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Synthesis and structure–activity relationships of novel, substituted 5,6-dihydrodibenzo[*a*,*g*]quinolizinium $P2X_7$ antagonists

Ga Eun Lee [a], Ho-Sung Lee [a], So Deok Lee [a], Jung-Ho Kim [b], Won-Ki Kim [c], Yong-Chul Kim [a,*]

[a] *Research Center for Biomolecular Nanotechnology, Department of Life Science, Gwangju Institute of Science and Technology (GIST), 1 Oryong-dong, Buk-gu, Gwangju 500-712, South Korea*
[b] *Biotech Research Division, Hanwha Chemical Research & Development Center, Taejon, South Korea*
[c] *Department of Neuroscience, College of Medicine, Korea University, Seoul, South Korea*

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ABSTRACT

Iminium quaternary protoberberine alkaloids (QPA) have been found to be novel $P2X_7$ antagonists. To assess their structure–activity relationships, these compounds were modified at their R^1 and R^2 groups and assayed for their ability to inhibit the 2′(3′)-*O*-(4-benzoylbenzoyl)-ATP (BzATP)-induced uptake of fluorescent ethidium by HEK-293 cells stably expressing the human $P2X_7$ receptor, and their ability to inhibit BzATP-induced IL-1β release by differentiated THP-1 cells. Compounds **15a** and **15d**, with alkyl groups at the R^1 position, and especially compound **19h**, with the 2-NO_2-4,5-dimethoxy-benzyl group at the R^2 position, had potent inhibitory efficacy as $P2X_7$ antagonists.

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The $P2X_7$ receptor ($P2X_7R$), a plasma membrane receptor for extracellular adenosine-5-triphosphate (ATP) dominantly expressed in inflammation-related cells has been known a crucial regulator of both IL-1 maturation and externalization.[1–3] Since interleukin-1 (IL-1), a pro-inflammatory cytokine, along with tumor-necrosis factor-α (TNFα) and IL-6 play central roles as the intercellular mediator in the generation and control of immune and inflammatory responses,[4] therapeutically controlling inflammation through blockade of IL-1 pathway through the inhibition of $P2X_7R$ has been actively studied for the clinical management of many human inflammatory and autoimmune disease such as rheumatoid arthritis (RA), atherosclerosis, osteoarthritis (OA) chronic obstructive pulmonary disease (COPD), and inflammatory bowel disease (IBD).[4,5]

Purinergic $P2X_7R$, the seventh member of the P2X receptor subfamily, is an ATP-gated non-selective cation channel[6] that is present on cells of hematopoietic origin,[7] including mast cells, macrophages, and the human monocyte cell line THP-1, and on brain glial cells,[8,9] including microglia and astrocytes. Like other P2X receptors, brief exposure of $P2X_7R$ to extracellular ATP results in a transient current, through opening of cation channels.[10] Unusually, however, the repeated or sustained activation of $P2X_7R$ converts it from an ion-channel to an enlarged pore, permeable to hydrophilic solutes of molecular mass up to 900 Da (e.g., ethidium, YO-Pro-1, and propidium ion).[10–13] In addition to its membrane properties, $P2X_7R$ mediates several other cellular events, including IL-1β release, L-selectin shedding and cell death or lysis.[2,14] In central nervous system, microglia lacking $P2X_7R$ do not release IL-1 after ATP stimulation,[15,16] which suggested that neuropathic pain could be mediated by the ATP activation of $P2X_7R$ which stimulates IL-1β release from microglia. Various $P2X_7$ antagonists have therefore been tested as potential therapeutic agents in inflammation-related diseases and neuropathic pain.[17,18]

KN62 (1-(*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl)-4phenylpiperazine) **1**[19,20] and its derivatives (compounds **2**,[21–23] **3**,[24,25] and **4**[26]) have been shown to be a potent, specific inhibitor of human $P2X_7R$. Other derivatives, including the amide **5**,[27,28] the 1-benzyl-5-phenyltetrazole **6**,[29,30] and the cyanoguanidine **7**,[31] also have been tested as candidates for orally-active drugs. Especially, the di-substituted tetrazole and cyanoguanidine derivatives showed significant antinociceptive activity in animal models of neuropathic and inflammatory pain through blockade of $P2X_7R$.[29,31] Moreover, chelerythrine **8**, a benzophenanthridine alkaloid, was reported to inhibit the 2′(3′)-*O*-(4-benzoylbenzoyl)-ATP (BzATP)-induced $^{86}Rb^+$ efflux by 74% in human B lymphocytes.[32]

In the effort to search a new $P2X_7R$ antagonist, we performed a cell based screening of the compound library of the Korea Chemical Bank,[33] and discovered the quaternary protoberberine alkaloids (QPA) **9** and **10** as the inhibitors of BzATP-activated ethidium$^+$

* Corresponding author. Tel.: +82 62 970 2502; fax: +82 62 970 2484.
E-mail address: yongchul@gist.ac.kr (Y.-C. Kim).

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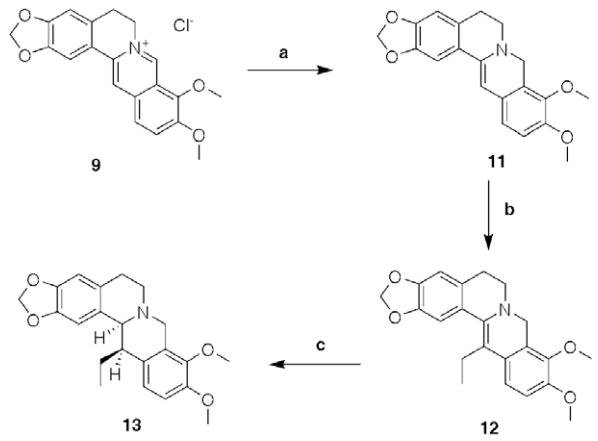
uptake by hP2X$_7$R-expressing HEK293 cells. The QPA, a large class of isoquinoline alkaloids, were derived from the 5,6-dihydrodibenzo[*a*,*g*]quinolizinium system, and have been shown to have several biological activities, including cytotoxicity, apoptosis, antimicrobial and anti-inflammatory activity.[34]

We have investigated the structure–activity relationship (SAR) of several novel QPAs, which were derived from 5,6-dihydrodibenzo[*a*,*g*]quinolizinium derivatives with substituted R^1 and R^2 groups. These compounds were evaluated for their ability to inhibit the uptake of fluorescent ethidium ion induced by BzATP, which activates the hP2X$_7$R stably expressed in HEK293 cells, and for their ability to inhibit IL-1β release from differentiated THP-1 cells.

The 9- and 13-positions of the iminium QPA skeleton (Fig. 1)—that is, the alkyl groups at the R^1 position and the substituted benzyl groups at the R^2 position—were systematically modified, as shown in Schemes 1 and 2. The commercially available starting material, compound **9** was reduced with $NaBH_4$ at 0 °C and subsequently alkylated with iodoethane under an autoclave condition. Following the condition in literatures,[35,36] reduction of compound **12** with $NaBH_4$ at rt afforded ethyltetrahydroberberine, (±)-**13** as shown in Scheme 1. As an efficient synthetic route for the introduction of R^1 group, compound **9** was first acetonylated by acetone in 45% aqueous NaOH, and refluxed with substituted alkyl or benzyl halides to modify the R^1 position with restoring the aromatic system at the same time. To transform into palmatine derivatives, compounds **15a–g** were dealkylated with the Lewis acid, $AlCl_3$, to afford 2,3,9-trihydroxy compounds **16a–c** selectively, which were subsequently O-methylated at the 2, 3, and 9-positions. 9-Methoxy group of compound **17a–c** could be selectively deprotected with LiCl in DMF. Finally, compounds **19a–n** were synthesized by the reaction with electrophile R^2X, which had substituted benzyl or pyridinylmethyl groups.

As mentioned previously,[32] the chelerythrine alkaloid **8** could not be assessed in dye uptake assays because of its fluorescent nature. However, protoberberine alkaloids have extremely weak fluorescence activity, with an emission spectrum having a λ_{max} at 550 nm when excited at 350 nm.[38] Therefore, the iminium QPA derivatives **15a–g, 17b–c**, and **19a–n**, as 5,6-dihydrodibenzo[*a*,*g*]-quinolizinium derivatives with substituted R^1 and R^2 positions, could be assessed in the ethidium$^+$ accumulation assay, using HEK293 cells stably transfected with cDNA encoding the hP2X$_7$R.

As shown in Table 1, the berberine and palmatine alkaloids (**9** and **10**) showed no or very week antagonism at 8 μM concentration in hP2X$_7$-expressing HEK293 cells, however the antagonistic activity of iminium QPA derivatives which were modified at the R^1 and R^2 position were determined in the range of 4–0.3 μM of IC_{50} values. Compound (±)-**13**, the tetrahydroberberine alkaloid, displayed a 1.6-fold decrease in antagonist activity relative to the corresponding quaternary amine compound **15a**. Compounds



Scheme 1. Synthesis of novel P2X$_7$ receptor antagonists consisting of tetrahydroberberine derivative (±)-**13**. Reagents and conditions: (a) $NaBH_4$, K_2CO_3, MeOH, 0 °C, 98%; (b) EtI, CH_3CN, autoclave, 130 °C, 75%; (c) $NaBH_4$, MeOH, rt, 90%.

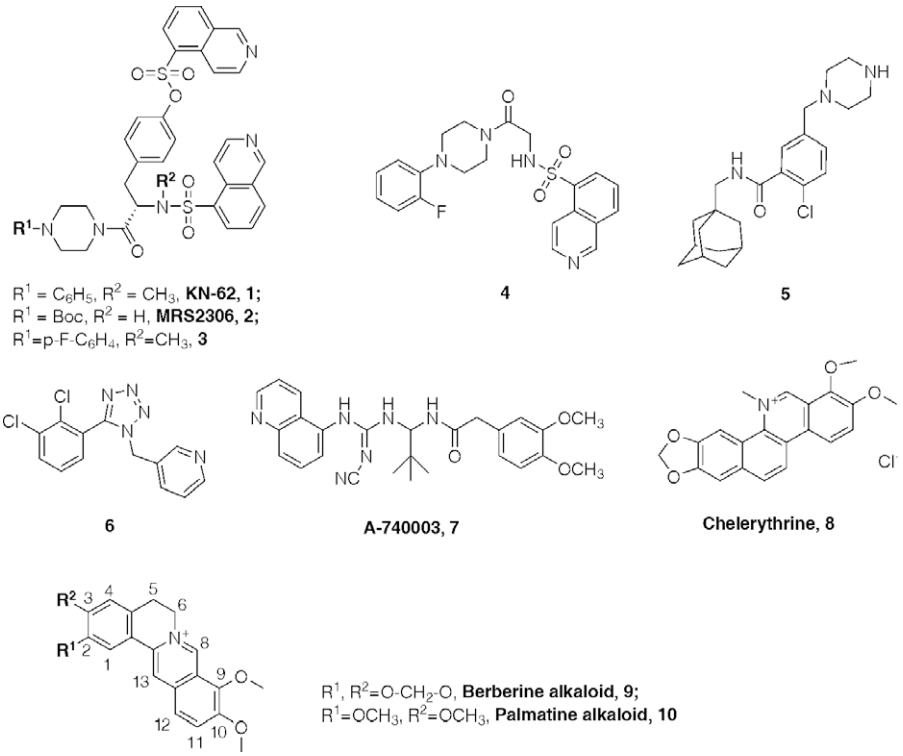
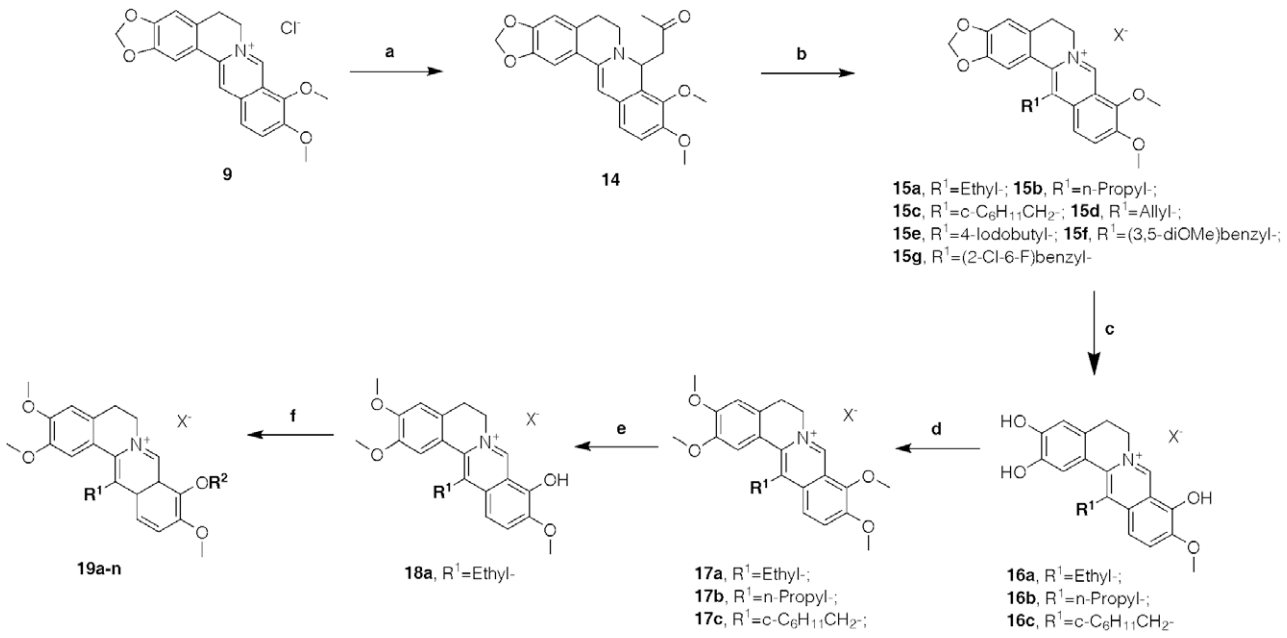
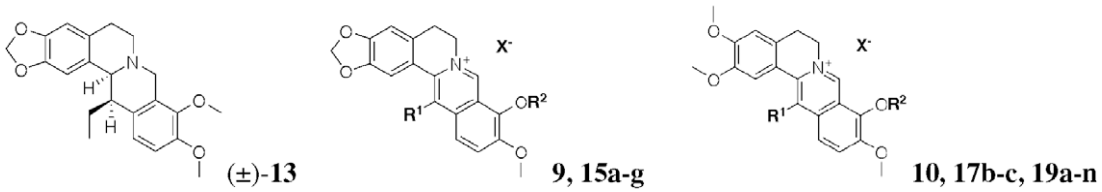


Figure 1. P2X$_7$ antagonists.



Scheme 2. Synthesis of 5,6-dihydrodibenzo[*a*,*g*]quinolizium derivatives of $P2X_7$ antagonist. Reagents and conditions: (a) acetone, aq 45% NaOH, H_2O, rt, 90%; (b) substituted alkyl halide or benzyl halide, acetonitrile or toluene, reflux, 47–54%; (c) i—$AlCl_3$, toluene, reflux; ii—0.8–1.2 N HCl, reflux, 96%; (d) dimethyl sulfate or methyl iodide, aq 50% NaOH, 81%; (e) LiCl, DMF, 140 °C, 90%; (f) R^2X, NaI, acetonitrile, reflux, 60–80%.

Table 1
Activities of 5,6-dihydrodibenzo[*a*,*g*]quinolizinium derivatives on ethidium accumulation in $hP2X_7$-expressing HEK293 cells[37]



Compound	R^1	R^2	X	IC_{50}[a] (μM)
		KN-62 (positive control)		0.34 ± 0.03
9	H	Me–	Cl	7.03 ± 3%[b]
10	H	Me–	Cl	NA[b,c]
(±)-13	Ethyl–	Me–		0.56 ± 0.26
15a	Ethyl–	Me–	I	0.35 ± 0.04
15d	Allyl–	Me–	I	0.44 ± 0.15
15e	1-Iodobutyl–	Me–	Cl	0.42 ± 0.08
15f	(3,5-$(MeO)_2$)benzyl–	Me–	Cl	0.26 ± 0.09
15g	(2-Cl-6-F)benzyl–	Me–	Cl	0.40 ± 0.19
17b	*n*-Propyl–	Me–	I	0.50 ± 0.28
17c	c-$C_6H_{11}CH_2$	Me–	Cl	1.12 ± 0.60
19a	Ethyl–	(2-Me)benzyl–	I	5.02 ± 0.72
19b	Ethyl–	(2-CF_3)benzyl–	I	2.56 ± 1.34
19c	Ethyl–	(2-Me-3-NO_2)benzyl–	I	5.40 ± 3.97
19d	Ethyl–	(2-CF_3-4-F)benzyl–	I	0.82 ± 0.40
19e	Ethyl–	(2-OMe-5-NO_2)benzyl–	I	0.42 ± 0.21
19f	Ethyl–	(2-NO_2-5-Me)benzyl–	I	0.73 ± 0.30
19g	Ethyl–	(2,5-diMe)benzyl–	I	1.53 ± 0.80
19h	Ethyl–	(2-NO_2-4,5-$(MeO)_2$)benzyl–	I	0.17 ± 0.07
19i	Ethyl–	(4-^{t}Bu)benzyl–	I	1.26 ± 0.73
19j	Ethyl–	(4-Br)benzyl–	I	2.60 ± 1.48
19k	Ethyl–	(4-Benzyloxy)benzyl–	I	2.81 ± 0.97
19l	Ethyl–	Biphenyl-4-ylmethyl–	I	3.22 ± 0.83
19m	Ethyl–	(3-Cl)benzyl–	I	2.66 ± 0.35
19n	Ethyl–	(6-Chloropyridin-3-yl)methyl–	I	4.41 ± 2.48

[a] Mean ± SD.
[b] The compound **9** and **10** represented the % inhibition at 8 μM concentration level.
[c] No activity.

15a, 15d, 17b, and **15e** with aliphatic groups (ethyl, allyl, *n*-propyl, and 1-iodobutyl, respectively) at the R^1 position had similar activities, whereas the compound with a 3,5-dimethoxybenzyl group (**15f**) showed increased antagonistic potency with an IC_{50} value of 0.26 μM. However, cyclohexylmethyl substitution at the R^1 position (**17c**) resulted in a significant decrease of the antagonistic activity.

Next, mono-, di-, and tri-substituted benzyl groups were introduced at the R^2 position. In general, electron-withdrawing groups at the 2-position of the benzyl moiety were preferable to electron-donating groups (e.g., CF_3- in **19b** vs CH_3- in **19a**; NO_2- in **19f** vs CH_3- in **19g**) for the antagonistic activity. Furthermore, as in the case of compound **19d**, introduction of a fluorine group at the 4 position of the benzyl moiety of compound **19b** containing a 2-CF_3 moiety increased the antagonistic activity more than threefold (**19d** vs **19b**). Among the di-substituted derivatives at the benzyl moiety of R^2 position, 2,4-and 2,5-disubsitutions resulted in more potent antagonists than 2,3-di-substitutions (e.g., **19c** vs **19d**, **19e**, **19f**, and **19g**). The tri-substituted analog, **19h**, containing 2-NO_2- and 4,5-dimethoxy groups at the benzyl moiety of R^2 position, turned out to be the most potent antagonist among all 5,6-dihydrodibenzo[*a*,*g*]quinolizinium derivatives tested in this study, showing twofold more potency than KN62 with an IC_{50} value of 0.17 μM. Neither halide (-Br and -Cl) nor bulky group substitutions at the 3- or 4-position at the benzyl moiety of R^2 position (**19i–n**) exhibited appreciable potency of single-digit micromolar IC_{50} values.

The functional antagonism of each of the 5,6-dihydrodibenzo[*a*,*g*]quinolizinium derivatives, at a concentration of 1 μM, was evaluated by assessing their ability to inhibit 1 mM BzATP-activated IL-1β release from LPS/IFNγ-differentiated human THP-1 cells (Table 2). The antagonism of compound **8** was determined with its effect on BzATP-mediated IL-1β release to compare iminium QPA derivatives, but compound **8** did not show an inhibitory activity at 1 μM concentration. ELISA assays of IL-1β showed that the 13-ethyl berberine alkaloid **15a** had twofold greater antagonistic activity than the tetrahydroberberine alkaloid (±)-**13**, a finding similar to the results of the ethidium$^+$ uptake assay. Also, compounds containing aliphatic groups (ethyl- (**15a**), allyl- (**15d**), and 1-iodobutyl- (**15e**)) at the R^1 position and those containing 2-, 4-, and 5-substituted benzyl groups (2-OMe-5-NO_2- (**19e**), 2-NO_2-5-Me- (**19f**), and 2-NO_2-4,5-dimethoxy- (**19h**)) at the R^2 position potently inhibited BzATP-induced release of IL-1β, similar to their activity in the ethidium$^+$ uptake assays. However, although compound **15f**, which has a (3,5-dimethoxy)benzyl moiety at R^1, had good antagonistic activity compared with KN62 in the ethidium accumulation assay (IC_{50}, 0.25 vs 0.34 μM), this compound showed much lower inhibitory activity than KN62 on IL-1β release. Compound **19h**, which showed a slightly more potent inhibitory effect than KN62 on IL-1β release induced by 1 mM BzATP, was further evaluated using full concentration–response curves to compare IC_{50} values (data not shown). We found that the IC_{50} value of **19h** (175 ± 51 nM) was twofold higher than that of KN62 (81 ± 12 nM).

In conclusion, a novel series of protoberberine-based $P2X_7$ antagonists with modifications at the R^1 and R^2 positions was synthesized and evaluated in ethidium accumulation and IL-1β release assays. Compounds with alkyl groups at the R^1 position (**15a** and **15d**) showed high potency in both assay systems as $P2X_7$ antagonists. In particular, compound **19h**, which contains an ethyl group at the R^1 position and a 2-NO_2-4,5-dimethoxy-benzyl group at the R^2 position, was discovered as the most potent antagonist of all QPAs tested, and the antagonistic potency was comparable to the positive control, KN-62.

Table 2
Antagonistic effects of protoberberine derivatives against BzATP-stimulated IL-1β release by LPS/IFNγ-differentiated human THP-1 cells[39]

Compound	% Inhibition[a]	Compound	% Inhibition
KN-62	79 ± 4	**19d**	13 ± 18
8	NA[b]	**19e**	42 ± 18
(±)-13	34 ± 4	**19f**	40 ± 7
15a	73 ± 14	**19g**	8 ± 6
15d	71 ± 0.30	**19h**	83 ± 3
15e	54 ± 10	**19i**	33 ± 13
15f	39 ± 25	**19j**	5 ± 16
15g	50 ± 8	**19k**	NA
17c	17 ± 12	**19l**	NA
19a	NA[b]	**19m**	7 ± 7
19b	13 ± 11	**19n**	33 ± 6

[a] Mean ± SD percent inhibition of 1 μM compound against 1 mM Bz-ATP-induced IL-1β release (n = 3).
[b] No activity.

Acknowledgments

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37. Ethidium accumulation in hP2X$_7$-expressing HEK 293 cells: All experiments were performed using adherent HEK293 cells stably transfected with cDNA encoding the human P2X$_7$ receptor. Cells were incubated for 30 min with 1 μM of each 5,6-dihydrodibenzo[*a,g*]quinolizinium derivative, re-suspended at 2.5×10^6 cells/ml in 20 mM HEPES (pH 7.4) buffer containing 140 mM KCl, 5 mM glucose, 1 mM ethylene diamine tetraacetic acid (EDTA), and 0.1 mM ethidium bromide, and added to wells of a 96 well plate. To each well was added BzATP, to a concentration of 1 mM, and the plates were incubated at 37 °C for 120 min. Cellular accumulation of ethidium$^+$ was determined by measuring fluorescence (excitation wavelength 530/20 nm, emission wavelength 590/20 nm) using a fluorescent plate reader.
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39. Effect on BzATP-mediated IL-1β release: THP-1 cells were differentiated by treatment for 3 h with 25 ng/ml LPS and 10 ng/ml IFNγ. The differentiated cells were incubated for 30 min with 1 μM of each 5,6-dihydrodibenzo[*a,g*]quinolizinium derivative, followed by stimulation with 1 mM BzATP for 30 min. Supernatants were collected by centrifugation at 1000 rpm for 5 min and assayed for the presence of mature human IL-1β using an ELISA kit.