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Benzenepolycarboxylic acids with potential anti-hemorrhagic properties and structure–activity relationships

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ABSTRACT

Previously, we reported the structural requirements of the cinnamic acid relatives for inhibition of snake venom hemorrhagic action. In the present study, we examined the effect of benzenepolycarboxylic acids and substituted benzoic acids against *Protobothrops flavoviridis* venom-induced hemorrhage. Pyromellitic acid (1,2,4,5-benzenetetracarboxylic acid) was found to be a potent inhibitor of hemorrhage, with an IC_{50} value of 0.035 μM. In addition, most of the antihemorrhagic activity of compounds tested in this experiment showed good correlation to acidity.

1. Introduction

Globally snakebites affect the lives of 4.5 million people annually. Conservative estimates suggest that at least 100,000 people die from snakebites, and another 250,000 are permanently disabled.[1] Envenomation from snakebites is an important public health hazard in many regions, particularly in tropical and subtropical areas.[2] The high fatality ratio of snakebites in tropical developing countries is the result of a combination of factors, including the scarcity of anti-venom, poor health services, and poor transportation from rural areas to health centers.[3] There are two main types of snake venoms, neurotoxins, which attack the central nervous system, and hemotoxins, which target the circulatory system. Snake venoms are usually complex mixtures of proteins including hemorrhagic metalloproteases, phospholipase A_2, myotoxins, and other proteolytic enzymes, cytotoxins, and cardiotoxins. Snake envenomation causes pathophysiological changes such as inflammation, increased body temperature, hemorrhage, necrosis, nephrotoxicity, cardiotoxicity, hemostatic changes and ultimately death.[4] Envenomation due to snakebite is commonly treated by parenteral administration of horse or sheep-derived polyclonal anti-venom that neutralizes toxins.[5] Although anti-serum is the only available medical antidote against snakebite, it does not provide enough protection against specific venom-induced symptoms, and it often produces adverse hypersensitivity reactions.[6–9] It is therefore important to search for other compounds which can effectively neutralize the hemorrhagic and other harmful activities of snake venoms, especially crotalid and viperid venoms.

We previously reported the inhibitory activities of cinnamic acid relatives against *Protobothrops flavoviridis* (Habu) venom-induced hemorrhage and clarified the structural requirements for this activity.[10] The structural features necessary for high potency snake venom inhibition included the presence of an *E*-enoic acid moiety in cinnamic acid relatives. Moreover, aliphatic acids, crotonic acid (IC_{50} 0.22 μM), sorbic acid (IC_{50} 0.21 μM) and *trans*,-*trans*-muconic acid (IC_{50} 0.11 μM), were also comparable to caffeic acid. This evidence suggests the phenyl group is not necessary for activity, but the enoic acid is. To better explore the structural features of CH=CH–COOH group compounds and to investigate the most potent snake venom anti-hemorrhage inhibitor, benzoic acid, benzenepolycarboxylic acids and substituted benzoic acids are discussed. Furthermore, the activity and the acidity relationships were examined to obtain information about their mechanism of actions.

2. Results and discussion

2.1. Anti-hemorrhagic activity of benzoic acid

The anti-hemorrhagic activity of benzoic acid (**1**), the smallest compound possessing a benzene ring and the enoic acid moiety in a molecule, was investigated. As a result, benzoic acid (**1**) (IC_{50} 0.20 μM) showed almost the same activity as caffeic acid (**2**)

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(IC_{50} 0.19 μM). Based on this result, it is estimated that benzoic acid analogues can also exhibit the same anti-hemorrhagic activity as compounds with an *E*-enoic acid moiety.

2.2. Anti-hemorrhagic activity of benzenepolycarboxylic acids

First, we tested three benzenedicarboxylic acids, phthalic acid (**3**), isophthalic acid (**4**), and terephthalic acid (**5**). As shown in Table 1, anti-hemorrhage potencies of these compounds were very similar and two-fold higher than those of benzoic acid (**1**) and caffeic acid (**2**). The inhibitory activity did not depend on the positions of carboxyl groups. Then the activities of two benzenetricarboxylic acids, trimesic acid (1,3,5-benzenetricarboxylic acid) (**6**) and trimellitic acid (1,2,4-benzenetricarboxylic acid) (**7**), were tested. Their IC_{50} values were almost the same and much smaller, respectively, than those of benzenedicarboxylic acids (**3**–**5**). This evidence demonstrated that the number of carboxyl groups on the benzene ring greatly influenced anti-hemorrhagic activity. Other benzenepolycarboxylic acids, pyromellitic acid (1,2,4,5-benzenetetracarboxylic acid) (**8**), benzenepentacarboxylic acid (**9**), and mellitic acid (benzenehexacarboxylic acid) (**10**), were revealed to have IC_{50} values of 0.035, 0.043, and 0.043 μM, respectively. The potency of the tetracarboxylic acid (**8**) was higher than those of tricarboxylic acids, as expected. But lower potency was observed for the penta- and hexacarboxylic acids (**9** and **10**) than for tetracarboxylic acid (**8**). The exact cause has not been confirmed yet, but it is estimated that the carboxyl groups caused steric hindrance to bind the active site of the snake venom (metalloproteinase) or the intramolecular hydrogen bonding among the carboxyl groups, though the mechanism of action of these compounds has not been fully understood.

2.3. Anti-hemorrhagic activity of benzenepolycarboxylic acids and acidities

As mentioned in the previous section, for the compounds with one to four carboxyl groups on the benzene ring, an increase in the number of carboxyl groups caused an increase of activity. It is also known that the acidity (pK_a) of benzenepolycarboxylic acid also increases according to the number of carboxyl groups. So we investigated the correlation between the activity and acidity of benzenepolycarboxylic acid. Figure 1 shows the correlation between IC_{50} values of benzenepolycarboxylic acids and their reported acidities (pK_a).[11] The effects of benzenepolycarboxylic acids on anti-hemorrhagic activity seem to be related to the acidity of their compounds, because more acidic compounds are more effective than less acidic compounds other than benzenepentacarboxylic acid (**9**) and mellitic acid (**10**).

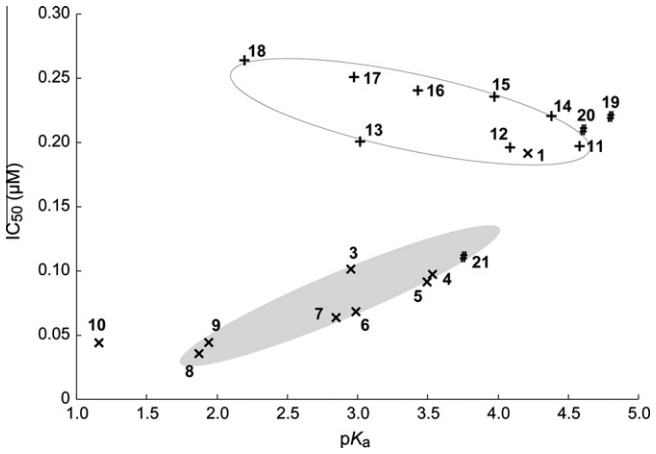
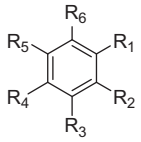


Figure 1. Correlation between acidity (pK_a) and anti-hemorrhagic activity (IC_{50}) of benzenepolycarboxylic acids and substituted benzoic acids. x, benzenepolycarboxylic acids; +, substituted benzoic acids; #, aliphatic carboxylic acids (**19**, crotonic acid; **20**, sorbic acid; **21**, *trans*,*trans*-mucoic acid), ● region of benzenepolycarboxylic acid; ○, region of substituted benzoic acids

2.4. Effect of a substituent on the benzene ring of benzoic acid to anti-hemorrhagic activity

To better understand the correlation between acidity and inhibitory activity, benzoic acids with electron-withdrawing groups and electron-releasing groups were investigated. Electron-withdrawing groups like Cl and NO_2 increase the acidity, while electron-releasing groups like OH, NH_2 and CH_3 decrease the acidity. The inhibitory activity of substituted benzoic acids along with

Table 1
Anti-hemorrhagic activity of benzenepolycarboxylic acids



Entry	pK_a	R_1	R_2	R_3	R_4	R_5	R_6	IC_{50} (μM)
Caffeic acid (**2**)	4.58	CH=CH–COOH	H	OH	OH	H	H	0.19
Benzoic acid (**1**)	4.20	COOH	H	H	H	H	H	0.20
Phthalic acid (**3**)	2.95	COOH	COOH	H	H	H	H	0.10
Isophthalic acid (**4**)	3.53	COOH	H	COOH	H	H	H	0.10
Terephthalic acid (**5**)	3.49	COOH	H	H	COOH	H	H	0.090
Trimesic acid (**6**)	2.98	COOH	H	COOH	H	COOH	H	0.067
Trimellitic acid (**7**)	2.84	COOH	COOH	H	COOH	H	H	0.062
Pyromellitic acid (**8**)	1.87	COOH	COOH	H	COOH	COOH	H	0.035
Benzenepentacarboxylic acid (**9**)	1.93	COOH	COOH	COOH	COOH	COOH	H	0.043
Mellitic acid (**10**)	1.15	COOH	COOH	COOH	COOH	COOH	COOH	0.043
4-Hydroxybenzoic acid (**11**)	4.57	COOH	H	H	OH	H	H	0.20
3-Hydroxybenzoic acid (**12**)	4.08	COOH	H	OH	H	H	H	0.20
2-Hydroxybenzoic acid (**13**)	3.01	COOH	OH	H	H	H	H	0.20
p-Toluic acid (**14**)	4.37	COOH	H	H	CH_3	H		0.22
4-Chlorobenzoic acid (**15**)	3.97	COOH	H	H	Cl	H		0.24
4-Nitrobenzoic acid (**16**)	3.42	COOH	H	H	NO_2	H		0.24
2-Chlorobenzoic acid (**17**)	2.97	COOH	Cl	H	H	H		0.25
2-Nitrobenzoic acid (**18**)	2.19	COOH	NO_2	H	H	H		0.26

their acidity are shown in Table 1. As a result, the activity was hardly correlated to acidity, but was with benzoic acid.

3. Conclusion

In this study, the inhibitory activity against snake venom-induced hemorrhage of benzenepolycarboxylic acids and substituted benzoic acids was tested, and the correlation between activity and acidity was investigated. Among the compounds tested in the study, pyromellitic acid (**8**) (0.035 μM) was the most effective, demonstrating that the four carboxyl groups on the benzene nucleus are important for inhibitory activity. Hemorrhages by snake venoms are principally caused by Zn^{2+}-dependent metalloproteinase enzymes that are responsible for degrading proteins of the extracellular matrix.[12] The inhibition of hemorrhagic activity induced by snake venom suggests an interaction between extract components or bioactive compounds and metalloproteinases, involving catalytic sites of these enzymes or essential metal ions, and thus, neutralizing their effects.[12–18] Many synthetic inhibitors of metalloproteinases are reported to act through mechanisms based on metal chelation, which is necessary for catalysis.[19,20] As for benzenepolycarboxylic acids, the carboxyl groups are expected to bind or chelate the metal ion and exhibit the inhibitory activity as the compound stated above, though the contribution to inhibition is still unclear. Alam and Gomes reported that 2-hydroxy-4-methoxybenzoic acid, isolated and purified from the methanolic root extract of *Hemidesmus indicus* neutralized the inflammation induced by *Vipera russelli* venom.[21] This pure compound potentiated the neutralization of the lethal effect of venom by commercial equine polyvalent antiserum in experimental models.[22] This is the first report of benzenepolycarboxylic acids with potential antihemorrhagic properties and their structure-activity relationship. The strong antihemorrhagic compounds described in this paper will lead to drugs that prevent hemorrhage induced by *P. flavoviridis* venom.

4. Experimental

4.1. Venoms and chemicals

P. flavoviridis venom (Okinawa) was purchased from Japan Snake Institute, Gunma. All other chemicals used herein were purchased from TCI, Tokyo, Japan.

4.2. Anti-hemorrhagic activity assay

Anti-hemorrhagic activity was examined under the modified method reported previously.[10,17] Male ddY mice of 20 g average weight were used for this experiment. Test solutions were prepared as follows. Crude snake venom solution of *P. flavoviridis* (0.14 mg/mL in saline, 50 μL) and sample solution in 10% dimethylsulfoxide (DMSO)–saline, 50 μL) were mixed and incubated at 37 °C for 10 min. The test solution (100 μL) was injected subcutaneously into the abdomen of the mice. Mice injected with the vehicle solution only served as a control group. After 24 h, mice were euthanized by inhalation of chloroform, the skin covering the abdomen was removed, and hemorrhagic lesions were determined as follows. We evaluated the lesions by calculating a value of major axes x minor axes, just as that of an ellipse, since the shapes of the lesions are always amorphous.

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