Evaluation of gambierol and its analogs for their inhibition of human K_v1.2 and cytotoxicity

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Keyword: gambierol, voltage-gated potassium channels, cytotoxicity

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Abstract

Gambierol and its heptacyclic and tetracyclic analogs were tested for inhibitory activity against the human voltage-gated potassium channel K_v1.2 (hK_v1.2), which was stably expressed in Chinese hamster ovary (CHO) cells. Gambierol, the heptacyclic analog, and the tetracyclic analog inhibited the potassium current evoked by a step pulse from -80 mV to 40 mV. The IC₅₀ values for the three compounds were 0.75 ± 0.15 nM, 7.6 ± 1.2 nM, and 28 ± 4.0 nM (the mean $\pm$ S.E.M., $n = 3$), respectively. The cytotoxic activity was examined in order to assess a relationship between cytotoxicity and inhibition of the hK_v1.2. The IC₅₀ values for gambierol, the heptacyclic analog, and the tetracyclic analog in the wild-type CHO cells were 95 ± 7.1 μ M, 6.5 ± 0.8 μ M (the mean $\pm$ S.E.M., $n = 3$), and >100 μ M ($n = 3$), respectively, whereas those in the CHO cells stably expressing hK_v1.2 were 78 ± 5.8 μ M, 6.0 ± 1.0 μ M (the mean $\pm$ S.E.M., $n = 3$), and >100 μ M ($n = 3$). These results suggested that cytotoxicity is not triggered by inhibition of the human K_v1.2. The electrophysiological recording at the resting potential in the presence of gambierol, the heptacyclic analog, and the tetracyclic analog revealed the dose-dependent leak current, which was largest when the heptacyclic analog was administered to the cells. We thus propose that the leak current induced by these compounds might cause a fatal effect on the cultured cells.

The ‘ladder-shaped polycyclic ethers’, including brevetoxins,^{1, 2} ciguatoxins,^{3, 4} gambieric acids,^{5, 6} gambierol,⁵ gymnocins,⁷ maitotoxin,⁸⁻¹⁰ prymnesins,^{11, 12} yessotoxins,¹³ have attracted research attention because of the organized array of the cyclic ethers and the biological activities.¹⁴ Their structures appear to resemble to each other, whereas their physiological targets are not identical. Brevetoxins were isolated from the dinoflagellate *Karenia brevis* and causes fatal effect on fish and mammals.^{15, 16} When a dinoflagellate outbreak occurs, shellfish accumulate a toxic substance, which depresses the local economy related to fish and farming industries. They bind to the voltage-gated sodium channels at Site 5,¹⁷ shift the activation potential, and delay inactivation.¹⁸ Additionally, ciguatoxins also bind to the voltage-gated sodium channel at Site 5 and exhibit competitive binding with the tritium-labeled brevetoxin analog.¹⁹ The acute toxicity of ciguatoxin in mice is 350 ng/kg,²⁰ which is far more potent than that of brevetoxins,²¹ although the ichthyotoxicity of ciguatoxin has not been specifically documented. Other polycyclic ethers weakly bind to the voltage-gated sodium channels,²² which implies that molecular recognition of the voltage-gated sodium channels is moderately regulated. The acute toxicity of maitotoxin in mice is 50 ng/kg,²³ which shows that maitotoxin is the most toxic secondary metabolite. Maitotoxin activates calcium influx,^{24, 25} which triggers various physiological events such as IP₃ breakdown,²⁶ hormone secretion^{24, 27} and neurotransmitter release.²⁸ Initially, the voltage-gated calcium channel was hypothesized to be the target of maitotoxin.^{24, 25, 29-31} Since then, electrophysiological recordings have ruled out that possibility and revealed that the maitotoxin-stimulated action is characterized by voltage-independent and nonselective cation influx.³²⁻³⁷ Nevertheless, the target of maitotoxin remains unknown. Yessotoxins are different from these toxins and interact with soluble proteins such as phosphodiesterases^{38, 39} and Rap1A.⁴⁰ Because of the low natural abundance, sufficient physiological studies including those on prymnesin⁴¹ and gymnosin⁴² have not been performed.

Gambierol (**Figure 1**) was isolated from the dinoflagellate *Gambierdiscus toxicus*.⁴³ It shows strong acute toxicity in mice (LD₅₀ 50 µg/kg, i.p.), and the neurological symptoms observed in the mice administered with gambierol resemble those shown by ciguatoxins.^{44, 45} Thus, a question has long been raised regarding its physiological

receptor. After the completion of the total syntheses of gambierol,⁴⁶⁻⁴⁸ physiological studies were performed using the synthetic material. It was reported that gambierol inhibited voltage-gated potassium currents in mouse taste cells⁴⁹ and enhanced muscle contraction and blocked a transient potassium current in skeletal muscle cells.⁵⁰ The voltage-gated sodium channels were excluded from the candidates for physiological receptor, whereas specific subtypes of the voltage-gated potassium channels were revealed to interact with gambierol.⁵¹ Systematic studies utilizing the chimera between gambierol-sensitive and insensitive subtypes of potassium channels postulated that the gambierol binding site was located at T427 in the Segment 6.⁵² Kopljar et al. further investigated and disclosed that gambierol affects the gating mechanism of K_v3.1 channels in the resting state.⁵³ While the interaction between gambierol and the potassium channel is theoretically based on multiple hydrogen bonds, other molecular determinants of gambierol for the inhibition of the potassium channel should be present. In this context, we previously elucidated the structure-activity relationship of the peripheral substituents on the octacyclic polyether core of gambierol using a series of synthetic analogs; this study has established the importance of the H-ring and the triene side chain for potent acute toxicity against mice.⁴⁵ Alternatively, the discovery of synthetic analogs that mimic the physiological activity of the natural product should be useful. We have already prepared the heptacyclic and tetracyclic analogs of gambierol on the basis of our structure-activity relationship study⁵⁴ (**Figure 1**). With gambierol, the two compounds were tested for cytotoxicity and inhibitory activity against the voltage-gated potassium channels expressed in the cultured cerebellar neurons.⁵⁵ It was revealed that the heptacyclic analog was the most toxic to the cultured cerebellar neurons and was the most potent inhibitor against the potassium channels expressing in the cultured cerebellar neurons. Because more than one subtype of voltage-gated potassium channels was expressed in the cerebellar neurons, it might be worth retrieving the structure-activity relationship using a cell line that only express the

specific subtype of the voltage-gated potassium channels.

Herein, we constructed CHO cells stably expressing hK_v1.2 because gambierol has been shown to inhibit this specific subtype most potently compared to other K_v1 subtypes.⁵¹ We then demonstrated the structure–activity relationship of gambierol against hK_v1.2 by patch clamp recordings using the gambierol and its heptacyclic and tetracyclic analogs. We also examined the cytotoxicity of the three compounds in the wild-type CHO cells and those stably expressing hK_v1.2 to evaluate the correlation between the inhibitory activity and the cytotoxicity of hK_v1.2.

We first constructed HEK293T cells stably expressing human K_v1.2 (hK_v1.2), and the electrophysiological recording revealed that the cells endogenously expressed the voltage-gated potassium channels.⁵⁶ The observed outward current was increased only by two- to three-fold when the hK_v1.2 was overexpressed. Because the contribution of the endogenous potassium current was not negligible, we used the CHO cells. We then prepared CHO cells stably expressing hK_v1.2, and the construction of each clone was confirmed by western blotting (**Figure 2A**). Although G418 is fatal to non-transfected CHO cells, the resistant clones were able to survive the selection process (Lane 1, duplicate). Positive clones were confirmed by the overexpressed hK_v1.2 using anti-c-myc monoclonal antibody (Lanes 2 and 3, duplicate), and were used for electrophysiological experiments. The whole cell patch clamp recording revealed that the cells also express endogenous potassium channels; the outward current was increased six- to seven-fold by overexpressing the hK_v1.2 and was inhibited by 4-aminopyridine, a specific blocker of the K_v1 channel (data not shown). The results convinced us of the establishment of the CHO cells stably expressing hK_v1.2.

The whole cell patch clamp recordings were conducted to investigate inhibitory action of gambierol against the hK_v1.2. Gambierol inhibited the potassium current in a dose-dependent manner (**Figure 2B**). The peak current was plotted against the concentration of gambierol (**Figure 3A**), and the IC₅₀ value was determined to be $0.75 \pm$

0.15 nM (the mean $\pm$ S.E.M., $n = 3$). The heptacyclic and tetracyclic analogs of gambierol (**Figure 1**)⁵⁴ were administered to the CHO cells expressing hK_v1.2, which displayed inhibitory action in a manner similar to that of gambierol (**Figures 2C and 2D**). The IC₅₀ values of the heptacyclic and tetracyclic analogs were 7.6 ± 1.2 nM (the mean $\pm$ S.E.M., $n=3$) and 28 ± 4.0 nM (the mean $\pm$ S.E.M., $n = 3$), respectively (**Figure 3A**). The inhibitory actions of gambierol and its analogs were in the order of gambierol > the heptacyclic analog > the tetracyclic analog, which did not coincide with the previous reports in which cerebellar neuron was selected in the electrophysiological experiments.⁵⁵ Pérez et al. showed that not only the inhibitory action of the heptacyclic analog against the potassium current in the cerebellar neuronal cells, and its cytotoxicity was the strongest among the three compounds. We analyzed the effect of gambierol and its analogs by adding designated concentrations (from lower to higher) to the same cells (**Figure 2**). According to Pérez et al., however, this administration method allows accumulation of compounds in the cells and brings a loss of function to the potassium channels, and the IC₅₀ values of gambierol, the heptacyclic analog, and the tetracyclic analog for inhibition of the total potassium current were markedly increased to 630 nM, 190 nM, 480 nM, respectively.⁵⁵ The distinctive difference in the IC₅₀ values obtained in this study disclosed that gambierol binds with high affinity to hK_v1.2, which might not express dominantly in the cerebellar neuron.

We questioned whether the inhibition of K_v1.2 is linked to cytotoxicity. We investigated the cytotoxicity of gambierol and the two analogs against CHO cells. The IC₅₀ values for gambierol, the heptacyclic analog, and the tetracyclic analog in the CHO cells stably expressing hK_v1.2 were 78 ± 5.8 μ M, 6.0 ± 1.0 μ M (the mean $\pm$ S.E.M., $n = 3$), and >100 μ M ($n = 3$) (**Figure 4A**), and those in the wild-type CHO cells were 95 ± 7.1 μ M, 6.5 ± 0.8 μ M (the mean $\pm$ S.E.M., $n = 3$), and >100 μ M ($n = 3$), respectively (**Figure 4B**). Similar results were obtained when HEK293T cells were used. The IC₅₀ values for gambierol, the heptacyclic analog, and the tetracyclic analog in the HEK293T

cells stably-expressing hK_v1.2 were $41 \pm 6.3 \mu\text{M}$, $2.2 \pm 0.9 \mu\text{M}$, and $47 \pm 7.2 \mu\text{M}$ (the mean $\pm$ S.E.M., $n = 3$) and those in the wild-type HEK293T cells were $55 \pm 9.6 \mu\text{M}$, $0.97 \pm 0.5 \mu\text{M}$, and $65 \pm 8.2 \mu\text{M}$ (the mean $\pm$ S.E.M., $n = 3$), respectively (data not shown). The heptacyclic analog displayed the smallest IC₅₀ values in the two different cell lines, which was compatible with the observation of Pérez et al.⁵⁵ In addition, the IC₅₀ values from the hK_v1.2-expressing cell line were comparable to those from the wild-type CHO cell line. The results postulated that the cytotoxicity is not triggered by inhibition of the hK_v1.2; it is triggered by other mechanisms, which are described as follows.

When the whole cell patch clamp recording was conducted using gambierol and its simplified analogs, we noticed the leak current in the CHO cells administered with gambierol and the two analogs. We then examined the effect of the leak current at the resting potential on the cytotoxic activity. **Fig. 3B** displays that gambierol, the heptacyclic analog and the tetracyclic analog induced the leak current in the wild-type CHO cells in a dose-dependent manner, and the heptacyclic analog clearly produced the largest leak current. Structural similarity between the marine polycyclic ethers are characterized by the polycyclic ether backbone contributing to Van der Waals bonding and the spatial orientation of ether oxygen contributing to hydrogen bonding with receptors.^{57, 58} The cytotoxicity of gambierol and the other two analogs could be derived from such intrinsic nature of these molecules. The ciguatoxin derivatives, P-CTX1 and CTX3C, also stimulated the leak current at the resting potential.^{59, 60} When HEK293 cells expressing Na_v1.4 or ND7-23 cells expressing Na_v1.8 were used for patch clamp recording, CTX3C distinctively activated the inward current through the TTX-sensitive sodium channel at the resting potential.⁶⁰ We propose that gambierol-activated and CTX3C-activated leak currents originate from a common mode of action shared by the polycyclic ether compounds.

Although the origin of the leak current could not be specified from this study, we

suspected that gambierol binds only to the hK_v1.2 in reference to the mode of action of maitotoxin. Maitotoxin, the largest polycyclic ether compound, is composed of a hydrophilic lower half and hydrophobic upper half and gains stability in the lipid bilayers.^{57, 61} Maitotoxin stimulates the nonselective cation influx, and one of the target molecules is the plasmalemmal Ca²⁺-ATPase (PMCA) pump.³⁷ Because maitotoxin exhibits cytotoxicity against most of the cells tested so far, the results did not rule out the presence of other receptors of maitotoxin. The amphiphilicity of maitotoxin might permit maitotoxin to bind to multiple membrane proteins and induce structural modification, which leads to the expression of cytotoxicity. Another explanation for the gambierol-induced leak current would be the pore formation by self-assembly. Matile et al. reported that brevetoxin molecules constitutes an oligomer and increases the ion permeability through the lipid bilayers.^{62, 63} Moran and Ilani conducted electrophysiological recording and found that prymnesin increases membrane conductance.⁴¹ Gambierol may stimulate the leak current, which is independent of the inhibition of hK_v1.2.

The results from the cytotoxicity test questioned the reasons that the heptacyclic analog displayed the strongest cytotoxicity among the tested compounds (**Figure 4**). The key to solving this intriguing phenomenon might be found in a previous study conducted by Torikai et al.⁵⁸ They demonstrated the synthesis of polycyclic ether models from monocyclic to dodecacyclic and an analysis of their interaction with the transmembrane region of the glycophorin A (GpA). The GpA dimer was dissociated in the presence of the polyether models, and the heptacyclic model bearing the two benzyl groups in the edge of the molecule (ALP7B) exhibited higher efficacy than the heptacyclic model without the benzyl groups (ALP7A). It was hypothesized the length of the hydrophobic region of ALP7B (ca. 25 Å) matches that of the low dielectric constant region of membrane, although the length of the hydrophobic region of ALP7A is not long enough. The scenario might fit the working hypothesis for explaining the

strongest cytotoxicity exhibited by the heptacyclic analog in this study. Our heptacyclic analog does not possess an aromatic group; however, the triene side chain is at the right terminus of the molecule. The functional group might provide sufficient rigidity to the side chain to help the molecule stretch in the hydrophobic environments of the lipid bilayers and reach the boundary region between the lipid bilayer phase and the external aqueous phase. Reduction of the double bond from the H-ring or the trienyl side-chain markedly reduced the acute toxicity of gambierol in mice.^{44, 45} We assume that our heptacyclic analog displayed a similar mode of action for the artificial heptacyclic polyether model described in the literature⁵⁸ and emphasize the importance of rigidity in the right end of the structure. Alternatively, the π -bond in the right end of the structure would be indispensable for interaction with the target protein.

Overall, we examined gambierol and its heptacyclic and tetracyclic analogs regarding whether to inhibit hK_v1.2 stably expressed in CHO cells; we revealed that gambierol is the most potent inhibitor of hK_v1.2 and that the tetracyclic analog is the smallest analog that mimics the gambierol-dependent biological activity. We demonstrated that the inhibitory activity of gambierol and its analogs against hK_v1.2 is independent of their cytotoxic activity. We thus proposed that there are multiple pathways for gambierol-dependent biological activity. The electrophysiological recordings using a planar lipid bilayer system to verify the source of the leak current induced by gambierol or using various K_v1.2 mutants to deduce molecular determinants other than T427 in Segment 6⁵² would help us to elucidate the mode of action of gambierol in the future.

Acknowledgments

The research studies were funded by the ERATO Murata Lipid Active Structure Project, Japan Science and Technology Agency, a Grant-in-Aid for Scientific Research on Innovative Areas 'Chemical Biology of Natural Products' from the Ministry of

Education,⁶⁴ Culture, Sports, Science and Technology, Japan (M. S., and K. K.), and the JSPS for Funding Program for the Next Generation World-Leading Researchers (LS012) (M. Y. Y.).

We thank Takayuki Oka and Masato Nishio of Bio Research Center Co. Ltd. and Atsushi Otsuki of Nanion Technologies GmbH for providing technical support for the electrophysiological recording with Port-A-Patch®. We thank RIKEN Bioresource Center through the National Bio-Resource Project of the MEXT, Japan, for providing the human embryonic kidney cell line 293T (RCB2202) and the Chinese hamster ovary cell line CHO-K1 (RCB0285).

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Figures and legends

Figure 1. Structures of gambierol and its analogs, including the heptacyclic analog and the tetracyclic analog.

Figure 2. Western blotting analysis of CHO cells stably expressing human K_v1.2 channel and inhibition of human K_v1.2 channel by gambierol, the heptacyclic analog, and the tetracyclic analog. (A) The cloned CHO cells transfected with human K_v1.2 cDNA were subjected to western blotting analysis using a monoclonal antibody against the c-Myc tag. Lane M represents the protein marker. Lane 1 represents the untransfected CHO cells, and Lanes 2 and 3 represent the CHO cells stably expressing human K_v1.2. (b-d) The whole cell patch clamp recording was conducted using the CHO cells stably expressing human K_v1.2. The potassium current evoked by a 200 ms step pulse from −80 mV to +40 mV was recorded in the presence of (B) gambierol (0.1, 0.3, 1, 3, 10, and 30 nM) and (C) the heptacyclic analog (0.1, 0.3, 1, 3, 10, 30, and 100 nM), and (D) the tetracyclic analog (1, 3, 10, 30, 100, 300, and 1000 nM).

Figure 3. Inhibition of human K_v1.2 channel and induction of leak current in the wild-type CHO cells by gambierol and its analogs. (A) The potassium current at the end of a 200-ms step pulse from −80 mV to +40 mV observed in **Figure 2** was normalized using potassium current in the absence of the compound: gambierol (closed circles), the heptacyclic analog (closed squares), or the tetracyclic analog (closed triangles). (B) Leak current at the resting potential of −80 mV was plotted against the concentration of gambierol (closed circles), the heptacyclic analog (closed squares), or the tetracyclic analog (closed triangles).

Figure 4. Growth inhibition of (A) CHO cells stably expressing human K_v1.2 and (B) wild-type CHO cells in the presence of gambierol, the heptacyclic analog, and the tetracyclic analog. After an aliquot of the cell suspension (50,000 cells/200 μL) was incubated with gambierol (closed circles), the heptacyclic analog (closed squares), or the tetracyclic analog (closed triangles) at the designated concentration for 72 h at 37 °C and 5% CO₂/air atmosphere. The relative cell viability was measured using WST-8 and plotted against the concentration of the compound.

Figure 1

Gambierol

Heptacyclic analog

Tetracyclic analog

Figure 2

Figure 3

Figure 4

***Graphical Abstract**

	Inhibition of human K _v 1.2	Cytotoxic activity
$0.75 \pm 0.15 \text{ nM}$	$78 \pm 5.8 \text{ }\mu\text{M}$
$7.6 \pm 1.2 \text{ nM}$	$6.0 \pm 1.0 \text{ }\mu\text{M}$
$28 \pm 4.0 \text{ nM}$	$> 100 \text{ }\mu\text{M}$