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Inhibition of Sanggenon G Isolated from *Morus alba* on the Metastasis of Cancer Cell

CUI Long[1*], LEE Hyun-sun[2], OH Won-keun[2], AHN Jong-seog[2]

1. College of Pharmacy, Beihua University, Jilin 132013, China
2. Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea

Abstract: **Objective** An organic layer prepared from the cortex of *Morus alba* (Moraceae) was studied in order to identify the active compounds for heparinase. **Methods** Bioassay-guided fractionation resulted in the isolation of sanggenon G. **Results** The compound showed inhibitory activity with IC_{50} of 3.7 μmol/L on heparinase *in vitro* as well as 24 μmol/L in invasion assay using MDA-MB231 cells. Sanggenon G also had the moderate cytotoxicity at SW 620 (colon) and ACHN (kidney) cancer cell lines with IC_{50} of 10.96 and 13.44 μmol/L, respectively. **Conclusion** This is the first time that prenylated flavonoid sanggenon G is described as heparinase inhibitor. Besides, this flavonoid would be expected to be a metastasis inhibitor of cancer cells and also a valuable reagent to explore the mechanism of heparinase/heparanase-mediated metastasis.

Key words: cytotoxicity; heparinase/heparanase; Moraceae; *Morus alba*; sanggenon G

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Introduction

Heparin and heparin sulfate (HS) are highly sulfate polysaccharides consisting of alternated uronic acid and *N*-acetyl-*D*-glucosamine residues. Although HS has a lower degree of sulfation and more glucuronic acid residues than heparin, it has been recognized that both biopolymers have similar biological functions (Gallagher, Lyon, and Steward, 1986). Cancer cells require the ability to degrade the extracellular matrix (ECM) in order to turn into invasive and metastatic cancer cells (Simizu, Ishida, and Osada, 2004). Heparanase, endo-beta glucuronidase that degrades the glycosamino-glycan HS on ECM, is associated with the metastatic potential of tumor cells (Gingis-Velitski *et al*, 2004). Interestingly, the pretreatment of the cells with heparinase which degrades heparin was also reported as a large reduction of basic fibroblast growth factor (bFGF) binding to its low-affinity receptors (Yang, Yanagishita, and Rechler, 1996). PI-88, known as a heparinase/heparanase inhibitor, is currently developed as an anticoagulant and antiproliferative agent (Demir *et al*, 2001). Thus, it was thought that heparinase which can also degrade HS, might play an important role as a regulator of various cellular functions including tumor cells (Bobek and Kovarik, 2004).

In the course of screening for heparinase inhibitors from medicinal plants, an ethyl acetate-soluble extract of *Morus alba* L. was found to have inhibitory activity. Further purification of the extract as bioactivity-guided fractionation afforded an active compound, sanggenon G. The extract of *M. alba* was reported to have a cancer cell apoptosis through inhibiting microtubule assembly (Nam *et al*, 2002), and some flavonoids with isoprenoid groups isolated from *Morus* sp. were reported to have cytotoxicity (Shi *et al*, 2001; Ni *et al*, 2010). However, to our knowledge, the inhibition of sanggenon G on the metastasis of cancer cells including heparinase inhibitory effect has never been described previously. In this paper, the cytotoxicity on several cancer cell lines, the inhibitory activity on heparinase, and anti-metastasis effect of sanggenon G isolated from *M. alba* were reported.

Materials and methods

Materials

* Corresponding author: Cui L Tel: +86-432-6460 8821 E-mail: jlcl1978@yahoo.com.cn
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Heparinase and heparin diagnostics kit (CRS106-A) were purchased from Sigma Co., fatal bovine serum (FBS) from GIBCO BRL, and Dulbecco's modified Eagle's medium (DMEM) from Biowhittaker Co. ^{1}H-NMR and ^{13}C-NMR spectra were measured on Bruker AMX—500 NMR. LC—10AT liquid chromatography, SPD—10A UV detector, and CBM—10A controller (Shimadzu Scientific Co.) were used for HPLC.

Isolation of sanggenon G

The whole sample of *Morus alba* L. was purchased from the Herbal Medicine Association of Taejon, Korea and was identified by Prof. BAE Ki-wan, College of Pharmacy, Chungnam National University (Voucher specimen, KRIBB 1132, has been deposited in the herbarium of the College of Pharmacy, Chungnam National University, Korea). The dried cortex of *M. alba* (3 kg) was extracted with MeOH (6 L) for 24 h. The EtOAc fraction (55 g) was chromatographed over silica gel (70－230 mesh) using a gradient of CH_2Cl_2-MeOH (10:1→3:1) to yield seven fractions (Fr. 1－Fr. 7). For further fractionation of active compounds, the Fr. 5 with the heparinase inhibitory activity (76% inhibition at 20 μg/mL) was rechromatographed on a RP-C_{18} column with a gradient of MeOH-H_2O (60:40→90:10). The active fractions eluted with MeOH-H_2O (60:40) were pooled, and the fractions eluted with CH_3CN-H_2O (73:27) on RP-C_{18} HPLC column (250 mm × 10 mm, 10 μm) was identified as the active principle (235 mg). The structure of active compound was identified as sanggenon G by analyses of MS, ^{1}H-NMR, ^{13}C-NMR data and by comparison of previously reported literature (Fukai *et al*, 1983).

Inhibitory activity against heparinase

The assay was carried out using the Sigma Diagnostics kit for the quantitative determination of heparinase in human plasma. The assay buffer (14 mmol/L NaOAc, 1.4 mmol/L $CaCl_2$, pH 7.0) containing 10 ng of porcine intestinal HP was loaded onto each well of 96-well microplate. The sample solution to be tested was added to the mixture, followed by 10 μL (0.2 Unit) of heparinase I from *Flavobacterium heparinum* (EC 4.2.2.7) solution. After 15 min of incubation, 25 μL of human antithrombin III was added to the mixture, and the mixture was incubated for 3 min to result in formation of an ATIII/HP complex. After 50 μL of factor Xa was added in reaction mixture, the reaction was terminated by adding 25 μL of glacial acetate, and then the absorbance was detected on spectrophotometer at 405 nm.

Cell culture

ACHN (kidney), LOX-IMVI (skin), SW 620 (colon), PC-3 (prostate), MDA-MB231 (breast), and NC-H23 (lung) cell lines were grown in RPMI-1640 medium supplemented with 10% FBS in 5% CO_2 incubator.

Invasion assay using MDA-MB231 cell line

The modified boyden chamber (Costar, Cambridge, USA) equipped with a 6.5 mm diameter membrane (pore size 8 μm) was used for invasion assay as previously described method (Kim *et al*, 2002). Matrigel solution (20 μg/membrane) was allowed to upper plate for coating and then rehydrated with a rehydration solution. After 0.75 mL of DMEM containing chemo attractant (10% FBS + 5 μg collagen type 1) was added to the plate well, 0.5 mL of cell suspension (1×10^5 cells) was added to upper layer. The plates with and without the compound from *M. alba* were incubated for 48 h, and non-invasive cells were removed with a cotton swap. The cells which migrated through the membrane were fixed and stained with hematoxylin and eosin. Cancer cells attached on the lower surface of membrane were counted under microscope.

Cytotoxicity assay

In vitro cytotoxicity against several cancer cell lines was tested by the Development Therapeutic Program of National Cancer Institute, USA in order to evaluate the drug selectivity for particular tumor types (Monks *et al*, 1991). Cells in exponential growth were trypsinized, dispersed in single suspension, dispensed in 100 μL volume into 96-well plates and grown overnight. After assay sample was added into each well as five different concentrations, the cells were further incubated for 2 d. Cytotoxicity was measured by the sulforhodamine B (SRB) method and IC_{50} value was calculated using Probits method (Skehan *et al*, 1990).

Results

Bioassay-guided fractionation on the EtOAc fraction of *M. alba* led to the isolation of active compound (Fig. 1). The structure of the isolated compound

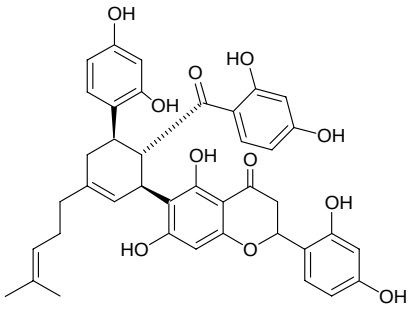

Fig. 1 Chemical structure of sanggenon G

was identified by physical and spectroscopic data measurement and by comparison with published values (Fukai *et al*, 1983).

Sanggenon G inhibited heparinase activity in a dose-dependent manner with IC_{50} value of 3.7 μmol/L. CRM-646A (IC_{50} = 3.0 μmol/L) known as heparinase inhibitor (Ko *et al*, 2000) and suramin (IC_{50} = 5.0 μmol/L) as potent inhibitor of melanoma heparanase (Vignon, Prebois, and Rochefort, 1992) were used as positive controls in this assay. When we examined whether the effect of sanggenon G was attributed by inhibition of any other components in the assay system or not, treated compound had no effect on the assay system (data not shown). To investigate the ability of the compound to inhibit invasive and metastatic activities of cancer cells, MDA-MB231 breast cancer cell line was treated with sanggenon G at different concentrations, and the number of cancer cells migrated through the membrane was determined. As expectation from functions of heparinase/heparanase in metastasis of cancer cell, sanggenon G inhibited invasion of cancer cell with IC_{50} value of 24 μmol/L (Fig. 2). In addition, when we tested the cytotoxicity of sanggenon G against several human cancer cell lines, i.e., LOX-IMVI, SW 620, ACHN, PC-3, and NC-H23, sanggenon G had moderate cytotoxicity against several cancer cell lines, and especially it had more potent activity in SW 620 (10.96 μmol/L) and ACHN (13.44 μmol/L) cancer cell lines (Table 1).

Discussion

Cancer metastasis is a malignant progression to distant organs by cancer cells. In these processes, the invasive cancer cells must attach on microvessel endothelial cells of other tissues, and degrade to basement membranes and ECM for invasion into other organs/tissues.

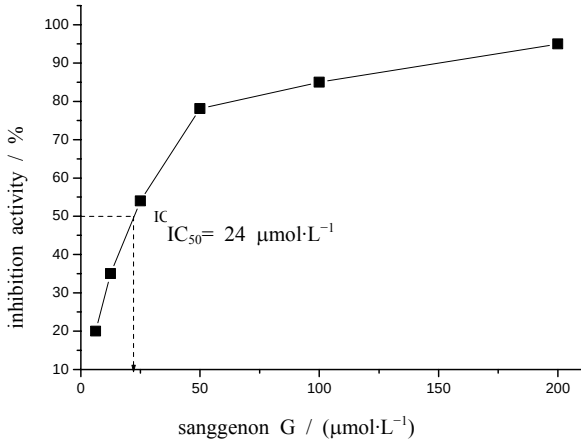

Fig. 2 Inhibitory activity of sanggenon G against MDA-MB231 cell invasion in matrigel

Table 1 Cytotoxicity of sanggenon G against several human tumor cell lines (*n* = 3)

Cancer cell lines	GI_{50} value / (μmol·L^{-1})
LOX-IMVI	> 20
SW 620	10.96
ACHN	13.44
PC-3	> 20
NC-H23	> 20

GI_{50} values represent inhibition concentration of 50% cell growth by sanggenon G

Expression levels of heparanase degraded HS on ECM have been known as association with the invasive, angiogenic, and metastatic potentials of diverse malignant cancer cell lines. However, usage of heparanase from human cancer cell lines is unpractical for analyzing a large number of samples because these assay systems were done basically radioactivity labeling with ^{35}S and chromatographic analysis of the HS molecular size (Rozenberg *et al*, 2001).

It is also reported that the treatment of heparin (unfractionated heparin, UFH), low molecular weight heparin (LMWH) and wafarin significantly reduced metastasis of primary or solid tumors (Drago, Weed, and Fralisch 1984). Furthermore, these heparin and heparin derivatives are known as more effective in inhibition stages of the metastatic cascade than in the influence on the primary tumors (Bobek and Kovarik, 2004). These evidences suggested that heparinase inhibitors could be used as potential agents for the treatment of cancer metastasis.

As part of our ongoing study to search for natural heparinase inhibitors from plants, the stem barks of *M.*

alba were chosen for detail investigation, since the MeOH extract was found to inhibit heparinase at 30 μg/mL. Bioassay-guided fractionation of the MeOH extract of the plant resulted in the isolation of prenylated flavonoid, sanggenon G as the active principle. Sanggenon G showed inhibitory activity against heparinase *in vitro*, and against invasion of MDA-MB231 breast cancer cells. In addition, sanggenon G had the moderate cytotoxicity at SW 620 and ACHN cancer cell lines with IC_{50} of 10.96 and 13.44 μmol/L, respectively.

Prenylated flavonoids including sanggenon G have been reported to possess a wide range of pharmacological activities that include anti-inflammatory, antiviral, antimicrobial, antitumor and antiangiogenic effects (Shi *et al*, 2001; Talla *et al*, 2003; Kim, *et al*, 2003). However, this is the first time that sanggenon G is described as a heparinase inhibitor. Because heparinase plays an important role in anticoagulation and cell proliferation, this flavonoid would be expected to be a metastasis inhibitor of cancer cells and also a valuable reagent to explore the mechanism of heparinase/heparanase-mediated metastasis.

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