifedipine. The antiaggregatory effect of beditine was observed when platelet aggregation was induced both by ADP and adrenaline. In contrast, idazoxane, a selective α_2-adrenoceptor antagonist, only decreases adrenaline-induced aggregation (Table 1). Beditine suppressed PMNL aggregation and $O_2^{-\cdot}$ generation, its EC_{50} being similar to that of nifedipine, while aspirin and idazoxane had no significant effect (Table 1).

It is known that PMNLs aggregate, degranulate, generate oxygen active species and release oxidation products of arachidonate exposed to LTB_4 [5]. In order to examine the mechanisms of beditine in inhibiting leukocyte functions, AA metabolism in PMNL was studied. Calcium ionophore A-23187 stimulates mediator release and formation of all AA metabolites in PMNL [12]. At low concentrations (0.1 and 1.0 μM), beditine reduced the generation of both lipoxygenase (5-HETE, LTB_4 and 12-HETE) and cyclooxygenase (HHT) products (Fig. 1). At higher concentrations (10 and 100 μM), inhibition of arachidonate 5-lipoxygenase was greater than that of 12-lipoxygenase and cyclooxygenase (Fig. 1). It should be emphasized that an important feature of this enzyme is its being a prerequisite for Ca^{+2}

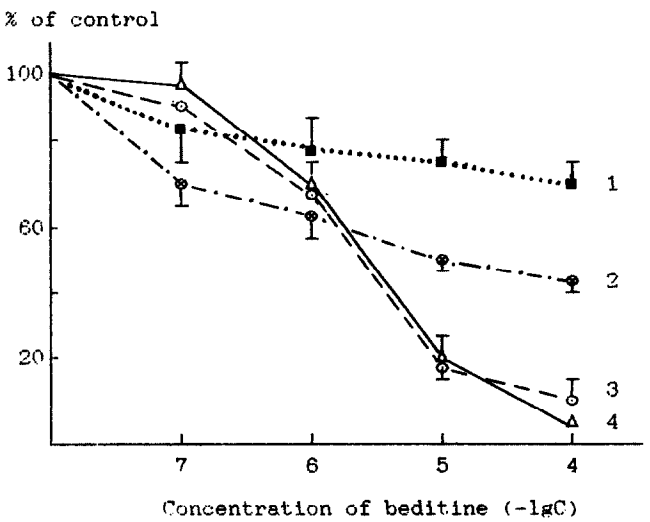

FIG. 1. Concentration-dependent effects of beditine on the biosynthesis of eicosanoids in PMNL activated by ionophore A-23187. 1—12-HETE; 2—HHT; 3—LTB_4; 4—5-HETE.

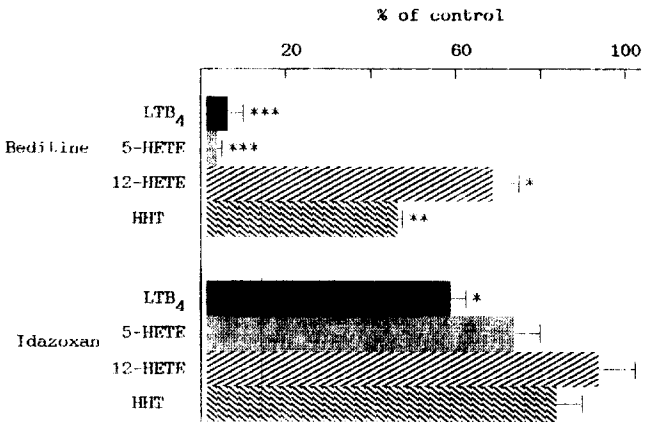

FIG. 2. Comparison of effects of beditine and idazoxan (both at a concentration of 10^{-4} M) on the biosynthesis of eicosanoids in PMNL activated by ionophore A-23187. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

activation [13]. Idazoxane did not significantly reduce AA metabolism (Fig. 2).

It has been proposed that arachidonate 5-lipoxygenase products and superoxide radicals contribute to ischemia-induced damage [2, 4, 5]. If this is so, the inhibition of their formation by beditine should help to prevent such injury. For variety of agents that are cardioprotective, their ability to reduce infarct size is correlated with their ability to inhibit arachidonate 5-lipoxygenase activity, reduce free radical generation and to increase intracellular cyclic AMP [2]. The fact that beditine reduces the activation of human platelet and PMNLs *in vitro* suggests that this drug could prevent their intravascular activation, aggregation and degranulation, which play an important role in the development of circulatory diseases [2–4, 14].

The results obtained support the use of beditine in the treatment of cardiovascular disorders. Our results indicate that beditine may be more effective in preventing the activation of intravascular blood cells and thrombosis than aspirin. The therapeutic effect of aspirin is associated with its ability to reduce platelet activation; however, it does not influence the leukocyte function, a factor which theoretically reduces its potential beneficial effects in circulatory diseases. Activation of PMNL provokes platelet aggregation and degranulation, therefore antiplatelet therapy may not be effective without the correction of the PMNL functional state [9, 15]. Beditine can reduce the activation of both platelets and PMNL simultaneously. Although beditine inhibits platelet and PMNL functions *in vitro* at relatively high concentrations, preliminary results obtained in our laboratory also show its ability to prevent the cell activation *in vivo*. Interestingly, other antiplatelet drugs, in particular calcium antagonists, also inhibit platelet activity *in vitro* at higher concentrations than *in vivo* [16].

The results of this work and the data obtained earlier [1] indicate that the effects of beditine cover many aspects of circulatory regulation, including changes in vascular tone, antihypoxic effects and effects on blood cells. While it is likely that the effects on vascular tone are largely mediated by α_2-blockade, the effect of beditine on circulatory blood cells is likely to be mediated by another mode of action. This hypothesis is confirmed by our observation that idazoxane, a selective α_2-adrenoreceptor antagonist, does not reduce ADP- and collagen-induced platelet aggregation, PMNL activation nor AA metabolism in PMNL, whereas beditine inhibits all of these responses.

We suggest that the effects of beditine might be explained by its ability to inhibit AA metabolism in PMNL.

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