Toxicon 119 (2016) 72–76

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Short communication

The voltage-gated sodium ion channel inhibitory activities of a new tetrodotoxin analogue, 4,4a-anhydrotetrodotoxin, and three other analogues evaluated by colorimetric cell-based assay

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ARTICLE INFO

Article history:
Received 29 February 2016
Received in revised form
14 May 2016
Accepted 18 May 2016
Available online 19 May 2016

Keywords:
Tetrodotoxin
Voltage-gated sodium ion channel
Neuro-2a
11-Oxotetrodotoxin
4,4a-anhydrotetrodotoxin
Maleimide

ABSTRACT

The voltage-gated sodium ion channel inhibitory activities of four tetrodotoxin analogues were evaluated for their ability to reduce the cytotoxicity of ouabain and veratridine in mouse neuroblastoma Neuro-2a cells. EC_{50} of the novel analogue, 4,4a-anhydrotetrodotoxin purified from pufferfish, was 750 fold larger than that of tetrodotoxin, supporting the implication of 4-OH in activity. The high activity of 11-oxotetrodotoxin was confirmed. Modification of C-6 of 11-nortetrodotoxin-6,6-diol to form an oxime derivative decreased the activity to 1/22.

Tetrodotoxin (TTX, **1**, Fig. 1A) (Goto et al., 1965; Tsuda et al., 1964; Woodward, 1964) is a potent and specific voltage-gated sodium ion channel (Na_v) blocker (Narahashi et al., 1960, 1964; Kao, 1966; Kao and Levinson, 1986; Catterall, 2000), that causes fatal pufferfish poisoning (Yasumoto et al., 1986; Yasumoto and Yotsu-Yamashita, 1996; Yotsu-Yamashita, 2001; Noguchi and Arakawa, 2008). Recently, TTX attracted particular attention as low concentrations of TTX were detected in European shellfish (Turner et al., 2015; Vlamis et al., 2015). The TTX molecule contains a guanidinium group and six hydroxyl groups including a hemilactal hydroxyl group as functional groups. Ogura and Mori (1968) examined local anesthetic action of many TTX derivatives and reported all their activities to be less than TTX. The recent structure-activity relationship of TTX was studied by us and by Nishikawa's group (Yotsu-Yamashita et al., 1999, 2003; Jang and Yotsu-Yamashita, 2007; Kudo et al., 2014; Satake et al., 2014), while binding models of TTX to Na_v were reported by Lipkind and Fozzard (1994) and by Tikhonov and Zhorov (2005). These studies revealed that the guanidinium group and the six hydroxyl groups at C-4, C-6, C-8, C-9, C-10 and C-11 are all involved in the Na_v blocking activity of TTX. However, the following points required clarification. Firstly, the contribution of 4-OH to inhibition activity was evaluated by comparing the activity of 4-*epi*TTX with that of TTX (Yotsu-Yamashita et al., 1999). However, 4-*epi*TTX is easily converted to TTX as these forms are in chemical equilibrium. In this paper, we isolated a novel analogue, 4,4a-anhydroTTX (**2**) (Fig. 1A) from pufferfish, suitable to evaluate the effect of 4-OH on inhibitory activity. Secondly, the affinity of 11-oxoTTX (**3**) to rat brain synaptosomes was almost the same as that of TTX (Yotsu-Yamashita et al., 1999), but the minimum lethal dose to mice (i.p.) was reported as 120 μg/kg (Khora and Yasumoto, 1989), much larger than that of TTX (TTX LD_{50}, mice, i.p. 10 μg/kg, Kao and Fuhrman, 1963). Here, we re-examined the activity of 11-oxoTTX by another assay method. In addition, we also evaluated the potencies of synthetic analogues, 11-norTTX-6,6-diol (**4**) and its C6-ketone modified derivative with a hydroxy amine compound with maleimide moiety (compound **5**, Fig. 1A). Compound **5** was prepared as a possible probe to detect Na_v, similar to saxitoxin derivatives used for PET-MR imaging (Hoehne et al., 2013). Inhibition of Na_v by these compounds was evaluated by reduction of the cytotoxicity of ouabain and veratridine in mouse neuroblastoma Neuro-2a cells (Kogure et al., 1988; Manger et al., 1993, 1995; Yasumoto et al., 1995; Yotsu-Yamashita et al., 2003). According to Lou et al. (2005), $Na_v1.7$ is robustly expressed in Neuro-2a cells, and

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http://dx.doi.org/10.1016/j.toxicon.2016.05.012
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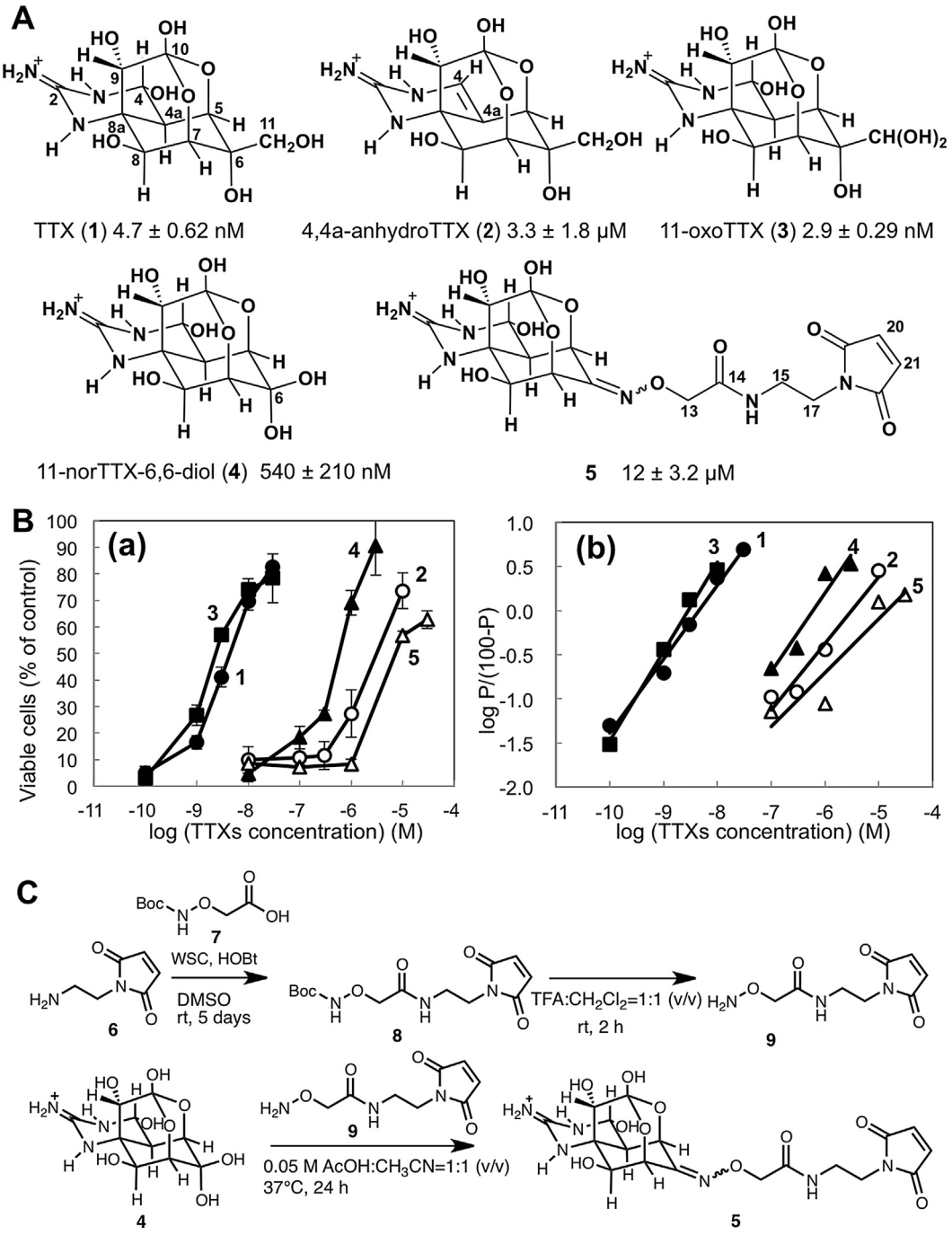

Fig. 1. The structures and synthesis of TTX analogues tested in this study and their Na_v inhibitory activities. **A**. EC_{50}s (mean ± SD) (n = 3 for all except **4** (n = 4)) obtained from the results shown in **B**. **1**, **2**, and **3** were purified from pufferfish, and **4** and **5** are synthetic analogues. **B**. Effects of **1**–**5** on cell viability in the presence of ouabain (100 μM) and veratridine (55 μM) on mouse neuroblastoma Neuro-2a cells. (**a**) Cell viability vs log TTXs concentration. Data points are set at means ± SD. (**b**) Hill plot of log *P*/(100-*P*) (*P* = viable cells, % of control) vs log TTXs concentration. Data shown are representative of three or four replicates. Symbols: TTX (**1**) (●); 4,4a-anhydroTTX (**2**) (○); 11-oxoTTX (**3**) (■); 11-norTTX-6,6-diol (**4**) (▲); compound **5** (△). **C**. The scheme of synthesis of **5**.

$Na_v1.2$, $Na_v1.3$ and $Na_v1.4$ are also detected.

TTX (**1**) and 4,4a-anhydroTTX (**2**) were isolated from a frozen stock of an activated charcoal treated extract of *Takifugu poecilonotus* ovary containing **1** and **2** (5:1, mol/mol). They were further purified by column chromatography on a Hitachi gel 3013C column (4.0 × 150 mm) with 10 mM ammonium formate buffer (pH 6.1) and a TSK gel Amide-80 column (4.6 × 250 mm) with 32 mM ammonium formate buffer (pH 3.1)-CH_3CN (15:85, v/v). Elution was monitored by liquid chromatography-fluorescence detection (LC-FLD) for TTXs (Yasumoto and Michishita, 1985). The retention times of **1**, **2**, 4-*epi*TTX, and 4,9-anhydroTTX were 19.4, 21.2, 23.6, and 26.4 min, respectively. Compound **2** (76 μg by LC-FLD), obtained as a colorless amorphous solid, also contained small amount of 6-deoxyTTX (2.3%, w/w) (Kudo et al., 2014) estimated by LC-MS. HR-ESI-MS: *m/z* 302.0992 $[M + H]^+$ (calcd. for $C_{11}H_{16}N_3O_7$ 302.0983). ^1H NMR (600 MHz, CD_3COOD–D_2O (4:96, v/v)) suggested that the ratio of hemilactal form (**2a**) and 10,7-lactone form (**2b**) was approximately 2:3 (mol/mol). Based on the COSY, HSQC