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Full Length Research Paper

Inhibitory activity of a water-soluble morin derivative on phosphatase of regenerating liver-3

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Morin sulphate, a novel water-soluble derivative was semi-synthesized from morin as a parent molecule. The aim of the current study was to examine the inhibitory activity of morin sulphate on the phosphatase of regenerating liver-3 (PRL-3), and its role in the metastatic potency of high metastatic ovarian carcinoma cell line (HO-8910PM) *in vitro*. Morin sulphate significantly inhibited PRL-3 activity in a dose-dependent manner with an IC_{50} value of 1.06 µM. Furthermore, morin sulphate inhibited the invasion and migration of HO-8910PM cells, suggesting that it is a PRL-3 inhibitor.

Key words: Protein tyrosine phosphatases, PRL-3 protein, enzyme inhibitors, morin hydrate, neoplasms, metastasis.

INTRODUCTION

Reversible phosphorylation of proteins on tyrosine residues is an important mechanism, by which cells regulate vital signaling pathways that control almost all aspects of cell physiology, including proliferation, differentiation, apoptosis, adhesion, invasion, and migration. The proper balance between tyrosine phosphorylation and dephosphorylation is maintained by a large family of protein tyrosine phosphatases and protein tyrosine kinases. The phosphatase of regenerating liver-3 (PRL-3), also known as PTP4A3, is a member of the small protein tyrosine phosphatases. The PRL-3 is consistently over expressed in colorectal cancer metastases versus the corresponding primary tumors and normal colorectal epithelium, and PRL-3 expression is generally correlated with colorectal carcinoma tumor progression (Saha et al., 2001). In addition, PRL-3 expression positively correlates with the progression of squamous cell carcinoma (Ooki et al., 2010), other tumor types, including gastric tumors, esophageal nasopharyngeal carcinoma (Zhou et al., 2009), melanomas (Kim et al., 2009), ovarian tumors, lung cancer (Ming et al., 2009), and breast carcinoma. A cause and effect relationship between PRL-3 and tumor metastasis has also been demonstrated by several recent observations *in vivo* or *in vitro* model systems (Zeng et al., 2003; Matsukawa et al., 2010). These observations strongly support the idea that PRL-3 may play an important role in tumor progression, particularly in the metastatic process. Therefore, PRL-3 is a tractable target for anticancer therapeutics, and regulating its expression or activity may be a new strategy to prevent or treat tumor metastasis (Al-Aidaroos and Zeng, 2010). Thus far, very few studies implicating small-molecule inhibitor against PRL-3 have been conducted. Moon et al. (2010) reported that two anthraquinone compounds from *Rubia akane* show inhibitory activity on PRL-3 in a dose-dependent manner, with IC_{50} values of 5.2 and 1.3 µg/ml, respectively. Choi et al. (2006) found that two biflavonoids; ginkgetin and sciadopitysin, isolated from the young branches of *Taxus*

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cuspidata, inhibit PRL-3 with IC_{50} values of 25.8 and 46.2 μM, respectively. Another study has reported that a rhodanine derivative can serve as a PRL-3 inhibitor with an IC_{50} value of 0.9 μM *in vitro*, showing a decreased invasion in cell-based assay (Ahn et al., 2006). In a trial to screen small molecule inhibitors from Chinese traditional and herbal drugs, morin sulphate, a novel water-soluble morin derivative, obviously inhibited PRL-3 activity and metastatic potency. The current paper describes the synthesis, structural characterization, and PRL-3 inhibitory activity of the morin derivative.

MATERIALS AND METHODS

Materials

Morin and fibronectin were purchased from Sigma, USA. Calf serum and RPMI-1640 were purchased from Invitrogen, Co. Transwell chamber (6.5 mm diameter, 8 μm pore size polyvinyl pyrrolidone-free membrane, PVPF) was obtained from Corning, Inc. Matrigel was obtained from BD Biosciences. The solvents chloroform, sulphuric acid, and methanol were of analytical reagent grade. The human highly metastatic ovarian carcinoma HO-8910PM cell line was grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2.

Synthesis, purification, and structure characterization of the desired morin derivative

We followed a representative procedure for the preparation of morin sulphate. First, 1 g morin was added into 4 ml sulphuric acid in a 50 ml glass beaker placed on an ice bath, and stirred for 2 h until complete sulfonation reaction was achieved. The mixture solution was then neutralized with 6 M sodium hydroxide, to which MeOH was added in order to dissolve the product at 4°C. After stirring gently for 3 h, the solution was centrifuged at 3,000 ×*g* for 15 min to remove precipitate containing mainly sodium sulphate, after which the supernatant fraction was collected. Methanol was evaporated in a rotary evaporator to obtain a yellow powder residue. The crude extract was applied to a Sephadex LH-20 column (50 × 1 cm i.d.). The column was respectively eluted with 30% methanol (v/v, Fraction 1), 50% methanol (v/v, Fraction 2), and 80% methanol (v/v, Fraction 3). Based on TLC ($CHCl_3$:MeOH = 3:1, v/v), the fraction 2 was further chromatographed by a series of Sephadex LH-20 columns, eluted with the eluents mentioned earlier, concentrated *in vacuo* (38°C), and then freeze-dried to yield a yellow powder with an approximate weight of 55 mg. The structure of morin sulphate was characterized by ^1H and ^{13}C NMR (Varian 500NB NMR spectrometry, USA). Mass spectra were obtained with Agilent 6340 ion trap LC/MS spectrometer.

3, 5, 2', 4'-Tetrahydroxy-7-sulphate flavone (morin sulphate) yellow powder (MeOH). ESI-MS: m/z 381 $[M+H]^+$. ^1H NMR (600 MHz, DMSO-d_6, δ ppm) : 12.59 (d, 5-OH), 10.64 (d, 2'-OH), 9.72 (d, 3-OH), 9.63 (s, 4'-OH), 6.19 (d, H_8), 6.29 (d, H_6), 6.36 (dd, $H_{5'}$), 6.43 (d, $H_{3'}$), 7.23 (d, $H_{6'}$). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm) : 148.9 (C_2), 136.1 (C_3), 176.1 (C_4), 163.6 (C_5), 102.9 (C_6), 160.9 (C_7), 93.3 (C_8), 156.8 (C_9), 109.2 ($C_{1'}$), 156.7 ($C_{2'}$), 98.0 ($C_{3'}$), 160.4 ($C_{4'}$), 106.8 ($C_{5'}$), 131.6 ($C_{6'}$).

Assay method phosphatase of regenerating liver-3

The cDNA molecule encoding PRL-3 was obtained from human highly metastatic ovarian carcinoma HO-8910PM cell line by polymerase chain reaction, and was inserted into the *BamH* I and *Hind* II sites of pCold II expression vector. PRL-3 (consisting of residues 1–168) lacking the C-terminal farnesylation site was expressed in *ArcticExpress Escherichia coli* for the native protein at 15°C after induction with 0.5 mM isopropyl-β-D-thio-galactoside. The cell pellets were resuspended in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5% (v/v) glycerol, and 0.04% (v/v) β-mercaptoethanol. After cell lysis by sonication, the His-tagged protein was purified by nickel affinity chromatography. The His-tag was removed by thrombin digestion, and the PRL-3 protein was further purified by ion exchange chromatography. For the PTPase activity assay (EnzChek Phosphatase Assay Kit, Molecular Probes), 4 μg purified PRL-3 was added to a reaction mixture containing 20 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 0.01% (v/v) Triton X-100, and 4 μM 6,8-difluoro-4-methy-lumbelliferyl phosphate (DiFMUP). The total reaction volume was 200 μl. The results were determined using a fluorimeter at an excitation/emission wavelength of 355/460 nm. The inhibition kinetics with morin sulphate was measured by adding increasing concentrations of morin sulphate ranging from 200 nM to 20 μM to the DiFMUP PTPase activity assay.

Cell cytotoxicity assay

The HO-8910PM cells were subcultured into a 96-well plate at 1 × 10^4 cells per well. Varying concentrations of morin sulphate diluted in the culture medium were added. Triplicate wells were used for each determination. The plates were incubated at 37°C in 5% CO_2 for 6 or 24 h when the control cells reached 90% confluence. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole (MTT) solution, was then added to each well after which the plates were incubated for another 4 h. The plates were read on enzyme-linked immunosorbent assay reader at 570 nm. The IC_{50} was defined as the concentration of the morin sulphate required to reduce the optical density by 50% in the treated cells to that of the control.

Cell migration assay

Cell migration assay was performed using the Transwell chamber system. The under surface of the PVPF membrane was coated with 5 μg Fibronectin. The 1 × 10^5 cells in the 0.5 ml serum-free medium containing 0.1% bovine serum albumin (BSA) were placed in the upper chamber. The lower chamber was filled with 0.6 ml RPMI-1640 culture medium with 0.1% BSA. After the treatments with 10, 20, and 40 μM morin sulphate for 6 h, the cells on the upper surface of the membrane were removed using cotton tips. The migrated cells attached to the under surface were fixed in methanol for 1 min, stained for 3 min with hematoxylin, washed with water, and then stained for another 3 s with eosin. The number of migrated cells on the lower surface of the membrane was counted under a microscope in 5 fields of 100-fold magnification. Triplicate samples were conducted, and data were expressed as the average cell number of 15 fields. The inhibitory rates were calculated according to the formula given by:

$$\text{Inhibitory rate}\,(\%) = (1 - \frac{\text{the mean of treated group}}{\text{the mean of control group}}) \times 100\%$$

Wound-healing assay

For the wound-healing assay, HO-8910PM cells (2 × 10^5 cells per well) were seeded in 6-well plates, and were allowed to grow to

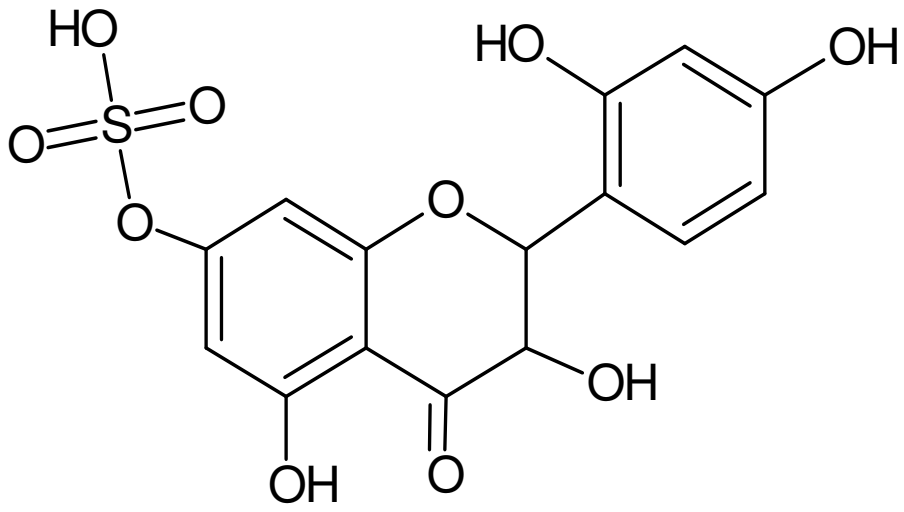

Figure 1. Structure of 3,5, 2',4'-tetrahydroxy-7-sulphate flavone.

complete confluence. A plastic pipette tip was used to scratch the cell monolayer to create a cleared area, and the wounded HO-8910PM cell layer was washed with fresh medium to remove loose cells. The cells were then re-fed with fresh medium containing 0.5% FBS, and then treated with 10, 20, and 40 µM morin sulphate for 24 h. Immediately following the scratch wounding (0 h), and after incubation of the cells at 37°C for 24 h, the phase contrast images of the wound healing process were digitally photographed with an inverted microscope (Olympus IX50). The experiments were repeated at least three times. The cells treated with phosphate buffered saline (PBS) served as the negative control.

Cell invasion assay

The Matrigel invasion ability of the cells was assayed using a Transwell chamber. The upper surface of the membrane was coated with 5 µg Matrigel; afterwards, the under surface was coated with 5 µg Fibronectin and then air-dried overnight. The next steps were performed according to the cell migration assay.

Statistical analysis

All data were expressed as means ± standard deviation of three independent determinations. One-way analysis of variance (ANOVA) was used, and the differences were considered significant at $P < 0.05$. All statistical analyses were performed with SPSS 13.0 for Windows.

RESULTS AND DISCUSSION

In the search for anticancer agents from active constituents of Chinese traditional and herbal drugs, morin has been discovered as a lead compound. Morin (3, 5, 7, 2', 4'-pentahydroxyflavone) is a flavonoid found in dietary fig and other *Moraceae* used as herbal medicines. Morin has wide-ranging biological activities, including antioxidant (Kim et al., 2010), anti-inflammatory, anti-angiogenic, and antinociceptive activities. It has anti-proliferative and antitumor effects in several human cancers (Manna et al., 2007). Structure-function experiments have demonstrated that its tumor specificity absolutely requires the 2', 4' hydroxyl configuration in the B ring as active sites. Hydroxylation at the 3, 5, or 7 positions of the A ring in morin is not required for its tumor specificity (Brown et al., 2003). However, morin is barely absorbed by rats, because it merely passes through the gastrointestinal tract to be degraded by the intestinal microflora. Therefore, our group synthesized a novel water-soluble derivative named 3, 5, 2', 4'-tetrahydroxy-7-sulphate flavone (Figure 1). As shown in the ^{1}H and ^{13}C NMR spectrum, the hydrogen atom on 7-hydroxyl was substituted with a sulphate group without destroying its 2', 4' hydroxyls, and morin was successfully sulfonated at 7-OH in A ring.

In the continuous screening of PRL-3, small molecule inhibitors from Chinese traditional and herbal drugs, morin exhibited moderate inhibitory effect on the PRL-3 activity of other flavonoids (He et al., 2010). In addition, morin sulphate exerted remarkable inhibition of the PRL-3 activity compared with its parent morin due to its water-solubility. The inhibitory effect of morin sulphate on the PRL-3 enzyme was assayed in the presence of various concentrations of morin sulphate (0, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 µM), and we found that morin sulphate inhibited the PRL-3 enzyme in a concentration-dependent manner (Table 1). The equation of linear regression was obtained using the logarithm of morin sulphate concentration and the probit of the inhibitory rate of corresponding morin sulphate concentration as the horizontal and vertical coordinates, respectively. The equation showed that $Y = 2.710\ X - 0.072$, r^2 was 0.985; in addition, IC_{50} was 1.06 µM according to the semi-effect-probit method.

To rule out the possibility that the inhibition of tumor cell metastatic potency *in vitro* was due to morin sulphate-induced cytotoxicity, the cell cytotoxicity of morin sulphate

Table 1. Inhibitory effect of morin sulphate on PRL-3.

Group	Value	Inhibitory rate	
		%	Probit
PBS (control)	-	0	-
0.2 µM morin sulphate	-0.70	6.6±0.6	0.07
0.4 µM morin sulphate	-0.40	20.3±1.3*	0.17
0.8 µM morin sulphate	-0.10	36.4±0.2*	0.40
1.6 µM morin sulphate	0.20	68.2±1.6*	0.70
3.2 µM morin sulphate	0.51	94.2±0.8*	0.91
6.4 µM morin sulphate	0.81	94.4±0.2*	0.98

Data are means ± SD (n=3). *P<0.05 *vs.* control group.

Table 2. Inhibitory effect of morin sulphate on migration ability of HO-8910PM cells.

Group	Cell count per field	Inhibitory rate (%)
PBS (control)	48.1±2.7	0
10 µM morin sulphate	34.4±3.3*	28.5±6.9*
20 µM morin sulphate	29.9±4.0*	37.8±8.3*
40 µM morin sulphate	21.2±3.4*	55.9±7.0*

Data are means ± SD (n=3). *P<0.05 *vs.* control group.

on the HO-8910PM cell line was investigated by MTT assay. The result shows that morin sulphate had low cytotoxicity against the HO-8910PM cell line at high concentrations compared with the control group. After the 6 and 24 h treatments, the IC_{50} values of morin sulphate were 183.29±4.31 and 105.32±3.84 µM, respectively. Up to 10, 20, and 40 µM morin sulphate were chosen which were much lower than IC_{50}, as the experimental concentrations in the further migration, invasion, and wound-healing assay. In the cell migration assay, treatment with 10, 20, and 40 µM morin sulphate resulted in a significant reduction of the migrated cell number (Table 2, Figure 2a and b), the inhibitory rates were 28.5±6.9, 37.8±8.3 and 55.9±7.0%, respectively. The inhibitory effect of morin sulphate on migration was confirmed by in wound-healing assays. Morin sulphate exerted an inhibitory effect on the closure of the wounded HO-8910PM cell monolayer compared with the control group (Figure 3). Morin sulphate also inhibited the invasive ability of the HO-8910PM cells. After the 6 h treatment of the cells with 10, 20, and 40 µM of morin sulphate, the invasion ability of HO-8910PM was significantly different from that of the control group (Table 3, Figure 2c and d), the inhibitory rates were 19.0±3.9, 33.5±8.1 and 49.1±8.9%, respectively.

Tumor cell migration, invasion, and adhesion are crucial steps in the metastatic cascade of cancer cells. The interruption of these steps is a potential strategy in the prevention and treatment of tumor metastasis. The phosphatase of regenerating liver-3, which belongs to the family of protein tyrosine phosphatases has gained attention as a crucial molecule in the multiple steps of metastasis. Zeng et al. (2003) showed that PRL-3 promotes cell motility, invasion activity and metastasis, which are directly dependent on its catalytic activity. These findings suggest that PRL-3 is a therapeutic target for metastatic tumors. Our current study demonstrated that morin sulphate inhibited PRL-3 enzyme activity and malignant tumor cell metastasis-associated behavior *in vitro*. This was achieved most likely by blocking these essential steps of tumor metastasis, including the invasion and migration of the HO-8910PM cell line. Further study on the anti-metastasis of the compound *in vivo* is in progress. The results shall be reported in due course.

Conclusion

We have successfully synthesized a novel water-soluble morin derivative, morin sulphate. The compound significantly inhibits PRL-3 activity and the metastatic potency of the tumor cell, suggesting that it is a PRL-3 inhibitor.

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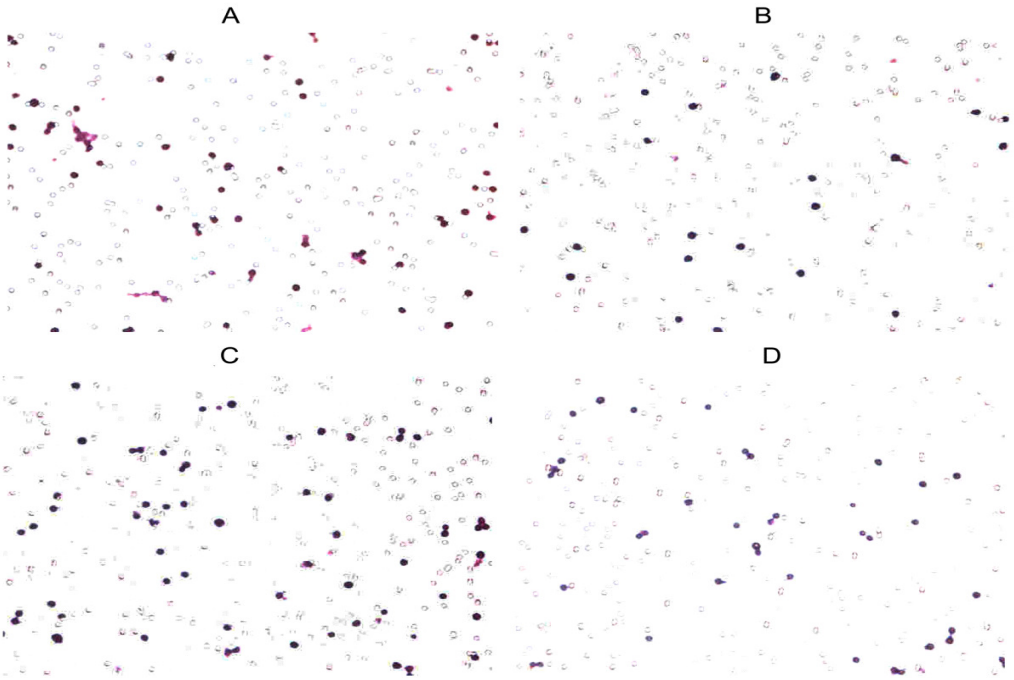

Figure 2. HO-8910PM cells passed through PVPF membrane after treatment with morin sulphate for 6 h; (A) Control group in migration test (PBS), (B) 40 µM morin sulphate treatment group in migration test, (C) Control group in invasion test (PBS); (D) 40 µM morin sulphate treatment group in invasion test. Results were representative of 3 independent experiments.

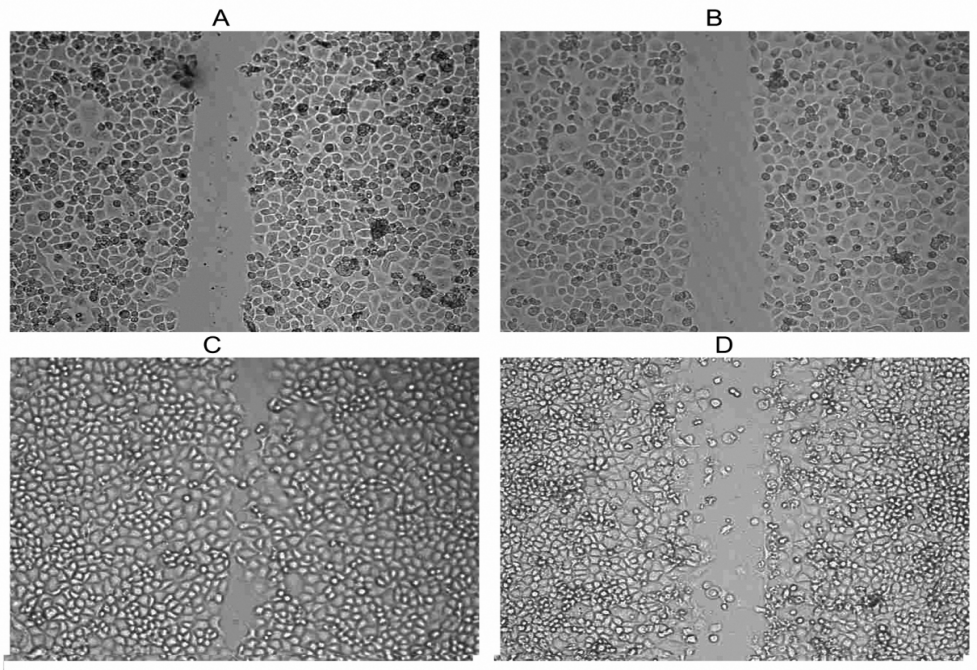

Figure 3. Morin sulphate inhibited HO-8910PM cell migration in wound-healing assay. HO-8910PM cells were seeded in a 6-well plate, streaks were made using a tip when the cells were grown to almost confluence. Streaks were photographed at 0 h or 24 h later at 100 ×. This showed that morin sulphate can prevent HO-8910PM cell wound healing; (A) Control group at 0 h (PBS), (B) 40 µM morin sulphate treatment group at 0 h, (C) Control group at 24 h (PBS), (D) 40 µM morin sulphate treatment group at 24 h. Results were representative of 3 independent experiments.

Table 3. Inhibitory effect of morin sulphate on invasion ability of HO-8910PM cells.

Group	Cell count per field	Inhibitory rate (%)
PBS (control)	59.4±6.5	0
10 μM morin sulphate	48.1±2.3	19.0±3.9
20 μM morin sulphate	39.5±4.8*	33.5±8.1*
40 μM morin sulphate	30.2±5.3*	49.1±8.9*

Data are means ± SD (n=3). *P<0.05 *vs*. control group.

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