

Protective Effect of KBT-3022, a New Cyclooxygenase Inhibitor, in Cerebral Hypoxia and Ischemia

Noriko Yamamoto¹, Koichi Yokota¹, Mikio Yoshidomi¹, Akira Yamashita¹ and Minoru Oda²

¹New Drug Research Laboratories, Kanebo, Ltd., 5-90, Tomobuchi-cho 1-chome, Miyakojima-ku, Osaka 534, Japan

²Research Laboratories, Torii Pharmaceutical Co., Ltd., 2-1, Ohnodai 1-chome, Midori-ku, Chiba 267, Japan

Received June 6, 1995 Accepted October 4, 1995

ABSTRACT—The protective effect of KBT-3022 (ethyl 2-[4,5-bis-(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetate), a new cyclooxygenase inhibitor, in cerebral hypoxia and ischemia was studied and compared with those of indomethacin and acetylsalicylic acid (ASA). Oral administration of KBT-3022 (3–100 mg/kg) and indomethacin (3 and 10 mg/kg) significantly prevented KCN-induced death in mice, while ASA (100 mg/kg) had no effect. KBT-3022 (3 and 10 mg/kg, p.o.) and indomethacin (10 mg/kg, p.o.) significantly prolonged the survival time of mice subjected to normobaric hypoxia, while ASA (100 mg/kg, p.o.) had no effect. KBT-3022 (3–30 mg/kg, p.o.) and indomethacin (3 mg/kg, i.p.) significantly ameliorated delayed neuronal death in the gerbil hippocampal CA1 sector after occlusion of bilateral carotid arteries for 5 min, while ASA (300 mg/kg, p.o.) had no effect. KBT-3022 (10 mg/kg, p.o.) significantly inhibited ATP depletion in the gerbil hippocampus after a 1-min occlusion of bilateral carotid arteries, but had no effect on ATP depletion after a 5-min occlusion and the recovery during recirculation. These results show that KBT-3022 exerts protective effects against cerebral anoxia and hypoxia and ameliorates delayed neuronal death in the hippocampus. KBT-3022 may therefore be useful for prophylaxis of ischemic cerebrovascular disorders.

Keywords: Cyclooxygenase inhibitor, KBT-3022, Hypoxia, Ischemia, Cerebrovascular disorder

Brain tissue is very vulnerable to oxygen deprivation. During cerebral anoxia, hypoxia or ischemia, depletion of the tissue stores of ATP causes dysfunction of ion transport systems and results in membrane depolarization with rapid elevation of the intracellular calcium concentration (1). This accumulation of intracellular calcium activates membrane phospholipase, resulting in the release of free fatty acids, especially arachidonic acid (AA) (2, 3). Recent studies have suggested that AA metabolites derived from both the cyclooxygenase and lipoxygenase pathways contribute to the development of cerebral injury through regulation of cerebral blood flow, vascular permeability and modulation of neurotransmission (4–6).

Moreover, the conversion of AA to prostaglandins (PGs) produces cytotoxic oxygen free radicals that induce cellular damage (7–10). Thus, an inhibitor of AA metabolism would be expected to be useful for preventing cerebral injury in cerebrovascular disorders. The beneficial effect of indomethacin, a cyclooxygenase inhibitor, in resistance to hypoxia and ischemia has been reported in several experimental studies (11, 12), but there are few

reports of clinical trials. Although the reason for this is not clear, adverse side effects, especially on the gastrointestinal tract, have been suggested (13–15).

Our newly synthesized compound KBT-3022, ethyl 2-[4,5-bis-(4-methoxyphenyl)thiazol-2-yl]pyrrol-1-ylacetate (Fig. 1), is a potent cyclooxygenase inhibitor (16). We

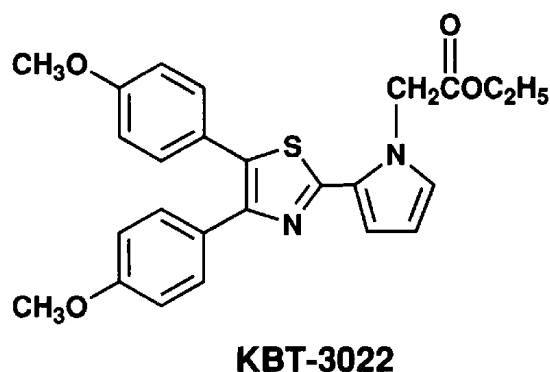


Fig. 1. Chemical structure of KBT-3022.

have previously reported that oral administration of KBT-3022 inhibits several platelet functions (17) and experimental thrombus formation (18), and this drug has low ulcerogenic properties at doses that were sufficient to produce anti-platelet and anti-thrombotic activity (17). KBT-3022 also inhibits lipid peroxidation in guinea pig brain homogenate and the development of ischemic brain edema in gerbils (19). Therefore, KBT-3022 is expected to have a cerebral protective effect with only weak side-effects. In this study, we have investigated the protective effect of KBT-3022 against cerebral hypoxia and ischemia in animal models and compared it with the effects of indomethacin and acetylsalicylic acid (ASA).

MATERIALS AND METHODS

Animals

Male ddY mice (Japan SLC, Hamamatsu) weighing 18–24 g and male Mongolian gerbils (Seiwa Experimental Animals, Fukuoka) weighing 58–85 g were used.

Materials

KBT-3022 was synthesized at the New Drug Research Laboratories of Kanebo, Ltd. (Osaka). Indomethacin was purchased from Sigma Chemical Co. (St. Louis, MO, USA), ASA from Wako Pure Chemical Industries (Osaka) and pentobarbital (sodium salt) from Nacalai Tesque, Inc. (Kyoto). For oral administration, the test drugs were dissolved or suspended in 0.5% polyoxyethylene sorbitan monooleate solution and given to animals after an overnight fast. Control animals received the vehicle alone.

Histotoxic anoxia by KCN injection

Using mice, histotoxic anoxia was produced by an intravenous injection of a lethal dose (3 mg/kg) of KCN-saline solution into the tail vein. Death was determined by respiratory arrest 30 min after the KCN injection. The test drugs were administered orally to the mice 1 hr before the KCN injection because the highest plasma level of the main active metabolite of KBT-3022 was obtained 1 hr after oral administration in mice (20).

Normobaric hypoxia

A method similar to that of Arnfred and Secher (21) was used. Mice were introduced into a 27-l plastic container in which nitrogen gas was circulated at a rate of 10 l/min. The oxygen concentration in the container was maintained at less than 0.5%. The time taken for respiratory arrest to occur was recorded as the survival time. The test drugs were administered orally to the mice 1 hr before the hypoxia.

Neuronal injury following ischemia

Gerbils were anesthetized with 2% halothane in 70% N₂ and 30% O₂. Bilateral carotid arteries were exposed and occluded with aneurysm clips for 5 min. During the occlusion, the animals were kept on a warming blanket at 37°C. After surgery, all animals were returned to their cages and permitted free access to food and water. After 7 days of ischemia, the animals were perfusion-fixed with heparin-containing saline followed by 10% formalin under pentobarbital anesthesia by transcardiac perfusion at a pressure of 120 cmH₂O. The brains were post-fixed with 10% formalin at 4°C for 24 hr. Two- or three-mm-thick coronal sections were cut, dehydrated and embedded in paraffin using standard procedures. Five- μ m-thick sections, which contained the dorsal hippocampus located 1.0–2.2 mm posterior to the bregma, were prepared and stained with 1% cresyl violet. The left and right dorsal hippocampi of each specimen were photographed using Panatomic-X film (Kodak, Rochester, NY, USA) at a magnification of 30 \times . On each photograph, the total linear length of the CA1 sector was measured by means of a digital curvimeter (D-scan, DT-3500; Seiko Instruments, Tokyo). The number of intact neurons in the striatum pyramidale within the CA1 subfield was counted by using a photomicroscope (Olympus, Tokyo) at a magnification of 400 \times . Neurons that had shrunken cell bodies surrounded by empty spaces were regarded as being dead. Based on these data, the number of CA1 neurons per 1-mm linear length of the striatum pyramidale observed in each 5- μ m section was calculated. The average of the left and right neuronal densities was regarded as the neuronal cell density of each animal. KBT-3022, ASA and vehicle were administered orally to the gerbils 3 hr before the occlusion because the highest plasma level of the main active metabolite of KBT-3022 was obtained 3 hr after oral administration in gerbils. In order to prevent gastric mucosal injury, indomethacin was administered intraperitoneally 30 min before occlusion according to the dosing schedule described by Sasaki et al. (22).

Cerebral metabolism following ischemia

Gerbils were anesthetized with 2% halothane in 70% N₂ and 30% O₂. Bilateral carotid arteries were exposed and occluded with aneurysm clips for 0, 1 or 5 min. In the recovery studies, the clips were released after 5 min of ischemia and recirculation continued for either 1 or 5 min. During the occlusion, the animals were kept on a warming blanket at 37°C. Each animal was then killed by microwave irradiation (TMW-4012A; Toshiba, Tokyo) (3.5 kW microwave, 3.2 sec), and the cortex, hippocampus and striatum dissected out and frozen in liquid nitrogen. The tissue samples were stored at –70°C until the time of sample preparation. KBT-3022 and vehicle were

administered orally to the gerbils 3 hr before the occlusion.

The procedure of sample preparation and fluorometric measurements of ATP, lactate, pyruvate and glucose with NADP and appropriate enzymes were done according to method of Lowry et al. (23). The samples were stored at -70°C until analyzed.

Statistics

Statistical significance was evaluated by Fishers exact probability test or one-way ANOVA followed by Dunnett's test. Differences with a P value of less than 0.05 were considered statistically significant.

RESULTS

Histotoxic anoxia by KCN injection

The protective effects of the test drugs against KCN-induced death are shown in Table 1. In control animals, all mice died within 3 min of the KCN injection. Oral administration of KBT-3022 (3–100 mg/kg) resulted in a decrease of mortality. Indomethacin (3 and 10 mg/kg, p.o.) and pentobarbital (30 mg/kg, p.o.) also prevented KCN-induced death significantly, while ASA (100 mg/kg, p.o.) had no effect. Although pentobarbital induced sedation, KBT-3022 and indomethacin did not.

Normobaric hypoxia

The effects of the test drugs on normobaric hypoxia are shown in Table 2. In control animals, all mice developed respiratory failure and died within 30 sec. Oral administration of KBT-3022 (3 and 10 mg/kg) significantly prolonged the survival time of the mice subjected to normobaric hypoxia. Indomethacin (10 mg/kg, p.o.) and pentobarbital (30 mg/kg, p.o.) also prolonged the survival time, while ASA (100 mg/kg, p.o.) had no effect. Although pentobarbital induced sedation, KBT-3022 and indomethacin did not.

Table 1. Effects of KBT-3022, indomethacin, acetylsalicylic acid (ASA) and pentobarbital on KCN-induced death in mice

Drug	No. of Survival/No. of Tested				
	Dose (mg/kg, p.o.)				
	1	3	10	30	100
KBT-3022	2/10	4/10*	4/10*	5/10*	5/10*
Indomethacin	3/10	4/10*	7/10**		
ASA			0/10		0/10
Pentobarbital			1/10	9/10***	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significant difference from the control (0/10) (Fisher's exact probability test).

Table 2. Effects of KBT-3022, indomethacin, acetylsalicylic acid (ASA) and pentobarbital on survival time of mice subjected to normobaric hypoxia

Drug	Dose (mg/kg, p.o.)	N	Survival time (sec)
Control		10	22.5 ± 0.2
KBT-3022	1	5	23.9 ± 0.6
	3	5	$25.6 \pm 1.0^*$
	10	5	$26.2 \pm 1.0^{**}$
Indomethacin	1	5	22.1 ± 0.4
	3	5	23.5 ± 0.4
	10	5	$26.9 \pm 1.3^{**}$
ASA	100	5	22.4 ± 0.5
Pentobarbital	10	5	22.1 ± 0.5
	30	5	$32.1 \pm 1.5^{**}$

Each value represents the mean \pm S.E. * $P < 0.05$, ** $P < 0.01$, significant difference from the control (Dunnett's test).

Neuronal injury following ischemia

The typical distribution patterns of the neuronal densities in the hippocampal CA1 sector following occlusion of bilateral carotid arteries in gerbils for 5 min are shown in Fig. 2. In control animals, extensive neuronal damage was observed. Oral administration of KBT-3022 (10 mg/kg) well-preserved the CA1 pyramidal cells. The effects of KBT-3022 and indomethacin on the neuronal cell density of the CA1 sector are shown in Table 3. In sham-operated animals, the neuronal cell density of the CA1 sector was $258.9 \pm 3.4/\text{mm}$ ($N=10$), ranging from 245/mm to 278/mm. In control animals, extensive neuronal damage was observed in the CA1 sector. The differences among the three control groups might have been due to the lots of the animals. KBT-3022 (3, 10 and 30 mg/kg, p.o.) significantly preserved the CA1 pyramidal cells. Indomethacin (3 mg/kg, i.p.) also showed a significant protective effect, while ASA (300 mg/kg, p.o.) had no effect.

Cerebral metabolism following ischemia

Figure 3 shows the time course of the changes in the concentration of ATP in the gerbil brain during ischemia and recirculation. Ischemia induced a rapid fall in ATP content that recovered almost completely after recirculation for 5 min. KBT-3022 had no effect on the ATP depletion after 5 min of occlusion and recovery after recirculation. However, the ATP content of the hippocampus after 1 min of ischemia was significantly higher in the KBT-3022-treated animals than in the control animals. In the cortex, the glucose content also fell rapidly during ischemia and recovered during recirculation.

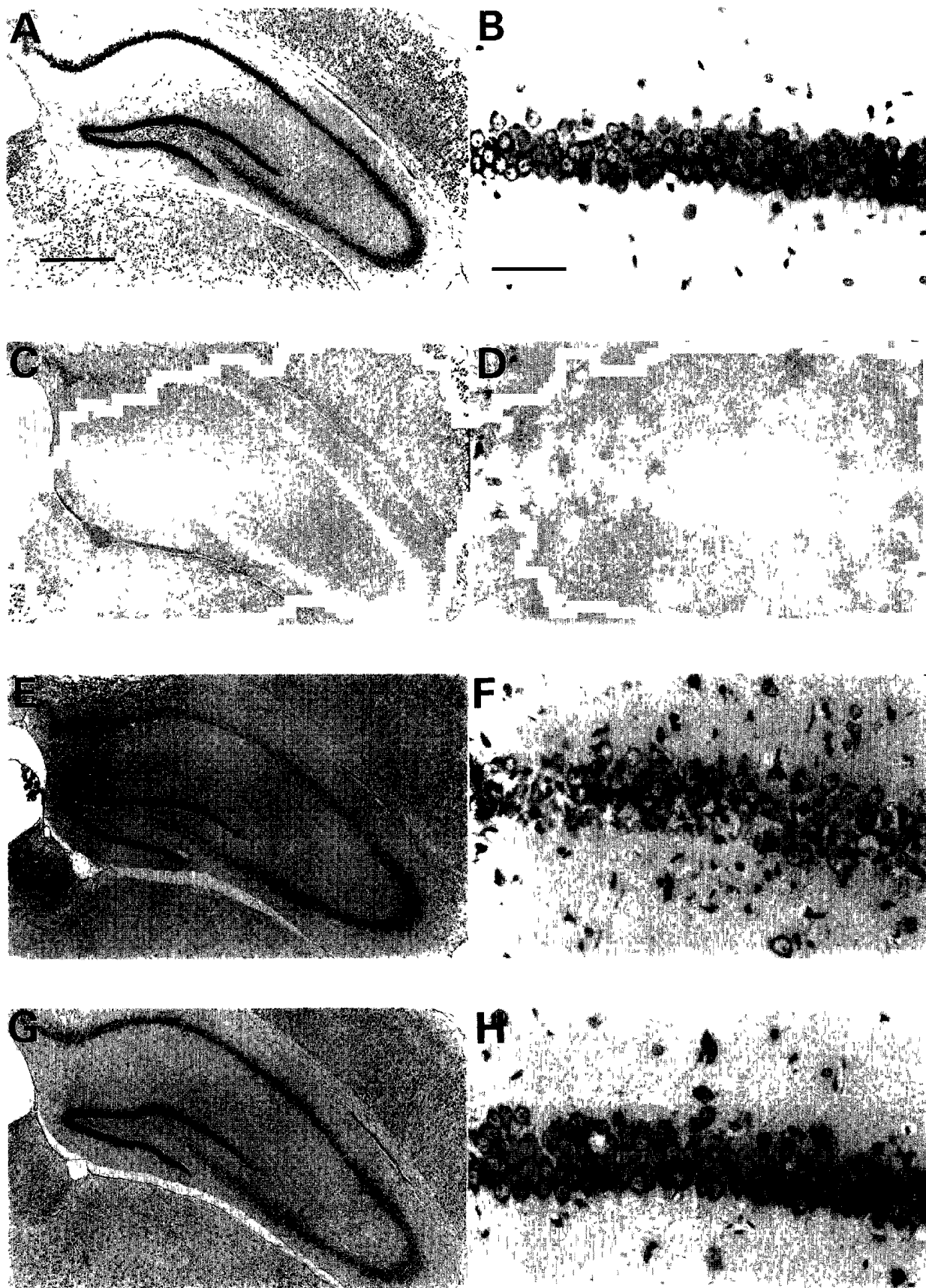


Fig. 2.

Table 3. Effects of KBT-3022, indomethacin and acetylsalicylic acid (ASA) on neuronal death of hippocampal CA1 neurons, 7 days after bilateral carotid artery occlusion for 5 min in gerbils

Drug	Dose (mg/kg)	Route	N	Neuronal density (No./mm)	% of sham
Sham-operation		p.o.	10	258.9 ± 3.4 ^{##}	—
Control		p.o.	10	62.9 ± 24.9	24.3
KBT-3022	1	p.o.	10	96.8 ± 26.7	37.4
	3	p.o.	10	160.7 ± 23.8*	62.1
	10	p.o.	10	181.6 ± 17.4**	70.2
	30	p.o.	10	171.0 ± 21.6**	66.0
Control		i.p.	10	114.5 ± 26.4	44.2
Indomethacin	0.3	i.p.	10	128.1 ± 26.2	49.5
	3	i.p.	10	204.6 ± 22.1*	79.0
Control		p.o.	8	9.3 ± 2.6	3.6
ASA	30	p.o.	8	35.6 ± 16.5	13.8
	100	p.o.	8	19.6 ± 8.0	7.6
	300	p.o.	8	13.4 ± 3.1	5.2

Each value represents the mean ± S.E. ^{##}P < 0.01, significant difference from each control. *P < 0.05, **P < 0.01, significant difference from the corresponding control (Dunnett's test).

tion (data not shown). The lactate content rose rapidly during ischemia and recovered during recirculation (data not shown). The pyruvate content was unchanged during ischemia and became elevated during recirculation (data not shown). KBT-3022 did not show any effect on the cortical glucose, lactate and pyruvate contents. The glucose, lactate and pyruvate contents in the hippocampus and striatum could not be determined due to the low sample volume.

DISCUSSION

When the brain is subjected to oxygen deprivation, free fatty acids, especially AA, are liberated from the cellular membrane and accumulate in the brain (2, 3). Recent studies have suggested that several products of AA metabolism are involved in the pathogenesis of brain injury (4–6). Indomethacin is a potent inhibitor of cyclooxygenase and is reported to reduce brain PG synthesis in rats (24). The beneficial effect of indomethacin in resisting hypoxia and ischemia has already been demonstrated in several experimental studies (15, 16). In this

Fig. 2. Representative photomicrographs of the hippocampal CA1 sector, 7 days after bilateral carotid artery occlusion for 5 min in gerbils assessed by Cresyl violet staining. A and B: sham-operation; C and D: control; E and F: KBT-3022 (1 mg/kg, p.o.)-treated animals; G and H: KBT-3022 (10 mg/kg, p.o.)-treated animals. Magnification of A, C, E and G: × 10; bar represents 500 μm. Magnification of B, D, F and H: × 100; bar represents 50 μm.

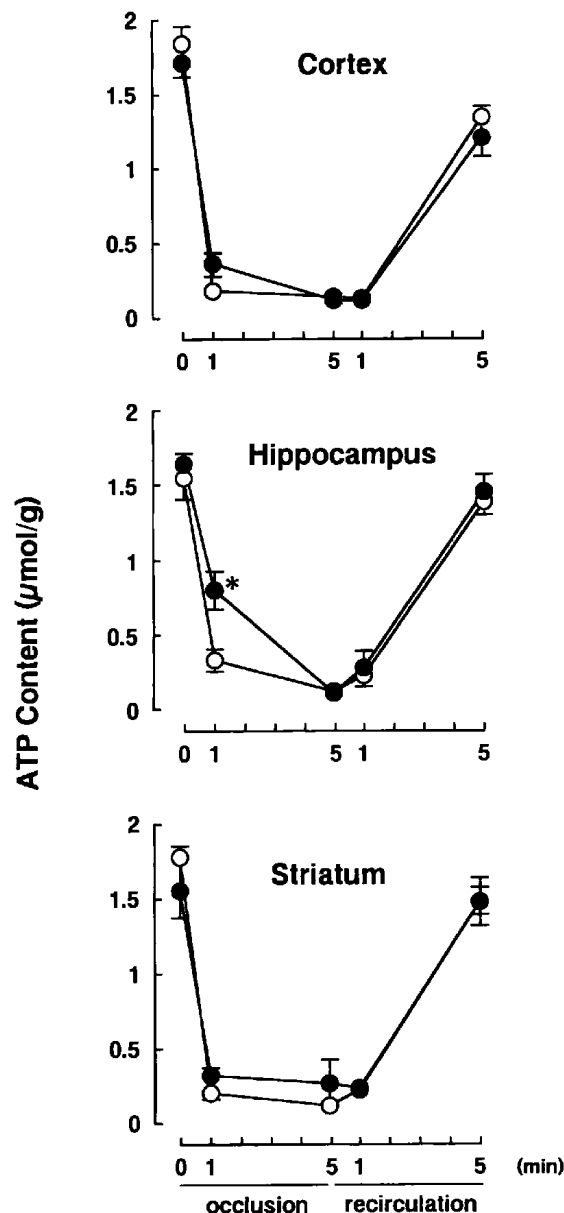


Fig. 3. Effect of KBT-3022 on changes in ATP contents in the cortex, hippocampus and striatum after ischemia and recirculation in gerbils. The ischemia was induced by occlusion of bilateral carotid arteries for 0, 1 or 5 min. The recirculation was restored for either 1 or 5 min after 5 min of ischemia. Each point represents the mean and vertical bar shows S.E. in μmol/g of brain tissue (N=5). *P < 0.05, significant difference from the control (Dunnett's test). ○: control, ●: KBT-3022 (10 mg/kg, p.o.).

study, we investigated whether KBT-3022 has a protective effect against cerebral hypoxia and ischemia, in comparison with the effects of indomethacin and ASA.

Our study demonstrated that KBT-3022 and indomethacin prevented KCN-induced death in mice and prolonged the survival time of mice subjected to normobaric hypoxia. KBT-3022 and its metabolite have been

reported to inhibit cyclooxygenase in ovine seminal tissue and are approximately 3 times more potent than indomethacin (16). Therefore, KBT-3022, like indomethacin, may exert cerebral protective actions against anoxia and hypoxia by inhibiting cyclooxygenase in the brain.

It is reported that sedative drugs such as barbiturates possess cerebral protective actions (21, 25). The cerebral protective activity of these drugs is due to cerebral metabolic depression or suppression of energy demand (26, 27). In this study, none of the animals treated with KBT-3022 was sedated. Furthermore, KBT-3022 had no effect on body temperature or pentobarbital sleeping (K. Yokota, unpublished data). Therefore, the cerebral protective effects of KBT-3022 might not be attributable to depression of the central nervous system.

We found that ASA had no effect on KCN-induced death and normobaric hypoxia, in spite of its inhibitory effect on cyclooxygenase. The ineffectiveness of ASA may be explained by its poor penetration into the brain. This is confirmed by the finding that ASA failed to reduce brain PG synthesis at a dose that completely inhibited PG synthesis in peripheral tissue in rats (24).

It has been demonstrated that transient cerebral ischemia in gerbils produces a selective pattern of delayed neuronal death (DND) in the hippocampal CA1 sector (28, 29). Our result has shown that DND in the hippocampal CA1 sector occurred at 7 days of reflow after occlusion of bilateral carotid arteries for 5 min in gerbils. The neurons of the hippocampal CA1 sector are highly susceptible (30, 31) and damaged by a few minutes of ischemia, whereas longer ischemic periods induce DND in other regions, including the cerebral cortex, thalamus and striatum (32, 33). Our result has shown that KBT-3022 and indomethacin ameliorated the DND in gerbil hippocampus. The result obtained with indomethacin was similar to that described previously by Sasaki et al. (22), suggesting cyclooxygenase products are involved in the development of DND.

Several pathophysiological mechanisms for DND have been proposed, such as an increased excitatory input to neurons, intracellular calcium overload and microcirculatory disturbance during postischemic reperfusion, but these are not fully understood. Extracellular accumulation of synaptically released neurotransmitters, such as glutamate and aspartate, may be a critical factor in the pathogenesis of DND (34, 35). $\text{PGF}_{2\alpha}$, PGE_2 and PGD_2 were reported to potentiate the excitatory actions of glutamate and aspartate on Purkinje cell dendrites (36). Moreover, free radicals produced by AA metabolism also cause an increase in the release of excitatory amino acids and trigger DND (37, 38). The protective effect of KBT-3022 on DND may thus result from inhibition of AA metabolism in the brain tissue. The ineffectiveness of

ASA on DND may again be explained by its poor penetration into the brain.

As shown in Fig. 3, the ATP content of the gerbil brain fell rapidly, and these changes were resolved completely after 5 min of recirculation. It is well-recognized that cerebral metabolic disturbance during ischemia and recirculation is related to the cellular damage (39–41). Arai et al. (42) examined the CA1 subfield in the same gerbil model and confirmed that there was no decrease of ATP during the period of cell deterioration. Also, it has been reported that there are no significant differences in metabolic levels during and after ischemia between the cerebral cortex and hippocampus and that neither lack of metabolite restoration nor secondary energy failure is a direct cause of CA1 neuronal loss (43, 44). Our result demonstrated that KBT-3022 had no effect on either the energy disturbance after 5 min of occlusion or the recovery after recirculation. Thus the prevention of DND by KBT-3022 might not be attributable to improvement of cerebral metabolic disturbance.

However, KBT-3022 significantly inhibited the depletion of ATP in the hippocampus after 1 min of ischemia. The effect was temporary and evident only in the hippocampus. KBT-3022 might minimize the consumption of ATP during the early period of ischemia when the initial influx of calcium may occur via the potential-dependent calcium channel. The neurons in the hippocampus play an important part in maintaining neuronal function with regard to the release of excitatory amino acids (45, 46), and KBT-3022 might suppress the excitative action of neurons by inhibiting PG production. It is conceivable that the preventive effect of KBT-3022 on ATP depletion during the early period of ischemia might contribute to the resistance to cerebral anoxia and hypoxia. Further investigations are needed to clarify this point.

There may be other possible explanations for the cerebral protective effects of KBT-3022. We have previously reported that KBT-3022 inhibited lipid peroxidation in guinea pig brain homogenate (19). Lipid peroxidation secondary to free radical formation may play a role in the neuronal damage sustained during cerebral hypoxia and ischemia (7), and thus the inhibitory effect of KBT-3022 on lipid peroxidation may contribute to its cerebral protective effects. KBT-3022 has also been reported to increase red blood cell deformability and decrease blood viscosity in guinea pigs (47). This hemorheological effect of KBT-3022 may also contribute to cerebral protective effects by improving microcirculation in the brain.

In conclusion, KBT-3022 exerted protective effects against KCN-induced death, normobaric hypoxia, and DND in the hippocampal CA1 sector. The mechanism may depend on inhibition of AA metabolism in the brain

among other possible mechanisms. These effects are as strong as those of indomethacin. Many non-steroid anti-inflammatory drugs have been reported to cause gastrointestinal damage when administered orally (14, 15). Laufer et al. (13) have reported that a single dose of indomethacin led to a dose-dependent increase in macroscopically visible gastric ulcers in rats at doses of 1.25 mg/kg, p.o. and higher. Oral administration of KBT-3022 had no effect on the gastric mucosa in mice at a dose of 30 mg/kg (17), which was sufficient to prevent cerebral damage. Thus, KBT-3022 is expected to be available for clinical trials, producing only weak side-effects in the gastrointestinal tract. The cerebral protective effect of KBT-3022, in addition to its well-established anti-platelet action, may be considered to be favorable for the treatment of patients with ischemic cerebrovascular disorders.

Acknowledgments

We wish to thank Dr. Takayuki Sukamoto and Dr. Tominori Morita (New Drug Research Laboratories, Kanebo, Ltd.) for reading and commenting on the manuscript. A preliminary account of this work was presented at the 65th Annual Meeting of The Japanese Pharmacological Society, Sendai, 1992 (19).

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