Organogermanium compound, Ge-132, forms complexes with adrenaline, ATP and other physiological cis-diol compounds

Background: In mammals, adrenaline and ATP are life-essential vicinal diol and cis-diol functional groups. Here, we show that interactions between a safe organogermanium compound and these cis-diol compounds have the potential to regulate physiological functions. In addition, we represent a possible new druggable target for controlling the action of cis-diol compounds. Results: We analyzed a single crystal structure of organogermanium 3-(trihydroxygermyl)propanoic acid (THGPA), a hydrolysate of safe Ge-132, in complex with catecholamine (adrenaline and noradrenaline), and evaluated the affinity between several cis-diol compounds and THGPA by NMR. An in vitro study using normal human epidermal keratinocytes was performed to investigate the inhibition of cis-diol compound-stimulated receptors by THGPA. At high concentration, THGPA inhibited the calcium influx caused by adrenaline and ATP. Conclusion: This study demonstrates that THGPA can modify cis-diol-mediated cell-to-cell signaling.

The element germanium belongs to the group 14 carbon family and generally exhibits 4 covalent bonds when incorporated into chemical compounds. Germanium is a metalloid and can act as a semiconductor. The inorganic germanium compound GeO₂ exhibits an erythropoietic effect, as reported by Hammett and Nowrey [1] and Hammett et al. [2] in 1922. However, inorganic germanium can be hazardous. In the 1990s, GeO₂ erroneously sold as organogermanium led to deaths by renal toxicity [3]. Sanai et al. described the toxicity of GeO₂ and deemed Ge-132, poly-trans-[(2-carboxyethyl)germasesquioxane], safe to consume [4]. Excessive GeO₂ intake leads to lethal nephropathy via accumulation in the nephrons of kidneys. Limited amounts of GeO₂ have physiological functions, while an abundance can be lethal.

Ge-132 is a water-soluble organogermanium compound that has been deemed safe in multiple toxic evaluations [5]. An acute toxicity test in male dogs revealed an LD₅₀ of 8500 mg/kg bodyweight. Ge-132 affects multiple physiological systems, including the immune response, and has anti-inflammatory, antiosteoporosis and antihypertensive effects, among others [5–10]. Our group previously revealed that Ge-132 induces antioxidant production and promotes heme synthesis [11,12]. Ge-132 was developed for medical use and has undergone multiple clinical evaluations. However, more recently, it has been categorized as a food supplement in Japan. Thus, Ge-132 is now mainly used to supplement food and cosmetics in Japan, China, Korea and USA.

When Ge-132 is used as a medical cream for peripheral burn care, pain decreases very quickly after treatment. Therefore, we were interested in the action of Ge-132 in the peripheral pain signaling system. Although the nociceptive effect of Ge-132 has been described using the acetic acid writhing method [13] and the tail–flick method [14] in mice, the mechanism of this pain inhibition remains unclear. In the recent literature, an extracellular ATP, a cis-diol compound, is well known as a nociceptive factor that can cause pain in the peripheral and central nerve systems [15]. When skin tissue is damaged by a peripheral burn, large quantities of ATP are...
Crystals of THGPA/Ge-132 are formed in the same direction from a plane of bonding carbon in a ring structure. Sugars possess many cis-diol structures (and/or trans-diol structures). However, the hydroxyl groups of catechol and catechin are located on the flat plane of the benzene ring plate. For convenience in this article, we also defined these flat diol structures as cis-diols.

Key terms

Ge-132: Water-soluble organogermanium compound poly-trans-(2-carboxyethyl)germasesquioxane (IUPAC name) is known by several names. In addition, 2-carboxyethylgermanium sesquioxide has been used in previous research reports. Ge-132 is one polymer form of (3-trihydroxygermyl)propanoic acid. Another polymer type is known as propagermanium, which differs from Ge-132 in function and use. Propagermanium is used as a drug component for treating chronic hepatitis. Repagermanium is the name of Ge-132 as a cosmetic use.

Cis-diols: Two vicinal surface OH groups are oriented in the same direction from a plane of bonding carbon in a ring structure. Sugars possess many cis-diol structures (and/or trans-diol structures). However, the hydroxyl groups of catechol and catechin are located on the flat plane of the benzene ring plate. For convenience in this article, we also defined these flat diol structures as cis-diols.

Hypervalent: In addition to transition metals, other types of atoms can possess extraordinary electron orbitals. In general, small atoms can form from one to four chemical bonds. However, large atoms with extraordinary orbitals can bind with more than five atoms. A molecule that contains these types of high coordinate bonds is known as a hypervalent coordinate molecule. In the case of organogermanium compounds, the molecule, which contains a germanium atom, can form between five and seven coordinate bonds. Such molecules are known as penta-, hexa- and hepta-coordinated germanium compounds, respectively.

leaked from the injured cells. We believe that the interaction between Ge-132 and leaked ATP can suppress the pain signal resulting from the burn.

Germainate interacts with D-fructose to promote the isomerization of D-glucose to D-fructose [16,17]. Recently, we reported an interaction between Ge-132 and certain monosaccharides [18]. GeO₂ also reacts with phenylfluorone, which contains a catechol skeleton, and this reaction is employed in GeO₂ analyses [19]. Complex formation between GeO₂ and catechol compounds has also been detected [20–22]. Furthermore, phenylfluorone can be used to detect Ge-132. Thus, Ge-132 can be chelated by catechol and other cis-diol compounds. Catechol compounds have important functions in humans. Specifically, some catecholamines act as neurotransmitters or hormones [23–27]. Previously, Ge-132 was found to relieved the suppressive effect of catecholamines against intestinal peristalsis [28]. This effect might be due to the formation of a chelate between Ge-132 and catecholamines. Cis-diol element-containing compounds are very important in mammals, particularly as components of some neurotransmitters. Several separation technics for cis-diol biomaterials have recently been reported based on a similar interaction by some organoboronates [29–31]. Every purified cis-diol biomolecule has been shown to be important to life. Therefore, we predict that Ge-132 can regulate the function of neurotransmitters and hormones possessing cis-diol structures.

In this study, we evaluated chelate formation between Ge-132 and adrenaline, the most well-known catecholamine. Crystals of the chelate complex were prepared, and structural analyzes were performed by X-ray diffraction and NMR. The rate of chelate formation between Ge-132 and several cis-diol compounds, such as catecholamines (accurately vicinal diol; however in this study we also express about catechol as cis-diol) and nucleotides, was studied. We also evaluated the effect of Ge-132 on the application of adrenaline and ATP to human skin keratinocytes. Here, we demonstrate that the chelation of cis-diol neurotransmitters may regulate homeostasis and neurotransmission through the adjustment of receptor binding activity. This is the first report of a direct reaction between an organogermanium compound and active biological compounds based on structural information.

Results

Complex of organogermanium with catecholamine

Ge-132 is a water-soluble organogermanium compound and is a polymer of 3-(trihydroxygermyl)propanoic acid, THGPA (Figure 1). THGPA can interact with cis-diol structures through its trihydroxy groups, and here, we evaluated the ability of THGPA to interact with several physiological cis-diol compounds. First, we evaluated the crystal formation of THGPA, a safe form of organogermanium, which possesses a trihydroxy structure, and adrenaline complexes. Equimolar aqueous solutions of THGPA and adrenaline were combined. The resulting mixture had a pH of ∼4. Other solutions were pH adjusted with NaOH or HCl and did not form crystals of the desired complex. At lower pH values, as adjusted with HCl, only Ge-132 crystals were formed. The crystals of the THGPA and adrenaline complex were planar and colorless (Supplementary Figure 1). We speculated that THGPA and adrenaline were dehydrolyzed at their hydroxyl moieties. Moreover, we predicted that a pentacoordinate structure had formed via lactone ring formation (Figure 1). Crystals of THGPA/dl-adrenaline and THGPA/l-noradrenaline were analyzed using X-ray diffraction, and their structures were determined (ORTEP in Figure 2A–D). The molecular formulae of the two complex compounds are C₃₀H₃₀GeN₂O₆ (Mw. 343.9) and C₃₁H₃₁GeN₂O₆ (Mw. 329.9), respectively. Dehydration resulted in bonding between the THGPA and adrenaline molecules. Two hydroxyl moieties from each compound formed a ring composed of C–C–O–Ge–O–. Moreover, two H₂O molecules were formed (Figure 2B & D). Note that the complex
atoms were hypervalent (pentacoordinate). The propanoic group originating from THGPA formed a lactone ring (butyrolactone) and formed a coordinate bond with the vacant d-orbital of germanium, resulting in the formation of a pentacoordinate germanium (V) atom. We speculated that a proton originating from the lactone ring might be found neighboring the germanium atom (Figure 1); however, the proton was actually bound to the nitrogen atom (Figure 2A & C). These data suggest that these complex molecules may have an electric polarization within each individual compound because germanium is negatively charged by the formation of a lactone ring; in addition, the protonation to nitrogen gives the germanium a positive charge. The THGPA and d-t-adrenaline compounds formed a complex with reverse chirality (d-form and L-form), found in an antiparallel position. This might originate from the polarization described above. The complex compounds lie in two layers (Supplementary Figure 3A), based on stacked benzene rings (Supplementary Figure 3B) that might form pi bonds. The distance between the two planes is 3.345 Å. For the complex formed by THGPA and L-noradrenaline, the 3D configuration of two complex molecules was twisted (Figure 2D). Two H2O molecules were observed per complex molecule.

The IR spectra of the THGPA and adrenaline crystals were measured by the KBr method. The spectrum of the pentacoordinate compound was different from the spectra of the two original compounds (Supplementary Figure 4A). The strong absorbance at 800 cm⁻¹ that originated from Ge–O–Ge in Ge-132 did not exist in the newly formed complex. Moreover, the strong absorbance at 1686 cm⁻¹ that originated from C=O of a carboxyl radical in Ge-132 was shifted to 1622 cm⁻¹. The shift may be related to the formation of a lactone ring.

The crystals of THGPA and adrenaline were soluble in methanol. However, their solubility in water was very low at room temperature. The dehydration of four hydroxyl moieties in the two original compounds led to a reduction of hydrophilicity. Therefore, structural studies and stability evaluations were conducted by dissolution in D3-methanol. The ¹H- and ¹³C-NMR CH-COSY spectra are shown in Supplementary Figure 4B. These signals did not change after 2 or 4 months, suggesting that this complex is stable at ambient conditions.

Affinity between organogermainium & catecholamine & mono nucleic acid

Using NMR, we investigated the interactions between THGPA and other physiologically relevant cis-diol compounds. At physiological pH (~7.4–7.6), THGPA

![Figure 1. Structural scheme of 3-(trihydroxygermyl) propanoic acid/d-t-adrenaline complex. A schematic illustration of complex formation between 3-(trihydroxygermyl)propanoic acid (THGPA) and adrenaline is shown. The germanium atom has a vacant d-orbital, and we predicted that the complex compound would have a lactone ring.](image-url)
Figure 2. ORTEP diagram of 3-(trihydroxygermyl)propanoic acid/catecholamine complexes. (A) ORTEP diagram of the crystal obtained from a mixed solution of THGPA and α-adrenaline. The structure of one molecule is drawn, and the X-ray analysis revealed the lactone ring and NH₂. (B) The complex compound had two crystallized water molecules. The symmetry of the complex compounds resulting from D formation and L formation is shown. (C) ORTEP figure of the crystal obtained from a mixed solution of THGPA and l-noradrenaline. (D) 3D diagram of the THGPA and l-noradrenaline complex compounds. The configuration of the two complex molecules was twisted. Two water molecules were present per complex molecule. THGPA: 3-(trihydroxygermyl)propanoic acid.
Figure 3. Complex formations of 3-(trihydroxygermyl)propanoic acid with cis-diol compounds. (A) NMR spectrum of THGPA and α-adrenaline in D₂O. The pH was adjusted to 7.4 with NaOD. The signals revealed that a mixture of complex compound and parental compounds was present. The complex formation rate was calculated from the ratio of the areas of the complexed and free compounds. (B) The complex formation pattern of THGPA and the cis-diol compound. Typical biological cis-diol compounds are aligned. (C) The ratio of complex formation between THGPA and four types of biological cis-diol compounds is shown. Noradrenaline exhibits a high affinity for THGPA. However, TMGPA did not exhibit any complex formation with ATP.

AD: Adrenaline; Ado: Adenosine; NA: Noradrenaline; THGPA: 3-(trihydroxygermyl)propanoic acid.
Figure 4. Inhibition of calcium signaling by cis-diol binding by 3-(trihydroxygermyl)propanoic acid in NHEK cells.

(A) The gene expression level of purinergic receptor subtypes in NHEK cells was evaluated by quantitative PCR.
(B) The suppressive effect of THGPA on the signaling of adrenaline and ATP by human keratinocytes, NHEKs, was evaluated. A calcium ion indicator was incorporated into the cells, and calcium ion influx was measured by the acceptance of adrenaline and ATP. (C) NHEKs incorporated fluo-8AM responded to ATP stimulation. (D) Calcium ion influx was suppressed by the addition of THGPA. (E) The calcium ion influx triggered by adrenaline was suppressed with THGPA treatment. A high concentration of THGPA (5 mM) yielded significant suppression. (F) The calcium ion influx triggered by ATP was suppressed by THGPA treatment. A high concentration of THGPA (10 mM) suppressed calcium ion influx significantly. All data are displayed as mean ± SEM and were analyzed using ANOVA and Tukey’s test.

* p < 0.05; ** p < 0.01.

ANOVA: Analysis of variance; NHEK: Normal human epidermal keratinocyte; SEM: Standard error of the mean; THGPA: 3-(trihydroxygermyl)propanoic acid.
not form complexes with ATP at a high concentration (100 mM), suggesting that the trihydroxy form is essential for this interaction.

**Inhibition effect of THGPA addition for calcium ion influx by cis-diols**

Normal human epidermal keratinocytes (NHEKs) have β-adrenergic receptors and P2X2/3 receptors. These receptors are G-protein coupled receptors and Ca\(^{2+}\) ion channels, respectively. It is known that NHEKs also contain P2Y1 and P2Y2 receptors, which are also categorized as G-protein coupled receptors. We evaluated the expression levels of P2 receptors. The mRNA expression levels of P2X3, P2Y1 and P2Y2 in NHEKs in normal culture conditions are shown in **Figure 4A**. P2X3 was not detected, whereas the expression of P2Y1 and P2Y2 was confirmed. The cell response to adrenaline and ATP administration was monitored with the calcium ion indicator Fluo-8 AM. To evaluate the effect of THGPA, the influx of calcium ions triggered by either adrenaline or ATP was measured (**Figure 4B**). Calcium influx by ATP addition to NHEKs was visualized and is shown in **Figure 4C**. The cells exhibited green fluorescence after ATP addition. The addition of adrenaline to the cells led to an influx of calcium ions, and THGPA inhibited this response (**Figure 4D**). Treatment with 1 μM adrenaline triggered calcium ion influx into NHEK cells. The response times of calcium influx after the addition of ATP and adrenaline were different. After the ATP addition, 15 s was a sufficient response time for emission (**Figure 4C**). However, the addition of adrenaline required a longer response time, and the maximal lighting by calcium influx was observed from a half minute to 1 min later (**Figure 4D**). THGPA inhibited the fluorescence intensity in a dose-dependent manner (**Figure 4E**). The percentage increases in fluorescence for the addition of 0, 2.5 and 5 mM THGPA were 41, 27 and 13%, respectively. Calcium ion influx was significantly inhibited by 5 mM THGPA (p < 0.01). Similar results were observed with the addition of ATP (**Figure 4F**). The increased fluorescence values obtained with THGPA additions of 0, 1 and 10 mM were 97, 75 and 32%, respectively. THGPA suppressed the calcium ion influx activated by ATP in a dose-dependent manner, and the difference between 0 and 10 mM was significant (p < 0.01). The difference between 1 and 10 mM was also significant (p < 0.05). THGPA suppressed the cell response to the acceptance of cis-diols at the high concentration.

**Discussion**

This study supposes novel targets for the control of cell–cell communication by cis-diol compounds. We confirmed that organogermandiium can form complexes with important cis-diol compounds, including hormonal compounds such as adrenaline, noradrenaline and ATP. Today, many current drugs of clinical value are used to target receptors. Furthermore, most drugs act by blocking binding sites, thus acting as antagonists. Many cis-diol compounds have important roles in human physiology, including neurotransmitters such as catecholamines, ATP and adenosine, hormones such as catecholamines, genetic information such as RNA and coenzymes such as NAD and NADP (**Figure 3B**). These molecules share common fundamental structures such as catechol and ribose. Catecholamines are known as stress hormones, and they are released from the adrenal gland during psychological stress; moreover, an induction of hyperglycemia by adrenaline has been reported [32]. Recently, the structures of β-adrenergic receptors bound to agonists were revealed [33]. Similarly, ATP is one of the most important components for life and is known as a biological energy compound [34,35]. However, the extracellular concentration of ATP is extremely low. Purine receptors have many roles, and some of these receptors sense the release of purines from punctured cells during pain signaling. ATP can act as a signal for cell damage. Extracellular ATP serves as a nociceptive signal in peripheral and central neuronal signaling through interactions with P2X2/3 and P2X3 or with P2X4, respectively [15,36–39]. Notably, the functions of both catecholamine and ATP are related to stress, and organogermandiium, Ge-132, can interact with cis-diol compounds that have stress-related physiological functions. In general, a receptor is depolarized by ligand binding. Therefore, an excess of ligand compounds decreases the intensity of the cellular response. Hence, spontaneous stress and cell damage may decrease the ability of the cell to respond. We evaluated interactions between THGPA and cis-diol compounds and whether ligand trapping by complex formation with THGPA suppressed binding and cell response. We crystallized the complexes of THGPA with adrenaline and noradrenaline (Figure 2). The crystals contained pentacoordinated germanium with a lactone ring. Germanium compounds have a carboxyethyl moiety that...
can form a lactone ring with pentacoordinated germanium in the formation of complexes. Moreover, if electron-donating groups exist on the delta position of the organogermanium compound (e.g., lactamo-N-methyl ligands), a cyclic structure with pentacoordinated germanium can form. NMR analyzes in deuterium oxide (Figure 3A) suggest an equilibrium state between tetra-coordinate (THGPA) and pentacoordinate (THGPA/catecholamine complex). However, X-ray analysis revealed that complexes with cis-diol structures only have pentacoordinated atoms. Therefore, we speculate that triols bonded to germanium are close to a diol when the pentacoordinate is formed (Figure 2). The distances of triols are estimated at 2.78–2.95 Å for the tetracoordinate. However, the distance of cis-diol for the THGPA/adrenaline complex was 2.57 Å in this study, which is a shorter distance than in the other compounds. Therefore, the pentacoordinate position may be suitable for this short distance.

These types of complexes are usually hydrolyzed under hydrophobic conditions. Therefore, an equilibrium state exists between the complex and substrates. If the complex formation is extremely strong, the signal transduction of the adrenaline and ATP ligands is reduced by Ge-132 intake. However, this equilibrium may simulate a decrease in ligand concentration. Although neurotransmitter and hormone signal transductions are essential molecules, an excess of these signals is not conducive to normal cell function. In this case, Ge-132 may change the apparent signal concentration in the receptors. In this study, we mainly focused on adrenaline and ATP. Previous reports indicate that Ge-132 can relieve the effect of catecholamine in the intestine [28]. Moreover, Ge-132 is useful for pain relief, and Ge-132 decreased pain in terminal cancer patients in double-blind clinical trials [40]. Extracellular ATP, adenosine, and catecholamine function in central or peripheral pain signal transduction or nociception [38,41–44]. Furthermore, in our previous study, THGPA suppressed a nociception threshold mediated P2X purinergic receptor by ATP signaling in mice (unpublished data). These facts suggest that interactions between THGPA and these compounds are involved in this pain relief. The interaction of THGPA with cis-diol compounds is of great interest because many kinds of cis-diol compounds are essential in mammals.

THGPA is a water-soluble compound and may not be readily incorporated into intercellular compartments. Therefore, a higher proportion of THGPA may be present in the extracellular space than in the intracellular fluid. For biological application, we demonstrate the inhibition of adrenaline and ATP binding to the receptors in Figure 4E & F. In this study, NHEKs expressed both P2Y1 and P2Y2 receptors in normal culture conditions. Mechanical stimulation evokes Ca²⁺ waves mediated by ATP signaling through P2Y2 receptors, and Koizumi et al. reported that these Ca²⁺ waves, as pain signals, are transmitted to sensory neurons [45]. Therefore, THGPA may suppress this Ca²⁺ wave signaling via ATP to peripheral neurons. In reality, the acute pain of a burn wound is blocked by the application of THGPA. A similar inhibition is expected for other cis-diol compounds in the extracellular space if THGPA is distributed within the area at high concentration. The receptors for catecholamine and purines are rich in variety and can be found in multiple cell types. For example, adrenergic receptors α₁, 2 and β1–3 and purine receptors P2X1–12 and P2Y1–13 have been known. These subtypes have different functions against the same compounds. When the cell membrane is harmed, ATP leakage occurs. Similarly, psychological or cold stress triggers the release of catecholamine. THGPA binds these cis-diol compounds and suppresses their binding to receptors. Ho et al. have reported that the intraperitoneal administration of Ge-132 lowered the mean arterial pressure in a dose-dependent manner in rat [46]. They supposed that Ge-132 acts by mediating the catecholamines in the brain. In this study, we evaluated Ge-132 by using the NHEK model; however, the same mechanism will be applicable to vascular smooth muscle cells. We speculate one aspect of Ge-132’s mechanism cis-diol compound mediation in our body, illustrating this concept schematically in Figure 5. Peripheral pain due to leaked ATP from injured cells is suppressed by THGPA trapping due to equilibrium complex formation. However, the formation of complexes between THGPA and catecholamines inhibits vasoconstriction mediating α1 receptors. Moreover, it has been reported that noradrenaline enhanced P2X3 expression in dorsal root ganglia in rat [43]. In this system, the pain signal mediates two different cis-diol molecules (noradrenaline and ATP), and both of them can interact with THGPA; therefore, THGPA treatment of peripheral skin may be beneficial for peripheral nociceptive pain. In addition, complex formation with adenosine, a metabolite of ATP, may inhibit its metabolism to inosine by adenosine deami-
Figure 5. Image illustrating cis-diol trapping by Ge-132. The Ge-132 monomer, 3-(trihydroxygermyl)propanoic acid, can interact with catecholamines and nucleotides. The suppression of pain by leaking ATP signaling and the inhibition of acute high blood pressure by catecholamines are expected after 3-(trihydroxygermyl)propanoic acid application or intake.

EKC: Epidermal keratinocyte; VEC: Vascular epithelial cell; VMC: Vascular muscle cell.

Ge-132 forms complexes with physiological cis-diol compounds. Multiple Ge-132 physiological functions have been reported, but the mechanism behind its action has not been determined. Differentiated cells have different receptor subtypes, and the same ligand can lead to different functions. Thus, the interaction of THGPA (Ge-132) with various cis-diol compounds may affect homeostasis, thereby explaining some of the functions of Ge-132. We anticipate that interactions with cis-diol compounds might be important. Different compounds can interact with cis-diol compounds, for example, such as phenyl borate and GeO₂. One borate compound, Bortezomib, is used to inhibit the proteasome in multiple myeloma. Recently, the inhibition of Bortezomib activity by epigallocatechin gallate was reported [47]. It has been suggested that the affinity for epigallocatechin gallate can be explained by its interaction with cis-diols in the body. However, Bortezomib exhibits several severe side effects, and some boronic acids and GeO₂ are also reportedly toxic. Therefore, the affinity of borate or GeO₂ for cis-diols may be too strong for use in physiological applications. THGPA has an equilibrium interaction with cis-diol compounds in hydrophilic conditions (in water). This equilibrium is shown as different spectra of ¹H-NMR for the THGPA and adrenaline complex in Supplementary Figure 4B (part of CH COSY in D₃-methanol) and Figure 3A (in D₂O). The proton signals from 1.5 to 2.5 ppm originated from THGPA and were detected as different triplet signals.
related to either free or complexed THGPA (Figure 3A). This hydrolysis equilibrium condition is related to the safety of Ge-132. THGPA possesses a second type of polymer structure, propagermanium, relative to Ge-132. Propagermanium is a CCR2 antagonist and is used as a drug for the treatment of chronic hepatitis. Recently, Yumimoto et al. revealed a cancer metastasis mechanism and the strong inhibition of cancer metastasis via propagermanium intake [48]. Although the structural mechanism of this inhibition has not been demonstrated, the interaction between THGPA and biological cis-diol compounds may affect CCR2 inhibition by propagermanium.

Although this study suggests that the physiological function of Ge-132 may be related to the interaction with bioactive cis-diol compounds, the concentration needed for effectiveness was considerable. Therefore, a micro regional distributational study of THGPA in vivo will be important. Recently, our group has developed a micro analysis method of THGPA with structural information by LC/MS/MS [49]. We expect to carry out further studies on the affinity of THGPA and cis-diol compounds in detail using this LC/MS/MS analysis method. In addition, we performed NHEK cell analyzes in normal conditions; therefore, a deviation from treatment by the complexes among THGPA and cis-diol compounds would be interesting to study in the future. In microglial cells of allodynia, the expression of P2X4 is very high [50]; hence, the effect of THGPA in such a model should also be revealed in the future.

Experimental

Chemicals and reagents

Ge-132 (Lot 006316A; purity over 99%), also known as poly-trans-([2-carboxyethyl]germasesquioxane), was synthesized at the manufacturing plant of Asai Germanium Research Institute Co., Ltd. The synthesis procedure for Ge-132 used by the Asai Germanium Research Institute is as follows. Ingots of germanium (purity greater than 99.9999%) were milled in powder. The powdered germanium was reacted with hydrochloride gas at high temperature, and trichlorogermane was produced. Next, the trichlorogermane was reacted with acrylic acid to form THGPA. The obtained 3-(trichlorogermnyl)propanoic acid was hydrolyzed to THGPA and dried to a polymer, Ge-132. An inactive form of the compound, TMGPA, was synthesized by Toru Yosihara at Asai Germanium Research Institute Co., Ltd. Adenosine, d1-epinephrine (adrenaline), l-noradrenaline and l-dopa (3-(3,4-dihydroxyphenyl)-l-alanine) were purchased from Wako Pure Chemical Industrial Co., Ltd. (Osaka, Japan). ATP was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). D4-methanol and deuterium oxide (D2O) were purchased from Merck, and D3-methanol was purchased from Isotec Laboratories, Inc. (Champaign, IL, USA).

Preparation of the adrenaline & Ge-132 chelate complex crystal

Equal moles each of adrenaline or noradrenaline and THGPA (0.5 mol of Ge-132) were reacted in 10 ml of pure water at room temperature. The solution was incubated at 4°C for several days. Single crystals began to form after half of a day. The crystals were observed with an optical microscope, and digital photographs were taken with a Moticam 2000 digital CCD scope (Shimadzu Corporation, Kyoto, Japan). The crystals were removed from the mother liquor, rinsed with pure water and dried. The crystals were subsequently used for structural analysis.

Structural analyzes of the complex crystal

X-ray structural analysis

A single crystal (~0.2 mm) was used for X-ray structural analysis with a Smart APEX II (Bruker AXS corporations, Billerica, MA, USA), which is a single crystal X-ray diffractometer with CCD detection. The data were processed with the APEX II software for reduction and cell refinement. X-ray diffraction analyzes were deposited with the Cambridge Crystallographic Data Centre. Deposition numbers are CCDC-894300 for Ge-132/d-t-adrenaline and CCDC-894301 for Ge-132/l-noradrenaline. Free copies of the data can be obtained via the Cambridge Crystallographic Data Centre website [51] or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK (Fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

FTIR analysis of the complex crystals

The spectra were measured using an IR 4000 spectrometer (Shimadzu) with the potassium bromide (KBr) method. A tablet was formed by adding 2 mg of pulverized crystals and 0.2 g of KBr to a press. The FTIR spectra were scanned from 4600 to 400 cm

NMR analysis of the complex crystals

Proton and 13C-NMR analyzes were conducted at 300 Hz (1H) and 75 MHz (13C) using a Gemini 300 spectrometer (Varian, Agilent Technologies, Inc., Santa Clara, CA, USA). Crystals of the THGPA and adrenalin complexes were dissolved in 800 μl of D3-methanol at saturation. The methanol signal was adjusted to 3.4 ppm in the 1H-NMR study. Measurements were acquired at 25°C, with 16 transients for 1H analyzes and 40,000 transients for 13C analyzes. CH-COSY and DEPT analyzes were used to assign each signal. The complex stability was evaluated by the collection of 1H-
NMR spectra over time. The assigned δ H signals are reported as follows (refer to Supplementary Figure 4B). A broad triplet at 1.77 ppm corresponds to a methylene (C1) neighboring germanium. A sharper triplet at 2.56 ppm confirms a methylene (C2) neighboring the ketone. Because Ge-132 does not dissolve in methanol, we posit that these are specific signals originating from THGPA incorporated into the complex with adrenalinone. One other methylene (C11), originally from adrenalinone, exhibits a doublet signal at 3.25 ppm. The bond between a methyl (C12) and the amine was detected as a singlet at 2.84 ppm. The hydrogen bonded to C10 was detected as a triplet signal at 4.88 ppm. Two hydrogen atoms bonded to C6 and C9 were detected at 6.75 ppm as a multiplet signal. The hydrogen bonded to C8 was detected at 6.88 ppm as a doublet signal. The assigned signals (refer to Supplementary Figure 4B) of δC are as follows: 16.6 (C1), 30.0 (C2), 33.8 (C12), 57.0 (C11), 70.4 (C10), 110.6 (C6), 112.5 (C9), 117.2 (C8), 131.5 (C7), 150.9 (C4), 151.4 (C5) and 181.2 (C3) ppm. The signals were assigned based on the known chemical shifts of adrenalinone from the 1D NMR spectral database of organic compounds [52] (National Institute of Advanced Industrial Science and Technology, November 13, 2014). Changes in spectral patterns were determined by comparing spectra acquired after 2 and 4 months at room temperature without inactive gas.

NMR analysis for affinity evaluation

For the affinity studies, each of adenosine, ATP, dl-adrenaline and l-noradrenaline, and THGPA were evaluated at equal molar amounts (0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 mmol/l each) in D_2O. ATP and TMGPA were also evaluated in D_2O at 100 mmol/l concentration each. For adrenaline, the 250 and 500 mmol/l solutions were too high (insoluble) to analyze. The solution was adjusted to pH 7.4 with NaOD. Measurements were acquired at 25°C, with 16 transients for each measurement with trypsin and were prepared as a suspension in culture medium to a final concentration of 6 × 10^5 cells/ml. Each well of a 96-well cell culture plate (black plate, catalog No.165305, Nunc, Thermo Fisher Scientific K.K., Yokohama, Japan) received 100 μl of cell solution. The cells were cultured for 24 h before the experiment. The cells were then harvested after treatment with trypsin. The NHEK cells were obtained from KURABO (Osaka, Japan). The cells were cultured in Humedia KB-2 (Kurabo) supplemented with insulin, hEGF, bovine pituitary extract and hydrocortisol. The purinergic receptor expression of NHEK cells was confirmed by quantitative PCR analysis. The cells were cultured in 12-well plates in Humedia KB-2. Confluent cells were lysed with 1 ml of Isogen (Nippon Gene Co., Tokyo, Japan), and then total RNA was extracted using the manufacturer’s protocol. One microgram of extracted total RNA was used as a template for cDNA synthesis via reverse transcription with an oligo dT primer and Super Script III (Invitrogen, Carlsbad, CA, USA). The synthesized cDNA was used for quantitative PCR analysis. Quantitative real-time PCR was performed for P2X3, P2Y1 and P2Y2 as target genes and for β-actin (ACTB) as a housekeeping gene. The PCR primer sets for each gene are listed in Table 1. The cDNA was amplified using SYBR Premix ExTaq II (Takara Bio, Ohtsu, Japan) on an Opticon 2 (Bio-Rad Laboratories, Hercules, CA, USA), which was programmed for 95°C for 30 s, followed by 40 cycles of denaturation (95°C for 5 s), annealing and extension (60°C for 30 s). Each expression value was calculated according to the threshold cycle value, and the data are displayed as the ratio of expression of each gene to β-actin.

Calcium influx measurement in human keratinocytes

A fluorescent reagent was used to measure the influx if calcium ions into cells. NHEK cells were cultured according to the supplier’s recommendations, as described above. The cells were harvested after treatment with trypsin and were prepared as a suspension in culture medium to a final concentration of 6 × 10^5 cells/ml. Each well of a 96-well cell culture plate (black plate, catalog No.165305, Nunc, Thermo Fisher Scientific K.K., Yokohama, Japan) received 100 μl of cell solution. The cells were cultured for 24 h before the experiment. The cells were then harvested after treatment with trypsin. The NHEK cells were obtained from KURABO (Osaka, Japan). The cells were cultured in Humedia KB-2 (Kurabo) supplemented with insulin, hEGF, bovine pituitary extract and hydrocortisol. The purinergic receptor expression of NHEK cells was confirmed by quantitative PCR analysis. The cells were cultured in 12-well plates in Humedia KB-2. Confluent cells were lysed with 1 ml of Isogen (Nippon Gene Co., Tokyo, Japan), and then total RNA was extracted using the manufacturer’s protocol. One microgram of extracted total RNA was used as a template for cDNA synthesis via reverse transcription with an oligo dT primer and Super Script III (Invitrogen, Carlsbad, CA, USA). The synthesized cDNA was used for quantitative PCR analysis. Quantitative real-time PCR was performed for P2X3, P2Y1 and P2Y2 as target genes and for β-actin (ACTB) as a housekeeping gene. The PCR primer sets for each gene are listed in Table 1. The cDNA was amplified using SYBR Premix ExTaq II (Takara Bio, Ohtsu, Japan) on an Opticon 2 (Bio-Rad Laboratories, Hercules, CA, USA), which was programmed for 95°C for 30 s, followed by 40 cycles of denaturation (95°C for 5 s), annealing and extension (60°C for 30 s). Each expression value was calculated according to the threshold cycle value, and the data are displayed as the ratio of expression of each gene to β-actin.

### Application for cellular study using human keratinocytes in vitro

**Gene expression analysis of P2 receptor in human keratinocytes**

NHEK cells were obtained from KURABO (Osaka, Japan). The cells were cultured in Humedia KB-2 (Kurabo) supplemented with insulin, hEGF, bovine pituitary extract and hydrocortisol. The purinergic receptor expression of NHEK cells was confirmed by quantitative PCR analysis. The cells were cultured in 12-well plates in Humedia KB-2. Confluent cells were lysed with 1 ml of Isogen (Nippon Gene Co., Tokyo, Japan), and then total RNA was extracted using the manufacturer’s protocol. One microgram of extracted total RNA was used as a template for cDNA synthesis via reverse transcription with an oligo dT primer and Super Script III (Invitrogen, Carlsbad, CA, USA). The synthesized cDNA was used for quantitative PCR analysis. Quantitative real-time PCR was performed for P2X3, P2Y1 and P2Y2 as target genes and for β-actin (ACTB) as a housekeeping gene. The PCR primer sets for each gene are listed in Table 1. The cDNA was amplified using SYBR Premix ExTaq II (Takara Bio, Ohtsu, Japan) on an Opticon 2 (Bio-Rad Laboratories, Hercules, CA, USA), which was programmed for 95°C for 30 s, followed by 40 cycles of denaturation (95°C for 5 s), annealing and extension (60°C for 30 s). Each expression value was calculated according to the threshold cycle value, and the data are displayed as the ratio of expression of each gene to β-actin.

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### Table 1. Primer sets for quantitative polymerase chain reactions for P2 receptor expression analysis.

<table>
<thead>
<tr>
<th>GenBank accession no.</th>
<th>Target gene</th>
<th>Amplicon size (bp)</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_002559.3</td>
<td>P2X3</td>
<td>109</td>
<td>5′-accaagtgcgtggttggaaga-3′</td>
<td>5′-aatggtgtgtcgcgctacg-3′</td>
</tr>
<tr>
<td>NM_002563.3</td>
<td>P2Y1</td>
<td>150</td>
<td>5′-ctctagctacacgatgagaata -3′</td>
<td>5′-ctaaagtgtggatggtggtg-3′</td>
</tr>
<tr>
<td>NM_176072.2</td>
<td>P2Y2</td>
<td>146</td>
<td>5′-tcctgctggttaatttgcgctc-3′</td>
<td>5′-ggggtcgagcactacgagaa-3′</td>
</tr>
<tr>
<td>NM_001101.3</td>
<td>ACTB</td>
<td>96</td>
<td>5′-cccaaggcggagagaagatgta-3′</td>
<td>5′-cagaggggtagcagag-3′</td>
</tr>
</tbody>
</table>

The cell–cell signaling control by the ligand with study using a one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons.

Safe organogermanium compound Ge-132, a polymer of 3-(trihydroxygermyl)propanoic acid (THGPA), can inhibit cell signaling mediating receptor for THGPA in inhibition of receptor binding of adrenaline and ATP and free ligand states. Moreover, we confirmed the biological application of organogermanium compounds, standing on the points of relation to cis-diols.

The complex crystal of THGPA/catecholamine has pentacoordinate germanium (V) and a lactone ring structure. The affinities of THGPA and catecholamine are higher than that of THGPA and mono nucleic acids.

THGPA inhibits cell signaling mediating receptor for cis-diols by ligand trapping at high concentration.

The cell–cell signaling control by the ligand with cis-diol structure trapping may be a novel draggable target.

Future perspective
The organogermanium compound, Ge-132, may represent a leading candidate for interaction with cis-diols toward establishing a novel type of medicine. In all likelihood, the affinity of Ge-132 with targeted cis-diols is too weak for use as a strong, effective drug. However, when we see the various physiological effects of THGPA and its polymers, we estimate that controlling cis-diol compounds with specific interactions has potential as a novel drug target. In the future, we anticipate that novel medicines will modulate receptor binding via complex formation with cis-diols. We expect advances in the study of the biological application of organogermanium compounds, standing on the points of relation to cis-diol compounds.

Conclusion
The structural details of the interaction between an organogermanium compound and active biological compounds were presented for the first time in this study. This finding accounts for the numerous physiological functions of Ge-132 while exhibiting low toxicity. Ge-132 is safe, and its effects are not potent. The low toxicity of THGPA may originate from its existence in equilibrium between bound and free ligand states. Moreover, we confirmed the inhibition of receptor binding of adrenaline and ATP by THGPA in vitro. However, the THGPA concentrations necessary to achieve significant inhibition were considerably high; therefore, whether effective cis-diol trapping can be achieved by THGPA may be highly restricted. Even so, the complex formed between THGPA and catecholamine may be beneficial for antioxidation applications in terms of melanin formation via catechol to quinone. Although the complex compound with an unstable catechol structure, the crystals of THGPA/adrenaline were very stable at conventional atmosphere for a few months, and the associated NMR spectrum did not change.

Statistical tests
The data are presented as the mean ± standard error of the mean (SEM). Statistical significance was evaluated for the in vitro study using a one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons, and p < 0.01 and p < 0.05 were considered significant for the cell experiments.

Executive summary
- The biological cis-diol compounds are very important for life.
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References

Papers of special note have been highlighted as:

• of interest; •• of considerable interest


Two different cis-diol signal of noradrenaline and ATP relate to nociceptive pain signalling in the peripheral neuron system.


Extracellular ATP signals as nociceptive signals mediating skin keratinocytes to peripheral sensory neurons are discussed.


Cambridge Crystallographic Data Centre. www.ccdc.cam.ac.uk/conts/retrieving.html

SDBSWeb. http://sdds.db.aist.go.jp