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Aminopeptidase N inhibition could be involved in the anti-angiogenic effect of dobesilates

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Abstract: Calcium, magnesium and zinc 2,5-dihydroxybenzenesulfonates (dobesilates) were synthesized by sulfonation of hydroquinone with sulfuric acid under mild conditions. To form the salts, neutralization with calcium carbonate followed by cation exchange by means of magnesium or zinc sulfates was performed. The dobesilates were characterized by standard spectral methods and by AAS for metal content and then tested for inhibitory activity against aminopeptidase N. The calcium and magnesium 2,5-dihydroxybenzenesulfonates exhibited rather weak inhibitory activity to aminopeptidase N, as demonstrated by the IC_{50} values of 978.0 and 832.1 μmol L^{-1}, respectively, while zinc 2,5-dihydroxybenzenesulfonate reached a more significant inhibitory activity characterized by an IC_{50} value of 77.4 μmol L^{-1}. The results of the inhibition activity suggest that the inhibition of aminopeptidase N could play a role in the anti-angiogenic activity of 2,5-dihydroxybenzenesulfonates.

Keywords: 2,5-dihydroxybenzenesulfonic acid salts; effect on CD13 activity; neovascularization; carcinogenesis; vasculopathies.

INTRODUCTION

Angiogenesis or the formation of new blood vessels is a process that rarely occurs in healthy adult tissues. It is typical either for growing embryonic tissues or for pathological conditions, such as carcinogenesis or proliferative vasculopathies, including diabetic retinopathy of nephropathy. The formation of new blood vessels or tubes capable of fulfilling their function in neoplasms is crucial for the survival, growth and metastasis of most solid tumors. Consequently, inhibition of angiogenesis is considered a promising approach in cancer treatment, and the design of novel angiogenesis inhibitors is currently underway.

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Angiogenesis is a complex multi-stage process, which is controlled by the balance between angiogenic factors and angiogenesis inhibitors.[1] The main factors that have been demonstrated to participate in angiogenesis regulation are growth factors, such as members of the vascular endothelial growth factor (VEGF) family, fibroblast growth factors (FGF), epidermal growth factors (EGF), platelet-derived growth factors (PDGF) and transforming growth factors (TGF). All these signaling proteins up-regulate angiogenesis. Interferon-gamma--inducible protein 10 (IP-10), which is an antagonist of VEGF, can serve as an example of the body's own angiogenesis inhibitor.[2] Some proteolytic enzymes also support angiogenesis. Among them, at least three aminopeptidases were reported to contribute to neovascularization. Aminopeptidases are ectopeptidases that cleave the N-terminal amino acid from a peptide chain. They are also metalloenzymes, as they are able to bind one or two divalent metal cations, such as Zn^{2+}, Co^{2+} or Mn^{2+}. Additionally, they exhibit broader or narrower substrate specificity. Methionine aminopeptidase type 2, containing a coordinated Co^{2+} and two zinc-aminopeptidases, adipocyte-derived leucine aminopeptidase (A--LAP) and aminopeptidase N (APN) were reported to be involved in angiogenesis control.[1] This paper discusses the effect of dobesilates on APN activity. APN (EC 3.4.11.2) is identical to CD13 antigen, which has been identified as a cell-surface marker for malignant myeloid cells.[3] It was also recognized as a cell entry receptor of some coronaviruses. In these viruses, the envelope spike glycoprotein mediates the attachment of the virus particles to APN and subsequent cell entry, which can be blocked by neutralizing antibodies.[4] It was also reported that bestatin, the most well-known APN inhibitor, stopped the invasion of tumor cells into reconstituted basement membrane.[5,6] Recently, studies on APN-null mice revealed impaired neoangiogenesis in model systems without cancer cells.[7] Moreover, it was demonstrated that a decrease of APN activity in tumor tissue results in a reduction in both the density and size of newly formed blood vessels.[8] VEGF, a key angiogenesis regulator, triggers the expression of APN at an early stage of tumor growth.[9] Similarly, the basic fibroblast growth factor (bFGF) and its downstream Ras-dependent signals, which mediate the formation of the endothelial cells network, induce APN expression in endothelial cells.[10] However, the precise mechanism by which APN regulates angiogenesis remains unclear.

Dobesilates are salts of 2,5-dihydroxybenzenesulfonic acid (**1**) with divalent or monovalent metal cations. They were first reported as early as 1880.[11] Calcium dobesilate (**2**) has been used for decades for the prevention and treatment of diabetic retinopathy,[12] nephropathy, chronic venous insufficiency,[13] hemorrhoids[14] and other related conditions. Potassium dobesilate (**3**) was successfully used for the treatment of actinic keratosis in an open-label clinical study.[15] **3** and magnesium dobesilate (**4**) were reported as the experimental drugs used in several *in vitro* studies.[16,17] Except in an early study of Seyda,[11] zinc dobesilate (**5**)

has only been mentioned in a U.S. patent.[18] Except for **2**, data in the literature concerning spectral and other identification information of dobesilates are poor. The anti-angiogenic activity of dobesilates was demonstrated in a mouse gelatin sponge assay using acidic fibroblast growth factor (aFGF) as an inducer of neovascularization. Here, **2** remarkably reduced vessel ingrowths in FGF-containing subcutaneous sponges in mice.[19] Another study showed that **2** inhibited angiogenesis in both choroidal explants cultured in collagen gels and the choroids of live diabetic rats. This effect was accompanied by inhibition of VEGF production in this tissue.[20] Furthermore, inhibition of neovascularization by dobesilates was discussed several times regarding its effectiveness in the treatment of angiopathies, neoplasms and other diseases in which angiogenesis plays an important role, such as chorioretinopathy,[21] malignant glioma[22] and rosacea.[23]

The aim of this study was to determine whether dobesilates inhibit APN and whether the subsequent APN inhibition plays a role in the antiangiogenic activity of dobesilates.

EXPERIMENTAL

All melting points were determined on a Büchi 535 melting point apparatus (Büchi, Flawil, Switzerland) and are uncorrected. The IR spectra were measured on a Nicolet Impact FTIR spectrometer using the ATR technique. The ^{1}H- and ^{13}C-NMR spectra were recorded on a 200 MHz Gemini 2000 instrument (Varian, Palo Alto, CA, USA) using DMSO-d_6 as the solvent at 200 MHz or 50 MHz, respectively. The UV–Vis spectra were recorded on an Agilent 8453 diode array UV–Vis spectrophotometer (Agilent Technologies, Waldbronn, Germany). The content of metals was determined by atomic absorption spectroscopy (AAS) on a NovAA 350 atomic absorption spectrophotometer (Analytik Jena, Jena, Germany) using the flame technique.

Dobesilates

Hydroquinone (15 g, 0.136 mol) was added portionwise under stirring into 60 mL of 95 % sulfuric acid over 1 h. Stirring was continued until the starting material was completely dissolved. The temperature during this process did not exceed 45 °C. This solution of crude 2,5--dihydroxybenzenesulfonic acid (**1**) in sulfuric acid was poured under stirring into 1500 mL of water and 150 g (1.5 mol) of calcium carbonate was then added portionwise under vigorous stirring. Stirring continued for an additional 15 min at room temperature. Then, the suspension was shortly heated to a boil and immediately filtered by suction filtration. The filtrate containing crude **2** was then divided into three equal volume portions.

*Calcium dobesilate (2).*The first portion was slowly concentrated by evaporation on a vacuum evaporator at a temperature that did not exceed 42 °C. A white precipitate that formed during evaporation was filtered off. The filtrate was evaporated to dryness. The residue was dried under reduced pressure (0.7 kPa) at 40–42 °C over diphosphorus pentaoxide for 7 days. The obtained calcium 2,5-dihydroxybenzenesulfonate (**2**) was identified by ^{1}H-NMR as the pentahydrate.

Magnesium dobesilate (4). Anhydrous magnesium sulfate (4.38 g, 0.0360 mol) was added under stirring to the second portion of the solution of crude **2** at room temperature. The mixture was stirred for an additional 30 min and then heated to boiling for a short period of

time. The formed calcium sulfate precipitate was removed by suction filtration of the hot suspension. The filtrate was slowly concentrated by evaporation on a vacuum evaporator at a temperature that did not exceed 42 °C. The white precipitate that formed during the evaporation was filtered off. The filtrate was evaporated to dryness. The residue was dried under reduced pressure (0.7 kPa) at 40–42 °C over diphosphorus pentaoxide for 7 days. The obtained magnesium 2,5-dihydroxybenzenesulfonate (**4**) was identified by ^{1}H-NMR as the decahydrate.

*Zinc dobesilate (**5**).* Zinc sulfate heptahydrate (6.53 g, 0.0230 mol) was added under stirring to the third portion of the solution of crude **2** at room temperature. The mixture was stirred for an additional 30 min and then heated to boiling for a short period. The formed calcium sulfate precipitate was removed by suction filtration of the hot suspension. The filtrate was slowly concentrated by evaporation on a vacuum evaporator at a temperature that did not exceed 42 °C. The white precipitate that formed during evaporation was filtered off. The filtrate was evaporated to dryness. The residue was dried under reduced pressure (0.7 kPa) at 40–42 °C over diphosphorus pentaoxide and protected from light for 7 days. The obtained zinc dobesilate (**5**) was identified by ^{1}H-NMR as the octahydrate.

Some physical, analytical and spectral data for the examined dobesilates are given in Supplementary material to this paper.

Biochemistry

Enzyme assay. Both L-leucine-*p*-nitroanilide, C.A.S. 4178-93-2, a substrate for APN, and APN, EC 3.4.11.2, were purchased from Sigma, USA. The other employed chemicals were of analytical or biochemical grade. The absorbance values at 405 nm, which is the absorption maximum of 4-nitroaniline, a product of substrate hydrolysis catalyzed by APN, were determined on a Helios Beta UV–Vis spectrophotometer equipped with a seven-position carousel (Unicam, UK). Every measurement was performed in triplicate. The results of the assays were evaluated and the IC_{50} values were calculated using QCExpert 2.5 statistical software (Trilobyte, Czech Republic). The colorimetric assay of enzyme inhibition was performed according to a previously described procedure that is considered a standard analytical method for this purpose,[24] although some alternative approaches, including RP–HPLC with fluorescence detection, have also been successfully tested.[25] The absorbance data together with the gradually increasing concentrations of dobesilates and their logarithms are given in Table I. The concentrations of dobesilates were recalculated into the concentrations of the anhydrous substances.

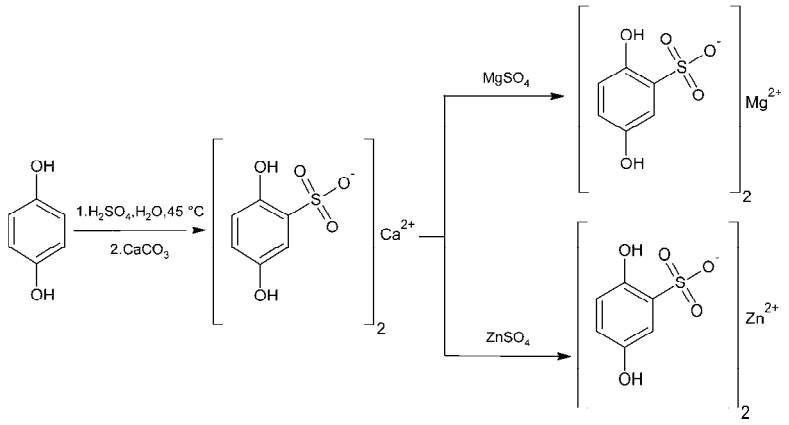
TABLE I. Absorbances of samples of dobesilates incubated with APN with gradually increasing concentrations of dobesilates

c(**2**)/10^{-3} mol L^{-1}	A(**2**)	c(**4**)/10^{-3} mol L^{-1}	A(**4**)	c(**5**)/10^{-4} mol L^{-1}	A(**5**)
0.000	0.965	0.000	0.965	0.000	0.965
0.720	0.959	0.605	0.753	0.189	0.956
0.823	0.929	0.691	0.702	0.283	0.918
0.926	0.747	0.777	0.610	0.377	0.782
1.029	0.366	0.864	0.537	0.472	0.682
1.131	0.071	0.950	0.344	0.566	0.656
1.233	0.050	1.040	0.158	0.660	0.533
–	–	–	–	0.755	0.460

RESULTS AND DISCUSSION

Chemistry

The target compounds were synthesized according to the steps outlined in Scheme 1. Calcium dobesilate (**2**) was prepared by sulfonation of hydroquinone under mild conditions followed by neutralization with calcium carbonate. It also served as an intermediate for the preparation of both magnesium and zinc dobesilates, which were produced from reactions of the appropriate metal sulfates with solutions of **2** under precipitation of the less soluble calcium sulfate. The synthesized compounds were characterized by melting points, IR, ^{1}H- and ^{13}C--NMR spectral data and metal content determined by AAS. Although these three dobesilates are not completely novel compounds, satisfactory spectral characteristics of two of them, **4** and **5**, have not hitherto been reported. Furthermore, the hydrates of **2**, **4** and **5** have also not been previously reported.



Scheme 1. Synthesis of salts of 2,5-dihydroxybenzenesulfonic acid.

The characterization data for **2**, **4** and **5** are given in the Supplementary material to this paper.

Enzyme assay

The linear regression analysis of the relationship of mean absorbance values at 405 nm with the dobesilate concentration, re-calculated into the concentration of anhydrous dobesilate, resulted in the following equations:

$$\log c(\mathbf{2}) = -0.2024A - 2.9120;\ R = 0.959 \quad (1)$$

$$\log c(\mathbf{4}) = -0.3688A - 2.9019;\ R = 0.943 \quad (2)$$

$$\log c(\mathbf{5}) = -1.1097A - 3.5761;\ R = 0.975 \quad (3)$$

The values of IC_{50} were then calculated as the concentration corresponding to 50 % of the maximum absorbance values.[26] IC_{50} values of 978.0, 832.1 and 77.4 μmol L^{-1} were obtained for **2**, **4** and **5**, respectively. Thus, **5** showed the highest

inhibitory activity. Compared to the most active APN inhibitors, **2** and **4** could be characterized as weak APN inhibitors, but the IC_{50} of **5** has a comparable order of magnitude with the values for known active compounds, such as the short-chain peptide bestatin, which, determined on the same pig kidney APN, reached 16 μmol L^{-1}.[27] These results suggest that the involvement of APN inhibition in the anti-angiogenic activity of dobesilates is possible because Zn^{2+} is available in blood plasma and divalent cations are freely interchangeable. As far as the mechanism of inhibition of APN by dobesilates is concerned, their inhibitory effect cannot be attributed to the replacement of Zn^{2+} in the active center of the enzyme with other divalent cations[28] because **5** has a greater inhibitory effect than **4** and **2**. The removal of Zn^{2+} from the active center through its simple complexation by the free phenolic groups of 2,5-dihydroxybenzenesulfonate also seems improbable. This does not occur even in compounds with the *o*-configuration of hydroxyls, which are much more suitable for such complexation, such as the flavonoid quercetin.[29] The Zn^{2+} is bound by His383, His387 and Glu406 residues, which belong to the active site of APN.[30,31] Zn^{2+} and Glu406 form a regular carboxylate ion pair, while donor–acceptor bonds exist between Zn^{2+} and the imidazole nitrogens of both His residues. These coordination bonds could be disrupted by the formation a 2,5-dihydroxybenzenesulfonate ion pair on a particular basic nitrogen of the His imidazole ring by interaction with any dobesilate. By coordination, the dobesilate metal cation can then interact with other suitable amino acid residues within the catalytic site, such as Arg, His, Tyr and Asp or Glu. This interaction could lead to a decrease in catalytic activity. The mode of the inhibitory action of dobesilates on APN could also be based on their reduction activity, as they are hydroquinone derivatives and can be readily oxidized to appropriate 1,4-benzoquinone derivatives – 3,6-dioxocyclohexa-1,4--diene-1-sulfonates. The possibility and reversibility of this reaction has been repeatedly demonstrated by cyclic voltammetry using various types of electrodes.[32] This oxidation must be accompanied by a complementary reduction, such as a change in a disulfide bridge between two cysteine residues into two independent thiol groups. Such bridges occur in APN between Cys758 and Cys765 and between Cys795 and Cys831.[4] Their removal could lead to the disruption of the tertiary structure of the enzyme protein, which could lead to decreased activity.

CONCLUSIONS

In the present paper, the synthesis of calcium (**2**), magnesium (**4**) and zinc (**5**) 2,5-dihydroxybenzenesulfonates is described. The results of inhibitory activity assays against APN showed that **2** and **4** are only weak inhibitors of this enzyme while **5** has medium inhibitory activity.

SUPPLEMENTARY MATERIAL

Some physical, analytical and spectral data for the examined dobesilates are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

ИЗВОД

ИНХИБИЦИЈА АМИНОПЕПТИДАЗЕ N ЈЕ МОЖДА УКЉУЧЕНА У АНТИ-АНГИОГЕНИ ЕФЕКАТ ДОБЕЗИЛАТА

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Сулфоновање хидрохинона сумпорном киселином и реакцијом одговарајућих соли добијени су калцијум- , магнезијум- и цинк-2,5-дихидроксибензенсулфонати (добезилати). Калцијумова со је добијена директном реакцијом киселине и калцијум-карбоната, док су магнезијумова и цинкова со добијене после јонске измене употребом магнезијум- или цинк-сулфата. Добезилати су окарактерисани стандардним спектроскопским поступцима и AAS за садржај јона метала и испитана им је инхибиторна активност према аминопептидази N. Калцијум- и магнезијум-2,5-дихидроксибензенсулфонати показују слабу инхибиторну активност према аминопептидази N, са IC_{50} вредностима 978,0 и 832,1 μmol L^{-1}, редом, док цинк-2,5-дихидроксибензенсулфонат показује знатно бољу инхибиторну активност, са IC_{50} од 77,4 μmol L^{-1}. Инхибиторне активности указују да инхибиција аминопептидазе N може да буде важна у анти-ангиогеној активности 2,5-дихидроксибензенсулфоната.

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