Antiprotease and Antimetastatic Activity of Ursolic Acid Isolated from *Salvia officinalis*

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Z. Naturforsch. **61 c**, 777–782 (2006); received December 2, 2005/February 3, 2006

Proteases play a regulatory role in a variety of pathologies including cancer, pancreatitis, thromboembolic disorders, viral infections and many others. One of the possible strategies how to combat with these pathologies seems to be the use of low molecular inhibitors. Natural products were evaluated in the *in vitro* antiprotease assay on serine proteases (trypsin, thrombin and urokinase) and on the cysteine protease cathepsin B. We found interesting results for β-ursolic acid isolated from *Salvia officinalis,* which significantly inhibited all tested proteases *in vitro* in the micromolar range. β-Ursolic acid showed the strongest inhibition activity to urokinase ($IC_{50} = 12\ \mu M$) and cathepsin B ($IC_{50} = 10\ \mu M$) as proteases included in tumour invasion and metastasis indicated possible anticancer effectivity. Therefore, we tested the ability of β-ursolic acid at doses of 50, 75 and 100 mg/kg given i.p. to inhibit lung colonization of B16 mouse melanoma cells *in vivo*. We found, that β-ursolic acid significantly decreased the number of B16 colonies in the lungs of mice at the dose 50 mg/kg ($p < 0.05$).

Key words: Inhibitors, Proteases, *Salvia officinalis*

Introduction

Salvia officinalis L. is an old medicinal plant widely used in traditional folk medicine (Lu and Foo, 2002; Slameňová *et al.*, 2004). *Salvia* species (Lamiaceae) are generally known for their various pharmacological effects including anti-inflammatory (Baricevic *et al.*, 2001), antioxidative (Wang *et al.*, 1999), antibacterial (Mašterová *et al.*, 1996) and hepatoprotective effects (Amin and Hamza, 2005). Various organic compounds of various biological activities were isolated from plants belonging to the genus *Salvia* L. like flavonoids (Lu and Foo, 2000), phenolic acids (Wang *et al.*, 1999), royleanones (Mašterová *et al.*, 1996), apianane terpenoids (Miura *et al.*, 2001), pentacyclic triterpenes (Mašterová *et al.*, 1989). A number of triterpenoids were shown to act as promising antineoplastic agents (Setzer and Setzer, 2003). Ursolic acid (Fig. 1) effectively inhibits angiogenesis, invasion of tumour cells and metastasis; however the mechanism of activity is poorly understood (Ovesná *et al.*, 2004).

One of the possibilities of cancer treatment is to suppress the activity of proteases (mainly urokinase, cathepsins, and metalloproteinases) that play an important role in tumour invasion and metastasis (Jedinák and Maliar, 2005). Therefore, proteases present an attractive target in therapy and pharmaceutical research (Tossi *et al.*, 2000; Maliar *et al.*, 2004). A very perspective strategy how to control proteases seems to be the development of selective low-molecular weight inhibitors from natural sources with possible lower toxicity. The selectivity of small-molecule inhibitors toward a protein or enzyme target is often of crucial importance in the development of therapeutically useful molecules (Mackman *et al.*, 2001). Thus inhibition of protease activity by selective low-molecular weight inhibitors represents a promising strategy for anticancer and antimetastatic therapy.

In this paper we studied the antiprotease activity of 16 natural products isolated from various plants on two neoplastic proteases, cathepsin B (cysteine protease) and urokinase (serine protease). We also studied the antiprotease activity of

0939–5075/2006/1100–0777 $ 06.00

natural products to trypsin and thrombin considering the selective effect of tested compounds to urokinase, because the serine proteases have a common catalytic triad and except chymotrypsin also a similar binding site (Hedstrom, 2002).

The strongest antiprotease activity to urokinase and cathepsin B was reported for ursolic acid with the inhibition activity IC_{50} = 12 μM to urokinase and IC_{50} = 10 μM to cathepsin B. Previously, an antiproliferative effect of ursolic acid against B16 melanoma cells (Es-Saady *et al.*, 1996) has been reported. Therefore, we were looking whether ursolic acid is able to suppress the metastasis behaviour of cancer cells *in vivo*. We found, that ursolic acid suppresses the lung colonization of B16 melanoma cells *in vivo.*

Materials and Methods

Compounds

The alkaloids including berberine, berbamine, jatrorrhizine, magnoflorine, tetrahydroberberine, tetrahydrojatrorrhizine, tetrahydropalmatin were previously isolated from *Mahonia aquifolium* (Košťálová *et al.,* 1981, 1986; Slavík *et al.*, 1985); the flavonoids isorhamnetin-3-*O*-β-D-glucopyranoside (isorhamnetin-3-*O*-Glc), isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside (isorhamnetin-3-*O*-Rha-Glc), isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→6)]-*O*-β-D-glucopyranoside (isorhamnetin-3-*O*-Rha-Rha-Glc) were isolated from *Calendula officinalis* L. (Mašterová *et al.*, 1991); patuletin and patulitrin were isolated from *Anthemis tinctoria* L. (Mašterová *et al.*, 1993); eupaline and eupatoline were isolated from *Rudbeckia bicolor* (Bukovský *et al.*, 1994), and the triterpenes β-ursolic acid and β-oleanolic acid were previously isolated from *Salvia officinalis* L. (Mašterová *et al.*, 1989). All tested compounds were isolated at the Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University in Bratislava, Slovakia and were identified by comparison of their ^{1}H, ^{13}C and mass spectra with published data. Purity of the tested compounds used in the present study was found to be higher than 96%.

Chemicals

Quercetin, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and dimethylsulfoxide (DMSO) were purchased from Fluka, Switzerland; Nα-benzoyl-D,L-arginine-4-nitroanilide hydrochloride (BApNA), trypsin from porcine pancreas (2000 BAEE units/mg), glycine-arginine paranitroanilide dihydrochloride (GapNA · 2HCl), urokinase from human kidney cells (10,000 Plough units/mg), Nα-benzoyl-phenylalanyl-valyl-arginine-paranitroanilide (BPVA-pNA), thrombin (150 NIH units/mg), thiobenzyl benzyloxycarbonyl-L-lysinate (Cbz-Lys-SBzl), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and cathepsin B (Cbz-Lys-*p*-nitrophenyl ester units/mg) were purchased from Sigma, St. Louis, USA. For the *in vitro* enzyme assays a photometric microplate reader MRX II (Dynex, Chantilly, Virginia, USA) and Revelation 2.01 software (Dynex) were used.

Cells

B16, a mouse melanoma cell line derived from spontaneous skin tumour in C57B1/6 mouse, was obtained from the Cancer Research Institute, Slovak Academy of Science, Bratislava, Slovakia. The cells were grown in Eagles minimum essential medium (MEM) (BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (BioWhittaker), sodium pyruvate (Koch-Light Laboratories, Haverhill Suffolk, UK), L-glutamine (Sebak, Aidenbach, Germany) antibiotic. The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. B16 cells at a concentration of 0.5×10^6 were plated and grown in a 25 cm^2 flask in 4 ml of complete culture medium.

Protease inhibition assay

Determination of natural products inhibition activity against trypsin, thrombin, urokinase and cathepsin B was done by simple photometry, using chromogenic substrates like BApNA for trypsin, GapNA · 2HCl for urokinase, BPVA-pNA for thrombin, and Cbz-Lys-SBzl in combination with DTNB for cathepsin B. The substrates were cleaved by trypsin, thrombin or urokinase according to the methods described earlier (Erlanger *et al.*, 1961; Nieuwenhuizen *et al.*, 1977) and for cathepsin B (Coleman and Green, 1981; Lottenberg *et al.*, 1981). Released chromogenic products are detectable photometrically at 410 nm. A convenient microplate screening system was used for the determination of protease inhibition activity of the compounds. The assay consisted of preparation of the microplate, start of the enzyme reaction and

microplate measurements and data processing. The microplates were prepared manually using 8-channel pipettes (Dynex, Chantilly, Virginia, USA; Socorex, Ecublens/Lausanne, Switzerland) by gradual dissolution of the substrate-inhibitor mixture. Each well (two parallel wells were used) contained buffer solution with 0.6 mM concentration of substrate with 1% dimethylsulfoxide (DMSO) (v/v) and the chosen concentration of the tested compounds. Plates were started by enzyme solution 0.0015 mg/ml of trypsin – 3 BAEE units, 0.05 mg/ml of urokinase – 500 Plough units, thrombin 150 NIH units/mg in 0.05 M Tris-HCl buffer, pH 7.6, without Ca^{2+} ions and 0.0106 mg/ml of cathepsin B – 0.0555 units Cbz-Lys-paranitrophenyl ester in 0.1 M Sörensen phosphate buffer, pH 5.0. The temperature was set at 36.5 °C, data scanning time was 1 min and 61 min. Measurement data after conversion via text format were sent to a PC. IC_{50} values were calculated for each compound, data processing was supported by statistic evaluation of the control wells (SD, %SD) and by the correlation factor. For each compound presented in this paper, several tests were performed. The average of IC_{50} values was calculated from at least three parallel tests to fulfil the chosen percentage criterions of the standard deviation (%SD) – lower than 10% and the correlation factor of semilogarithmic plot % inhibition = f(log C) better than 0.95.

Animals

Male C57BL/6N mice (5 to 6 weeks old) were obtained from Velaz (Prague, Czech Republic). Mice were randomized into experimental groups ($n = 6$) receiving vehicle or daily i.p. treatments at the doses of 50, 75 and 100 mg/kg. All procedures were in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare.

Lung colonization assay

Mice were injected with $5–7.5 \times 10^4$ tumour cells in 0.1 ml PBS in the lateral tail vein. Treatments were administered i.p. daily, immediately after tumour cell injection during consecutive 16 d. Mice were killed by cervical dislocation 29 d after tumour cell injection. Lungs were fixed in Bouin's solution, and black tumour cell colonies counted.

Statistical analysis

All results are expressed as means ± standard deviation. Statistical evaluation of the data was done using Student's *t*-test.

Results and Discussion

The paper reports an *in vitro* antiprotease activity of 16 natural compounds previously isolated from five medicinal plants, *Mahonia aquifolium*, *Calendula officinalis*, *Anthemis tinctoria*, *Rudbeckia bicolor*, *Salvia officinalis*, which are used in traditional medicine in Slovakia. The antiprotease activities of natural compounds (7 alkaloids, 7 flavonoids and 2 triterpenes) are presented in Table I. The compounds were tested on two neoplastic proteases, cathepsin B and urokinase. We also studied the antiprotease activity of natural products to trypsin and thrombin considering the selective effect of tested compounds to urokinase. From the results a considerable protease inhibition activity of selected compounds and a selective inhibition activity to one of tested proteases are evident. In fact, compounds were tested as perspective building blocks of potential anticancer agents taking into account the size of the molecules. The flavonoid quercetin was used as a standard for all tested proteases (Maliar *et al.*, 2004; Mantle *et al.*, 1999).

The collection of the evaluated compounds includes several chemical groups: alkaloids (berberine, berbamine, jatrorrhizine, magnoflorine, tetrahydroberberine, tetrahydrojatrorrhizine, tetrahydropalmatin), flavonoids (isorhamnetin-3-*O*-β-D-glucopyranoside, isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-*O*-β-D-glucopyranoside, isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→6)]-*O*-β-D-glucopyranoside, patuletin, patulitrin, eupaline, eupatoline), and triterpenes (β-ursolic acid and β-oleanolic acid). The best candidates from the antiprotease efficiency point of view are the triterpenes isolated from *Salvia officinalis* with relatively high efficiency, related to the results of complete collection. The highest inhibition activity to urokinase as a rational drug target for the treatment of cancer and metastasis (Katz *et al.*, 2000) was reported for β-ursolic acid ($IC_{50} = 12\,\mu M$). The flavonoid quercetin used as a standard was little less effective ($IC_{50} = 14\,\mu M$) to urokinase than β-ursolic acid. Moreover, β-ursolic acid showed also high inhibition activity to cathepsin B ($IC_{50} = 10\,\mu M$), which

Table I. Inhibition activities of natural products on proteases expressed as IC_{50} (mM).

Compound	IC_{50} [mM]			
	Trypsin	Thrombin	Urokinase	Cathepsin B
Alkaloids				
Berberine	>1	>1	>1	0.55
Tetrahydroberberine	>1	>1	>1	>1
Magnoflorine	>1	>1	>1	0.08
Tetrahydropalmatin	>1	>1	>1	>1
Berbamine	>1	>1	>1	0.017
Jatrorrhizine	>1	>1	>1	>1
Tetrahydrojatrorrhizine	>1	>1	>1	>1
Flavonoids				
Eupatoline	>1	>1	>1	>1
Eupaline	>1	>1	>1	>1
Patuletin	0.0067	0.06	0.03	>1
Patulitrin	–	0.68	0.23	>1
Isorhamnetin-3-*O*-Glc	>1	>1	0.35	>1
Isorhamnetin-3-*O*-Rha-Glc	>1	>1	0.29	>1
Isorhamnetin-3-*O*-Rha-Rha-Glc	>1	>1	0.15	>1
Triterpenes				
β-Oleanolic acid	0.02	0.05	0.07	0.09
β-Ursolic acid	0.010	0.03	0.012	0.010
Quercetin (standard)	0.015	0.026	0.014	0.011

represents another possible drug target for the suppression of tumor invasion and metastasis (Jedinák and Maliar, 2005). Inhibition activity of β-ursolic acid to cathepsin B was comparable to authentic quercetin (IC_{50} = 11 μM). On the other hand, the highest inhibition activity to the protease trypsin, which is involved in pathogenesis of pancreatitis (Liddle and Nathan, 2004), was reported for the flavonoid patuletin (IC_{50} = 6.7 μM) isolated from *Anthemis tinctoria*. Patuletin showed more than two times higher activity than authentic quercetin (IC_{50} = 15 μM). β-Ursolic acid showed weaker inhibition activity (IC_{50} = 10 μM) than patuletin, but a stronger activity than the standard quercetin. In addition, the natural compounds also showed inhibition activity to thrombin as one of the possible drug targets in the treatment of thromboembolic disorders (Katira *et al.*, 2005). β-Ursolic acid exhibited comparable inhibition activity to thrombin (IC_{50} = 30 μM) as the standard quercetin (IC_{50} = 26 μM).

The effect of β-ursolic acid (Fig. 1) that inhibited *in vitro* cell growth of a large list of cancer cell lines including mouse melanoma B16 cells (Es-Saady *et al.*, 1996) was evaluated *in vivo* at daily i.p. doses of 50, 75, and 100 mg/kg. No mortality and no body weight changes were observed at chosen doses. The most perspective compound that potently inhibited proteases was tested for its ability to inhibit lung colonization. The evaluation was made on the 29th day after tumour cell application. In all controls and treated groups of animals 6 animals/group were evaluated except for dose group 50 mg/kg (n = 5). In spite of a relative smaller number of lung colonies in the control group, the lungs of all animals with the lowest dose (50 mg/kg) were free of colonies. The macroscopic appearance of the lungs from untreated and treated mice clearly showed that ursolic acid at the dose of 50 mg/kg reduced the number of lung metastases after i.v. injection of B16 cells (Fig. 2). We suggest that one of the possible explanations of lower effect at higher doses may be that the

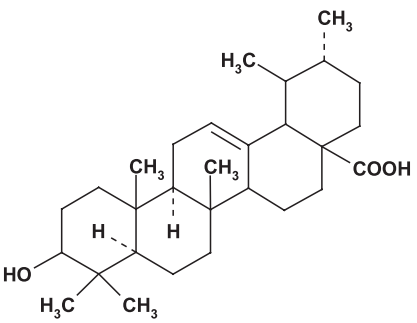

Fig. 1. Chemical structure of β-ursolic acid (3β-hydroxy-urs-12-en-28-oic acid).

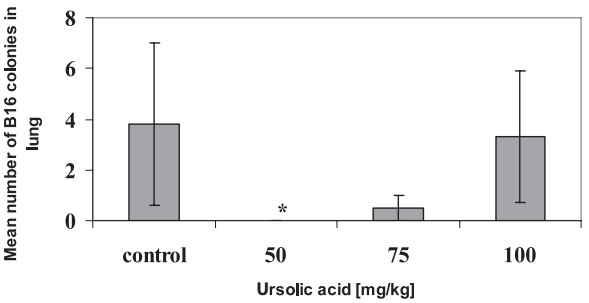

Fig. 2. Inhibition of lung colonization of B16 cells by β-ursolic acid. Colonies were counted 29 days after tumour cells injection. Results are expressed as means of B16 colonies of lung from all treated animals in group. * Level of statistical significance $p < 0.05$.

compound in the context of *in vivo* conditions can have opposite effects at different doses. This may be associated with a wide range of effects of ursolic acid in tumorigenesis, tumor promotion and angiogenesis. However, the mechanism of ursolic acid activity is still poorly understood (Ovesná *et al.*, 2004) and remains largely unknown (Setzer and Setzer, 2003; Hsu *et al*., 2004).

This reported antiprotease and antimetastatic effect of ursolic acid *in vivo* reflects the growing interest in research of triterpenoids as chemotherapy perspectives. The mechanism of ursolic acid action should be further investigated.

Acknowledgements

This work was supported by Slovak Grant Agency for Science VEGA (Grant No. 1/1185/04 and 2/4058/04) and by Slovak Agency for Science and Technology Assistance (Grant No. APVT-99-019402).

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