BIOLOGICAL ACTIVITY OF THE MAIN METABOLITES OF UBENIMEX† IN HUMANS

FUMINORI ABE, HIROSHI KURAMOCHI, KATSUTOSHI TAKAHASHI, MASAAKI ISHIZUKA†† and TOMIO TAKEUCHI††

Research Laboratories, Pharmaceuticals Group, Nippon Kayaku Co., Ltd., Shimo 3-31-12, Kita-ku, Tokyo 115, Japan
††Institute of Microbial Chemistry, Kamiosaki 3-14-23, Shinagawa-ku, Tokyo 141, Japan

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The biological activity of the two main metabolites of ubenimex in humans, (−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-(4′-hydroxy)phenylbutyryl]-L-leucine (OH-ubenimex) and (2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutyric acid ((2*S*,3*R*)-AHPA) was examined. OH-Ubenimex was almost identical in inhibitory activity against mouse peritoneal resident macrophage aminopeptidases (APases) and the growth of IMC carcinoma in mice to ubenimex. In contrast, the inhibition of; mouse spleen cell APase activities *in vitro*, blastogeneses of mouse T and B cells *in vitro*, delayed cutaneous hypersensitivity in mice, and the growth of C1498 leukemia and HeLa S_3 cells *in vitro* was weaker than ubenimex. Macrophage APase activity was only slightly inhibited by (2*S*,3*R*)-AHPA which also had practically no activity in the other biological assays.

Ubenimex, (−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxyphenylbutyryl]-L-leucine, is a dipeptide discovered in the culture filtrate of *Streptomyces olivoreticuli*[1] and can inhibit aminopeptidase (APase) activities[2~4] and cause immunomodulation[5~14]. Ubenimex also has antitumor activities[6,11,15,16] together with enhancing the effects of antitumor activities of other cytotoxic agents[17]. It is believed that ubenimex interacts with membrane bound APases to activate macrophages and T cells[18]. About 10^7 and 10^6 molecules of ubenimex bound to each of these cell types[19]. Recently, two main metabolites, (−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-(4′-hydroxy)phenylbutyryl]-L-leucine (OH-ubenimex) and (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutyric acid ((2*S*,3*R*)-AHPA), were identified in human urine[20]. In the present study we examined whether these metabolites retain any biological activity.

Materials and Methods

Chemicals

Ubenimex, OH-ubenimex and (2*S*,3*R*)-AHPA were kindly synthesized by Dr. SAINO, Research Laboratories, Nippon Kayaku Co., Ltd., as reported previously[21,22]. Concanavalin A (Con A) was purchased from Pharmacia Fine Chem., Inc. and lipopolysaccharide of *Escherichia coli* 055; B5 (LPS) from Difco Lab. L-Arginine-β-naphthylamide (Arg-NA), L-leucine-β-NA (Leu-NA) and L-phenylalanine-β-NA (Phe-NA) were obtained from Sigma Chemical Co. Oxazolone (4-ethoxymethylene-2-oxazolone-5-one) was purchased from Aldrich Chemical Co., Ltd. [6-^{3}H]Thymidine ([^{3}H]TdR) with a specific activity of 15.5 Ci/mmol was obtained from New England Nuclear.

Animals

Specific pathogen free inbred BALB/c, C57BL/6 and CD(BALB/c×DBA/2)F1 mice were obtained from Charles River Japan, Inc. These mice were 8 to 10 weeks old and housed here in an

† By recommendation of WHO, the name of ubenimex is used for bestatin.

Isorack with a clean air system. Each group consisted of at least 5 mice.

Tumors

Myeloid leukemia C1498 and IMC carcinoma have been maintained in ascites form in C57BL/6 mice and CDF1 mice, respectively. HeLa S_3 cell line was maintained as monolayers in EAGLE's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Gibco Island, NY), MEM non essential amino-acid, streptomycin and benzylpenicillin. Kato III cell and K562 cell lines were maintained in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS.

Cell Preparation

Spleen cells were prepared as described previously[15]. T cells were separated from the spleen cells by passing through a Nylon column. Resident macrophages were washed out from the mouse peritoneal cavities with HANKS' balanced salt solution (HBSS).

Enzyme Inhibition

APase activity was measured as reported previously[4] by measuring β-naphthylamine liberated from amino acid-NA after incubation. Briefly, HBSS containing amino acid-NA and an enzyme source was incubated for 30 minutes at 37°C and then cooled to 0°C to terminate the reaction. The liquid phase of the mixture was separated by centrifugation, heated for 5 minutes on boiling water and subjected to fluorescence spectrophotometry. APase activity was calculated from the amount of β-naphthylamine formed by the added enzyme.

Blastogenesis

Spleen T cells or unseparated spleen cells were used for testing T cell or B cell blastogenesis, respectively. Both cell types were incubated in microplates in the presence of an optimal concentration of Con A and LPS. Cultures were set up in triplicate. Each well contained 1×10^5 cells in a volume of 0.2 ml of RPMI 1640 supplemented with 20% of FBS and antibiotics. After 48 hours of incubation at 37°C cultures were pulsed with 1 μCi [^{3}H]TdR per well and the cells collected 6 hours later.

DCH to Oxazolone

Delayed cutaneous hypersensitivity (DCH) to oxazolone was tested as described previously[23]. BALB/c mice were sensitized by painting on the shaved abdomens with 0.1 ml of 5% oxazolone in absolute ethanol. Three days later the sensitized mice were challenged by painting a hind footpad with the same oxazolone solution. DCH was evaluated 24 hours after the challenge by measuring the thickness of the footpad with calipers.

Antitumor Effect

HeLa S_3, Kato III and K562 cells were cultured at the initial density of 5×10^4 cells/2 ml/plate. Four hours after incubation of Kato III and K562 cells or 48 hours after incubation of HeLa S_3 cells, ubenimex or ubenimex metabolites were added at various concentrations and cultured for a further 3 days. The cell numbers before and after treatment were counted by a coulter counter (model ZB1). The IC_{50} value, which is the concentration of each sample required for 50% inhibition of growth, was obtained graphically by a plot of its log concentration *versus* the probit of inhibition rate. Antitumor effects against mouse syngeneic tumor, IMC carcinoma and C1498 leukemia in mice were also examined as described previously[15]. The effect of ubenimex administration was evaluated by comparing the average tumor weight in treated mice with that of non-treated mice 20 days after tumor inoculation.

Results

We examined the effects of two ubenimex metabolites, OH-ubenimex and (2*S*,3*R*)-AHPA, on the APase activity of peritoneal resident macrophages and spleen cells (Table 1). Consistent with previous reports[4], ubenimex inhibited the Arg-NA, Leu-NA and Phe-NA hydrolyzing activities of both

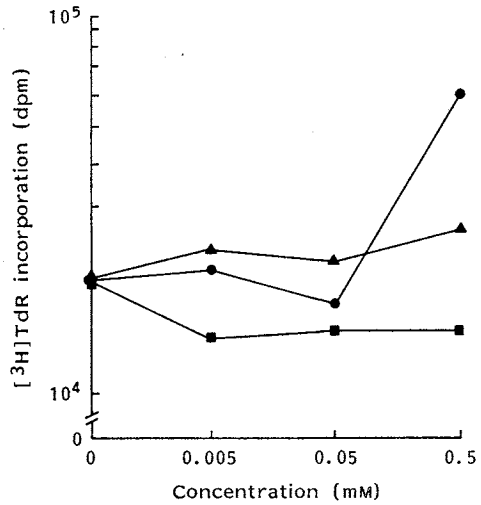
Table 1. Inhibition of aminopeptidase activities of peritoneal resident macrophages and spleen cells by OH-ubenimex and (2*S*,3*R*)-AHPA.

Substrate	Compound	IC_{30} (μg/ml)	
		Macrophages	Spleen cells
Arg-NA	Ubenimex	<0.1	0.18
	OH-Ubenimex	<0.1	<0.1
	(2*S*,3*R*)-AHPA	145.0	>1,000.0
Leu-NA	Ubenimex	20.0	143.0
	OH-Ubenimex	21.5	290.0
	(2*S*,3*R*)-AHPA	1,000.0	>1,000.0
Phe-NA	Ubenimex	1.3	46.0
	OH-Ubenimex	0.98	300.0
	(2*S*,3*R*)-AHPA	50.0	203.0

The incubation was done in HBSS including 2×10^6 spleen cells from female BALB/c mice with an age of 9 weeks or 2×10^5 macrophages from the same mice in a total volume of 1.0 ml. Arg-NA, Leu-NA or Phe-NA was added at 0.05 mM as the substrate. IC_{30} value means the concentration of each sample required for 30% inhibition of the enzyme activity.

Fig. 1. Effect of OH-ubenimex and (2*S*,3*R*)-AHPA on T cell blastogenesis.

● Ubenimex, ▲ OH-ubenimex, ■ (2*S*,3*R*)-AHPA.



T cells from BALB/c mice with an age of 12 weeks were incubated with or without Con A for 3 days. Ubenimex or one of metabolites was added as indicated at the beginning of incubation. Concentration of 0.5 mM for ubenimex, OH-ubenimex and (2*S*,3*R*)-AHPA corresponds to each of about 154, 164 and 100 μg/ml, respectively.

Table 2. Effects of OH-ubenimex and (2*S*,3*R*)-AHPA on delayed cutaneous hypersensitivity in mice.

Group (n=5)	Dose (mg/kg)	Footpad-swelling	
		$\times10^{-2}$ mm ±SD	%
None	0	62±32	100
Ubenimex	0.5	108±26	174*
	5.0	94±19	152
OH-Ubenimex	0.5	78±14	126
	5.0	58±40	94
(2*S*,3*R*)-AHPA	0.5	60±43	97
	5.0	37±37	60

Female BALB/c mice of 15 weeks old were used. Mice were immunized by giving 0.1 ml of 5% oxazolone in absolute ethanol to abdominal skins. Ubenimex and metabolites were intraperitoneally given at the time of immunization. The immunized mice were challenged 3 days later by painting the oxazolone solution to footpads. The swellings of footpads were measured with a caliper 24 hours after the challenge.

* $P<0.05$ (Student's t-test).

Table 3. *In vitro* growth inhibition of ubenimex and its metabolites against human tumor cell lines.

Sample	IC_{50} (μg/ml)		
	HeLa S_3	K562	Kato III
Ubenimex	108	<12.5	>200
OH-Ubenimex	>200	82	>200
(2*S*,3*R*)-AHPA	>200	>200	>200

IC_{50} value means the concentration of each sample required for 50% inhibition of growth.

immunocompetent cells. OH-Ubenimex also inhibited these three macrophage activities, and the Arg-NA hydrolyzing activity of spleen cells to almost the same extent as ubenimex. However, the inhibition of the Leu-NA and Phe-NA hydrolyzing activities of spleen cells was weaker than that of ubenimex. (2*S*,3*R*)-AHPA inhibited the Phe-NA hydrolyzing activity of spleen cells to a similar extent as OH-ubenimex but had practically no inhibitory activity on the other amino acid-NA hydrolyzing activities examined.

The effects of OH-ubenimex and (2*S*,3*R*)-AHPA on three immune functions, that is, T

Table 4. Antitumor effect of OH-ubenimex against syngeneic tumors transplanted in mice.

Tumor	Group	Dose (mg/kg)	Mice (*n*)	Tumor weight (g±SD)	Inhibition (%)
IMC carcinoma	Control	0	11	2.98±1.71	
	Ubenimex	0.5	11	1.08±0.72*	63.8
		5.0	11	1.41±1.05*	52.7
	OH-Ubenimex	0.5	11	0.96±0.04*	67.8
		5.0	10	1.11±0.38*	62.8
C1498 leukemia	Control	0	9	0.844±0.607	
	Ubenimex	0.5	8	0.332±0.128*	60.7
		5.0	8	0.184±0.197*	78.2
	OH-Ubenimex	0.5	7	0.549±0.400	35.0
		5.0	7	0.497±0.421	43.2

IMC carcinoma (1×10^6 cells) and C1498 leukemia (1×10^4 cells) were subcutaneously inoculated in CDF1 mice and C57BL/6 mice, respectively. Ubenimex and OH-ubenimex were administered 5 times once a day starting from day 7. Tumor weights were determined 20 days after the inoculation.
* $P<0.01$ (Student's t-test).

and B cell blastogeneses and DCH to oxazolone were also examined. Ubenimex increased [^{3}H]TdR incorporation into Con A-stimulated T cell blastogenesis at 0.5 mM (Fig. 1). OH-Ubenimex also enhanced T cell blastogenesis at the same concentration but to a lesser degree. (2*S*,3*R*)-AHPA had no effect on this blastogenesis. Neither metabolite affected LPS-stimulated B cell blastogenesis using spleen cells as the B cell preparation although ubenimex clearly enhanced this blastogenesis (data not shown). Additionally, both metabolites had practically no enhancing effect on DCH (Table 2).

Examining the antitumor effects of OH-ubenimex and (2*S*,3*R*)-AHPA, we found the IC_{50} values of OH-ubenimex and (2*S*,3*R*)-AHPA against *in vitro* growth of K562 cells were 82 μg/ml and more than 200 μg/ml, respectively, whereas the IC_{50} value of ubenimex was less than 12.5 μg/ml (Table 3). Metabolites IC_{50} values were more than 200 μg/ml against HeLa S_3 cells, whereas that of ubenimex was 108 μg/ml. This indicates that both metabolites have almost no direct cytotoxicity *in vitro*. Against IMC carcinoma OH-ubenimex exerted *in vivo* antitumor effect similar to that of ubenimex but against C1498 leukemia the effect was less than that of ubenimex (Table 4).

The acute toxicity in mice was also examined. Both metabolites did not exhibit toxicity when administered either intraperitoneally or orally at the maximum dose of 2 g/kg.

Discussion

The pharmacokinetics and biotransformation of ubenimex have been examined in detail[14,20,24]. KOYAMA reported that ubenimex was rapidly and well absorbed from the gastro-intestinal tract and then excreted mainly into urine following oral administration to rat, dog and human. When ubenimex was administered to human, relatively small amounts of two metabolites, (2*S*,3*R*)-AHPA and OH-ubenimex and more than 70% of unchanged ubenimex were excreted into urine[20]. We examined the biological activity of these two metabolites.

OH-Ubenimex, the main human metabolite of ubenimex, had APase-inhibitory activity, immunomodulating activity and antitumor activity. Some of these activities were similar to those of ubenimex, while some were less. Namely, the inhibitory effects of macrophage APases and IMC carcinoma growth were similar, while the enhancing effects on T and B cell blastogenesis and DCH, the inhibitory effect on C1498 leukemia and the direct cytotoxicity of tumor cells were less. Therefore, OH-ubenimex has similar biological activity to ubenimex but was less potent. (2*S*,3*R*)-

AHPA lacked almost all of the activity of ubenimex.

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