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Evaluation of gambierol and its analogs for their inhibition of human $K_v1.2$ and 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 IC_{50} values for the three compounds were 0.75 ± 0.15 nM, 7.6 ± 1.2 nM, and 28 ± 4.0 nM (the mean ± SEM, n = 3), respectively. The cytotoxic activity was examined in order to assess a relationship between cytotoxicity and inhibition of the $hK_v1.2$. The IC_{50} values for gambierol, the heptacyclic analog, and the tetracyclic analog in the wild-type CHO cells were 95 ± 7.1 μM, 6.5 ± 0.8 μM (the mean ± SEM, n = 3), and >100 μM (n = 3), respectively, whereas those in the CHO cells stably expressing $hK_v1.2$ were 78 ± 5.8 μM, 6.0 ± 1.0 μM (the mean ± SEM, n = 3), and >100 μM (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 IP_3 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 prymnesin[41] and gymnosin[42] have not been performed.

Gambierol (Fig. 1) was isolated from the dinoflagellate *Gambierdiscus toxicus*.[43] It shows strong acute toxicity in mice (LD_{50} 50 μg/kg, ip), and the neurological symptoms observed in the mice administered with ganbierol 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

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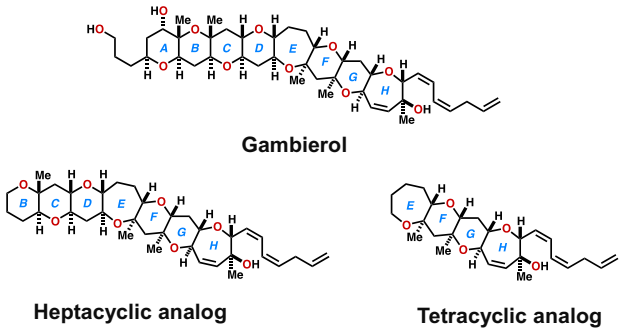

Figure 1. Structures of gambierol and its analogs, including the heptacyclic analog and the tetracyclic analog.

synthetic material. It was reported that gambierol inhibited voltage-gated potassium currents in mouse taste cells[49] 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 study[54] (Fig. 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 2- to 3-fold when the $hK_v1.2$ was overexpressed. Because the contribution of the endogenous potassium current was not negligible, we then prepared CHO cells stably expressing hKv1.2, and the construction of each clone was confirmed by western blotting (Fig. 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 (Fig. 2B). The peak current was plotted against the concentration of gambierol (Fig. 3A), and the IC_{50} value was determined to be 0.75 ± 0.15 nM (the mean ± SEM, $n = 3$). The heptacyclic and tetracyclic analogs of gambierol (Fig. 1)[54] were administered to the CHO cells expressing $hK_v1.2$, which displayed inhibitory action in a manner similar to that of gambierol (Fig. 2C and D). The IC_{50} values of the heptacyclic and tetracyclic analogs were 7.6 ± 1.2 nM (the mean ± SEM, $n = 3$) and 28 ± 4.0 nM (the mean ± SEM, $n = 3$), respectively (Fig. 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 (Fig. 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 IC_{50} 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 IC_{50} 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 $hK_v1.2$ is linked to cytotoxicity. We investigated the cytotoxicity of gambierol and the two analogs against CHO cells. The IC_{50} values for gambierol, the heptacyclic analog, and the tetracyclic analog in the CHO cells stably expressing $hK_v1.2$ were 78 ± 5.8 μM, 6.0 ± 1.0 μM (the mean ± SEM, $n = 3$), and >100 μM ($n = 3$) (Fig. 4A), and those in the wild-type CHO cells were 95 ± 7.1 μM, 6.5 ± 0.8 μM (the mean ± SEM, $n = 3$), and >100 μM ($n = 3$), respectively (Fig. 4B). Similar results were obtained when HEK293T cells were used. The IC_{50} values for gambierol, the heptacyclic analog, and the tetracyclic analog in the HEK293T cells stably-expressing $hK_v1.2$ were 41 ± 6.3 μM, 2.2 ± 0.9 μM, and 47 ± 7.2 μM (the mean ± SEM, $n = 3$) and those in the wild-type HEK293T cells were 55 ± 9.6 μM, 0.97 ± 0.5 μM, and 65 ± 8.2 μM (the mean ± SEM, $n = 3$), respectively (data not shown). The heptacyclic analog displayed the smallest IC_{50} values in the two different cell lines, which was compatible with the observation of Pérez et al.[55] In addition, the IC_{50} values from the $hK_v1.2$-expressing cell line were comparable to those from the wild-type 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