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Silver(I)/6-hydroxyiminolumazine compounds differently modify renin–angiotensin system-regulating aminopeptidases A and N in human neuroblastoma and glioma cells

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ABSTRACT

We have described that local tissue renin–angiotensin-system (RAS) is involved in tumor growth in a rat model of experimental glioma *in vivo*, through the modification of their corresponding local proteolytic regulatory enzymes. Thus, we have found a time-dependent significant decrease in aminopeptidase N (APN) and a significant increase in aminopeptidase A (APA) activities concomitantly with tumor growth in tumor tissue whereas no changes were found in circulating aminopeptidase activities; we suggested that angiotensin peptides may play an essential step in both tumor infiltration and associated angiogenesis. Here we analyze *in vitro* the antiproliferative efficacy, apoptotic properties and effects of three new disilver complexes containing *E*-6-(hydroxyimino)ethyl-1,3,7-trimethyllumazine (lumazine = pteridine-2,4(1*H*,3*H*)-dione) on RAS-regulating APA and APN specific activities in human neuroblastoma and glioma cell lines NB69 and U373-MG. Disilver compounds showed cytotoxicity against both cell lines, although their potency was different for each cell type. Furthermore, NB69 cells need higher concentrations of silver complexes than U373-MG cells to obtain a 50% growth inhibition. All compounds showed apoptotic effects, with U373-MG cells being more susceptible. The three silver complexes tested also show a dose-dependent inhibitory effect on APA activity in NB69 and U373-MG cells, although U373-MG cells are more sensitive. On the contrary, none of them showed effects on APN activity in NB69 neuroblastoma cells whereas the three compounds showed a dose-dependent stimulatory effect on APN activity in U373-MG glioma cells with a similar potency. Disilver complexes show specific antitumor activity against brain tumor cells acting through the paracrine regulating system mediated by local tissue RAS.

1. Introduction

There has been little improvement in the efficacy of adjuvant therapies against malignant glioma and neuroblastoma, and currently noncurable central nervous system neoplasias [1]. A therapeutic possibility in the treatment of cancer is through inhibition of angiogenesis and invasiveness [2,3] to avoid the recruitment of new blood vessels which provide the principal route by which tumor cells exit the primary tumor site and enter to the circulation [4]. Tumor vessels have an aberrant response to constrictor hormones such as angiotensins. Angiotensin peptides of the renin–angiotensin system (RAS), are involved in the control of cell growth and vascular permeability [5]. In fact, angiotensin II (AngII) stimulates angiogenesis and tumor growth [6,7]. Classically, AngII has been considered as the effector peptide of the RAS, but AngII is not the only active peptide. Several of its degradation products, including angiotensin III (AngIII) and angiotensin IV (AngIV), also possess biological functions. These peptides are formed *via* the activity of several RAS-regulating aminopeptidases (Fig. 1).

Thus, AngIII is obtained from AngII by deletion of the N-terminal aspartic residue by aminopeptidase A (APA; EC: 3.4.11.7) and AngIII further is converted to AngIV by aminopeptidase N (APN; EC 3.3.11.14) [8–10]. We have previously described a strong relationship between RAS-regulating aminopeptidase activities and tumor growth in a rat model of experimental glioma. Thus, we found a time-dependent

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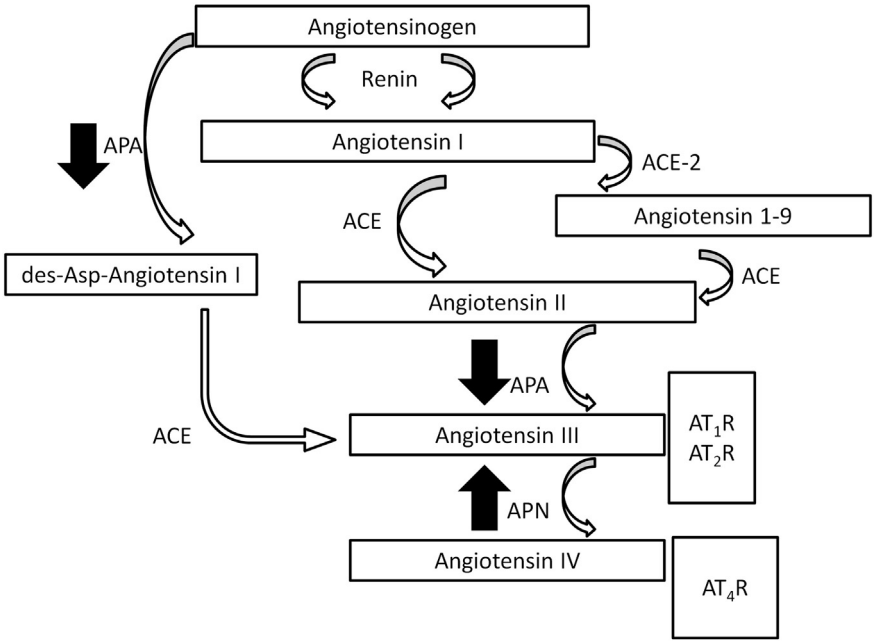

Fig. 1. Angiotensin peptides are derived from the precursor protein angiotensinogen through several enzymatic conversion pathways. The decapeptide angiotensin I (AngI) is formed by renin acting upon the amino terminal of angiotensinogen. AngI serves as a substrate for angiotensin converting enzyme (ACE), a zinc metalloprotease that hydrolyzes the carboxy terminal dipeptide His-Leu to form the octapeptide angiotensin II (AngII). AngII is converted into heptapeptide angiotensin III (AngIII) by aminopeptidase A (APA) that cleaves the Asp residue at the N-terminal. Aminopeptidase N (APN) cleaves Arg at the N-terminal of AngIII to form the hexapeptide angiotensin IV (AngIV). AngIV can be further converted to inactive peptide fragments and amino acid constituents. Angiotensins exert their actions through the different angiotensin receptor subtypes AT_1, AT_2 and AT_4. Black arrows show the effects exerted by silver complexes tested here on U373-MG glioma cells.

significant decrease in APN activity, and a significant increase of APA activity concomitantly with tumor growth in tumor tissue [11].

The use of silver(I)-based metal-organic frameworks (MOFs) as building blocks to make structures of different dimensionalities is a continuous growing research field with interesting applications in medicine [12–17]. Their bioactivity could be related to the weakness of the metal–ligand bonds; in biological systems, this might result in further substitution with bioligands as proteins, enzymes, and membrane-containing thiol groups in their active sites. In this way, the high cytotoxic activity against A549 and MCF-7 cancer cell lines shown by a number of disilver–saccharinate complexes has been recently reported, which may be closely related to their DNA binding affinities [18].

Since angiotensin peptides might play an essential step in both brain tumor infiltration and associated angiogenesis, the present study reports the effects of three new polymeric disilver complexes ($[Ag_2L_2(ClO_4)_2]_n$ (**1**), $[Ag_2L_2(NO_3)_2]_n$ (**2**) and $[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n \cdot nEtOH$ (**3**); L = *E*-6-(hydroxyimino)ethyl-1,3,7-trimethyl-pteridine-2,4(1*H*,3*H*)-dione) on the activity of the RAS-regulating APA and APN activities in the human neuroblastoma and glioma cell lines NB69 and U373-MG, to analyze their potential ability as antitumor compounds.

The silver compounds were previously characterized using elemental analysis, FT-IR, solution ^1H and $^{13}C\{^1H\}$ NMR spectroscopies, X-ray crystallography and AIM calculations [19]. The polymeric structures are built from disilver MOFs as depicted in Fig. 2; individual MOFs consist of disilver units with two head-to-head pteridine bridging ligands $[(N_2O_3)Ag–Ag(N_2O_2)]$. In the crystal structures, MOFs are linked to each other through bridging counteranions affording chain-like monodimensional arrangements; it is noteworthy that the related silver(I) complex containing the free tetrafluoroborate anion [19], which consists of mononuclear AgL_2^+ units, does not show the biological activity reported in this paper. Bearing in mind that the polymeric structures are difficult to be retained in the solutions used for the biological assays, additional studies have been carried out in order to acquire some information on the metallic fragments present.

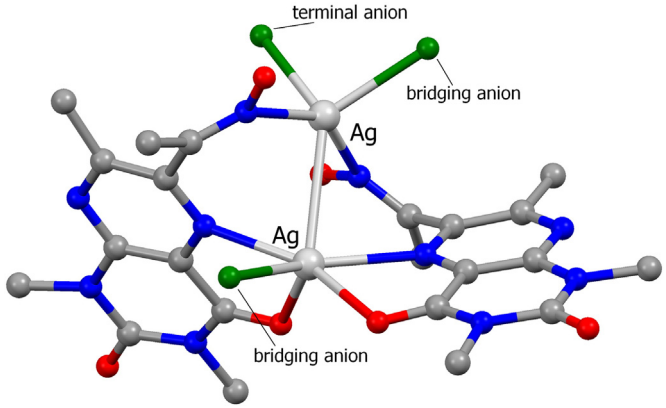

Fig. 2. Ball and stick view of the head-to-head disilver-oxime MOFs (ball color as usual).

2. Experimental

2.1. Synthesis of silver compounds and NALDI-ToF measurements

Silver complexes $[Ag_2L_2(ClO_4)_2]_n$ (**1**), $[Ag_2L_2(NO_3)_2]_n$ (**2**) or $[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n \cdot nEtOH$ (**3**) were isolated following methods previously reported [19]. Nano-assisted laser desorption/ionization-time of flight (NALDI-ToF) experiments were performed in the Scientific Instrumentation Center of the University of Granada, using an Autoflex III MALDI-ToF/ToF mass spectrometer (Bruker Daltonics, Bremen, Germany) with a Smartbeam™ laser as the ionization source (laser repetition rate 5 Hz). The acceleration voltages were 19 (ion source 1) and 16.6 kV (ion source 2). For each spectrum, 11 (**1**), 40 (**2**) and 63 (**3**) shots in the positive reflection mode were accumulated (pulsed ion extraction (PIE) delay, 30 ns; lens voltage, 7.8 kV; linear detector voltage, 1.622 kV). Data were collected in a *m/z* range of 300–1300 Da.

For the analysis, 6 μL of 50 mg · L^{-1} DMSO solutions (*ca.* 50 μM) of the disilver compounds were deposited and dried on NALDI™ plates (Bruker, Bremen, Germany).

2.2. Cell culture

Human neuroblastoma NB69 and astroglioma U373-MG cells were grown in 5% fetal bovine serum (FBS)-supplemented Dulbecco's Modified Eagle Medium (DMEM) without antibiotics. Cells were incubated at 37 °C in a modified atmosphere of 5% CO_2/95% air until reaching confluence. Freedom from mycoplasma contamination was checked regularly by testing with Hoechst 33528.

2.3. Colorimetric cytotoxic assay

To set up the colorimetric cytotoxic assay (CCA), cells were trypsinized from monolayer and diluted to 4×10^4 cells · mL^{-1}. Cells were in exponential phase of growth during the whole experiment. Aliquots of 1 mL of cells were pipetted into wells of 24-well tissue culture plates (Nunc) and the plates were incubated for 24 h. Silver complexes $[Ag_2L_2(ClO_4)_2]_n$ (**1**), $[Ag_2L_2(NO_3)_2]_n$ (**2**) or $[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n$·nEtOH (**3**) were then added in triplicate to the wells in a volume of 1 mL per well at a range of concentrations 1–5 μM in 5% DMSO aqueous solution, each dose being used in at least four replicate wells. After 72 h incubation, the media were removed and the cultures were washed with PBS (phosphate buffered saline) prior to fixation with 10% trichloroacetic acid (TCA) (4 °C) for 30 min and then washed with tap water to remove TCA. Plates were air dried and then stored until use. TCA-fixed cells were stained for 20 min with 0.4% (w/v) sulforhodamine B (SRB) dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and cultures were rinsed with 1% acetic acid to remove unbound dye. The cultures were air dried and bound dye was solubilized with 10 mM Tris base (pH 10.5). Optical density (OD) was read in a Tecan Genios Plus plate reader at 492 nm. The photometer response was linear with dye concentration and it was proportional to the cell numbers counted in parallel with an automatic cell counter (TC-10, Bio-Rad).

2.4. Annexin V and dead cell assay

The assay utilizes annexin V to detect phosphatidylserine on the external membrane of apoptotic cells and 7-amino-actinomycin D (7-AAD) as dead cell marker and indicator of cell membrane structural integrity [20]. Thus, cells were trypsinized from monolayer and diluted to 4×10^4 cells · mL^{-1}. Cells were in exponential phase of growth during the whole experiment. Aliquots of 1 mL of cells were pipetted into wells of 24-well tissue culture plates (Nunc) and the plates were incubated for 24 h. The silver complexes $[Ag_2L_2(ClO_4)_2]_n$ (**1**), $[Ag_2L_2(NO_3)_2]_n$ (**2**) and $[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n$·nEtOH (**3**) were then added in triplicate to the wells in a volume of 1 mL per well at a range of concentrations 1–5 μM in 5% DMSO aqueous solution, each dose being used in at least four replicate wells. After a 1 h

Table 1
Dose–effect relationship parameters of the silver complexes $[Ag_2L_2(ClO_4)_2]_n$ (**1**), $[Ag_2L_2(NO_3)_2]_n$ (**2**) and $[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n$·nEtOH (**3**) on growth of NB69 human neuroblastoma cells and U373-MG human glioma cells.

Drug dosage (μM)	Fa	m	Dm (μM) (lower 95%–upper 95%)	R
NB69 cells				
$[Ag_2L_2(ClO_4)_2]_n$				
1	0.0067			
2	0.0375			
3	0.1126			
4	0.1454		921	
5	0.1602	2.2 ± 0.2	(6.80–12.47)	0.981
$[Ag_2L_2(NO_3)_2]_n$				
1	0.0010			
2	0.0451			
3	0.2192			
4	0.2683		5.18	
5	0.2751	3.8 ± 0.7	(3.83–7.01)	0.958
$[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n$·nEtOH				
1	0.1497			
2	0.2401			
3	0.2682			
4	0.3061		16.37	
5	0.3139	0.59 ± 0.06	(10.77–24.87)	0.982
U373-MG cells				
$[Ag_2L_2(ClO_4)_2]_n$				
1	0.0280			
2	0.1585			
3	0.3162			
4	0.3757		5.25	
5	0.3854	2.0 ± 0.3	(4.09–6.74)	0.971
$[Ag_2L_2(NO_3)_2]_n$				
1	0.0044			
2	0.1260			
3	0.2357			
4	0.2859		7.90	
5	0.2946	1.4 ± 0.1	(6.13–10.18)	0.984
$[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n$·nEtOH				
1	0.0539			
2	0.1517			
3	0.2807			
4	0.3246		7.39	
5	0.3004	1.4 ± 0.2	(5.16–10.59)	0.966

The parameters Fa, m, Dm and R are the fractional inhibition, slope coefficient of the straight line, dose at 50% inhibition (equivalent to IC_{50} value), and the linear correlation coefficient of the median–effect plot (Fig. 3).

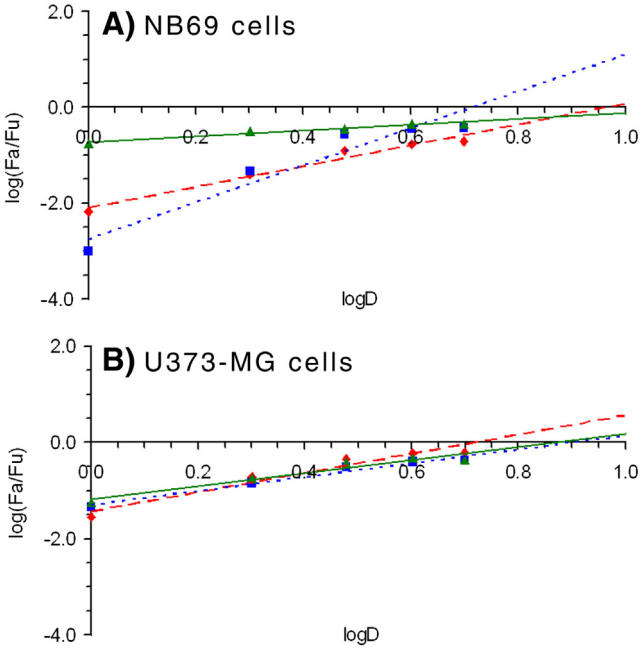

Fig. 3. Median–effect plot of cytotoxicity data for (A) human NB69 neuroblastoma and (B) U373-MG glioma cells for the silver complexes tested. The median–effect plot displays log(Fa/Fu) *versus* logD, where Fa and Fu are the affected and unaffected fractions, respectively, and D is the dose of the drug (long dashed line, red ♦, $[Ag_2L_2(ClO_4)_2]_n$; short dashed line, blue ■, $[Ag_2L_2(NO_3)_2]_n$; solid line, green ▲, $[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n \cdot nEtOH$).

incubation, the media were removed and the cultures were washed with PBS. Cells were then trypsinized, washed twice in PBS containing 1% FCS and resuspended in PBS with 1% FCS at a concentration of 1×10^6 cells · mL^{-1}. The cells were stained with an equal volume of a ready-to-use solution of 7-AAD and annexin V (Millipore) for 20 min at room temperature while protecting from light. Samples were analyzed within 1 h in a Muse Cell Analyzer (Millipore). Data were further analyzed with the Muse 1.3.1 analysis software. Percent of viable, early apoptotic, late apoptotic and dead cells were considered according to their annexin V (−)/7-AAD (−), annexin V (+)/7-AAD (−), annexin V (+)/7-AAD (+) and annexin V (−)/7-AAD (+) status.

2.5. Aminopeptidase A (APA) activity assay

APA activity was analyzed in whole cells, using aspartyl-β-naphthylamide (AspNNap) as substrate, in accordance with previously described methods [9]. After removal of the culture medium, the cells were incubated in triplicate for 30 min at 37 °C with 100 μL of the assay medium containing the silver complexes $[Ag_2L_2(ClO_4)_2]_n$ (**1**), $[Ag_2L_2(NO_3)_2]_n$ (**2**) or $[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n \cdot nEtOH$ (**3**) at a range of concentrations 1–5 μM in 5% DMSO aqueous solution, 100 μM of AspNNap and 0.65 mM dithiothreitol (DTT), in artificial cerebrospinal fluid (NaCl 116 mM, KCl 5.4 mM, $MgCl_2$ 0.9 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 25 mM, glucose 10 mM) pH 7.2.

2.6. Aminopeptidase N (APN) assay

APN was also measured fluorometrically in whole cells using alanyl-β-naphthylamide (AlaNNap) as substrate. After removal of the culture medium, the cells were incubated in triplicate for 30 min at 37 °C with 100 μL of the substrate solution containing the silver complexes $[Ag_2L_2(ClO_4)_2]_n$ (**1**), $[Ag_2L_2(NO_3)_2]_n$ (**2**) or $[Ag_2L_2(CF_3SO_3)_2(EtOH)]_n \cdot nEtOH$ (**3**) at a range of concentrations 1–5 μM in 5% DMSO aqueous solution, 100 μM of AlaNNap and 0.65 mM DTT in artificial cerebrospinal fluid (as described in Section 2.4).

The reactions was stopped by addition of 100 μL of acetate buffer 0.1 M (pH 4.2) and the amount of β-naphthylamine released as the result of the enzymatic activities was measured fluorometrically at 412 nm emission wavelength with excitation wavelength of 345 nm. Specific enzyme activities were expressed as picomoles of the corresponding aminoacyl-β-naphthylamide hydrolyzed per min per 10^6 cells, by using a standard curve prepared with the latter compound under corresponding assay conditions.

2.7. Statistics

We used one-way analysis of variance (ANOVA) to analyze differences between groups. Post-hoc comparisons were made using Newman–Keuls test. P-values below 0.05 were considered significant.

3. Results and discussion

3.1. Physicochemical information on the title compounds

Since only small differences between the solid NMR spectra of free ligand and silver complexes indicate the existence of weak Ag-L interactions, the most likely is that the initial polymeric structures are not retained in aqueous DMSO solution; actually, the NMR (DMSO) spectra of complexes are virtually coincident with that of the metal-free ligands. Furthermore, in order to get additional physicochemical information on the title compounds in solution, in particular to distinguish if there are AgL or Ag_2L_2 entities, new evidences about the Ag–L interactions, based on UV–visible spectroscopy, potentiometric measurements and mass spectrometry, have also been looked for. Unfortunately, these techniques have not been able to reach useful results at all, because at the concentrations used in the biological experiments ($1–5 \times 10^{-6}$ M), no significant metal–ligand interactions were perceived and, when the concentrations were increased up to usual values for potentiometry (*ca.* 10^{-3} M in water), solid complexes precipitated immediately. Moreover, the mass spectra of the silver compounds obtained from methanol solutions were not neither helpful because they indicated the presence (in increasing order of abundance) of $Ag_2L_2^+$, AgL_2^+ and AgL^+ molecular fragments, which only drive us to suggest that, under the MS spectrum recording conditions, the highest stability corresponds to the AgL^+ monomer. Thus, while the structures of the crystalline title compounds – isolated from clear approx. 0.01 M ethanolic solutions (Ag/L = 1) – were fully known, there were still doubts about the mono- or dimetallic nature of the silver MOFs existing in the micromolar aqueous (5% DMSO) solutions used to perform the biological tests.

Furthermore, we have tried to overcome this inconvenience using NALDI-ToF measurements, thinking that a soft ionization technique might give some useful information about the molar mass distribution of the species in solution. The spectra clearly show the appearance of a strong doublet at m/z = 370.0 and 372.0 which corresponds, in both intensity and multiplicity, to mononuclear $[AgL]^+$ moieties, whereas the expected triplet at m/z about twice as assignable to $[Ag_2L_2]^+$ does not appear at all. Therefore, the presence of either polymetallic or dimetallic entities in the tested solutions must be refused and, at least in principle, the biological activity attributed to monometallic AgL species.

3.2. Biology

The dose–effect relationship of the title silver complexes were determined on the growth of human NB69 and U373-MG cells from the values of fraction of cell growth affected (Fa) by drug dosage; these were used to compute the dose required for 50% inhibition of cell growth or IC_{50} (Dm) and subjected to the median–effect analysis [21,22] to determine their potency (Dm), shape (coefficient of the sigmoidicity of the dose–effect curve, m) and conformity (linear correlation coefficient of the median–effect plot, R) (Table 1, Fig. 3).

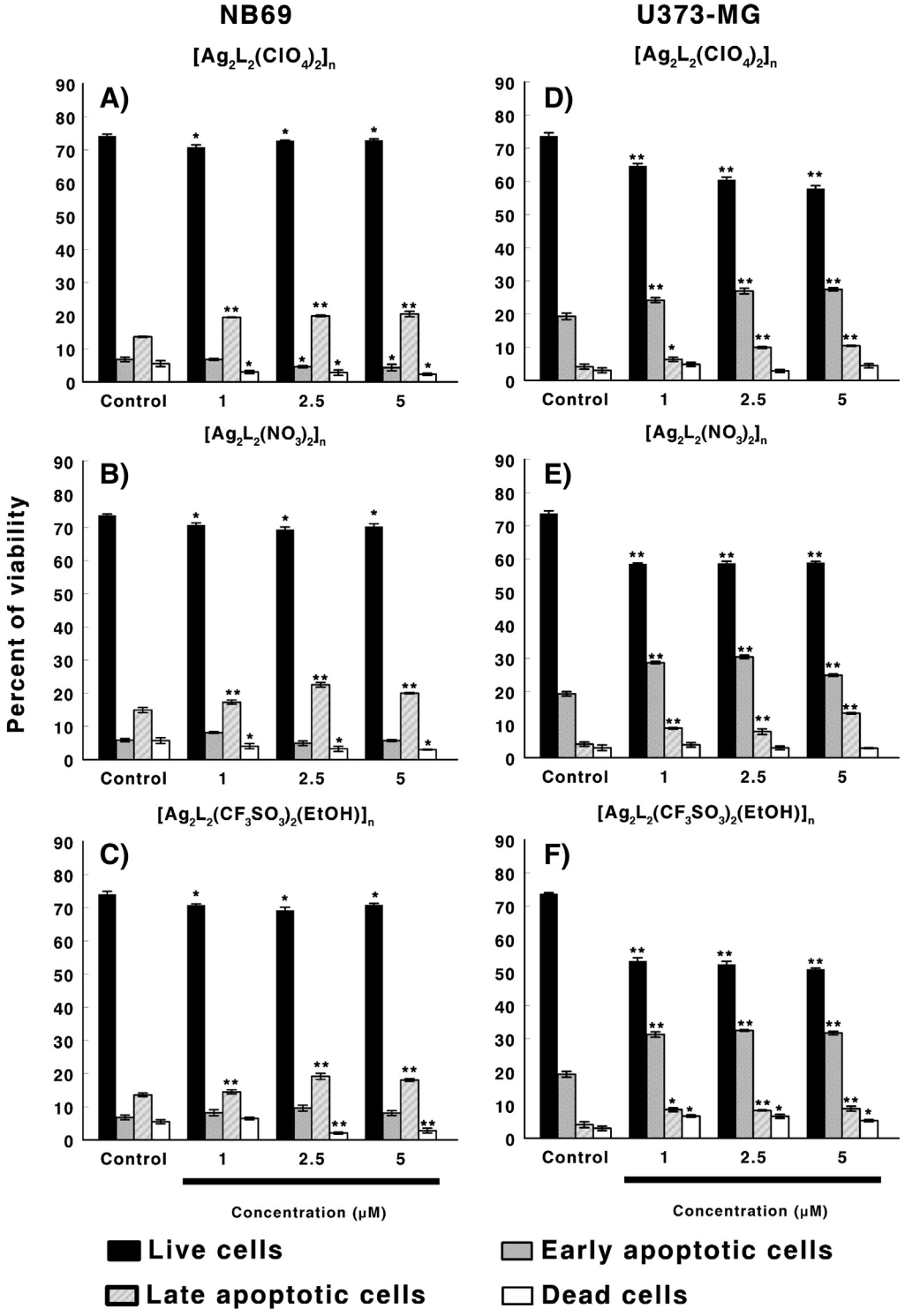

Fig. 4. Percent of live, early apoptotic, late apoptotic and dead human NB69 neuroblastoma and U373-MG glioma cells after the treatment with the silver complexes **1** (A, D), **2** (B, E) and **3** (C, F). Results are expressed in percent of viability (mean ± SEM; *P < 0.05; **P < 0.01; n = 4).

All tested compounds showed cytotoxicity against both cell lines, although their potency was different for each cell type. Thus, for NB69 neuroblastoma cells, the order of potency was **2** > **1** > **3**, with Dm values of 6.59, 9.21 and 16.37 μM, respectively. For U373-MG glioma cells, the order of potency was **1** > **3** > **2**, with Dm values of 5.25, 7.39 and 7.90 μM. As a whole, NB69 cells need higher concentrations of silver complexes than U373-MG cells to obtain 50% growth inhibition. Furthermore, although both silver salts and metal-free ligands showed cytotoxicity to a certain extent, none of them modify aminopeptidase activities, which indicates an inespecific toxic effect on cell growth. However, it is noteworthy that the reported Dm values are still preliminary because the values were determined using only a narrow range of compound concentrations.

Regarding apoptotic properties of tested compounds, all of them promotes apoptosis in U373-MG cells in a dose-dependent manner, mainly with the 1–2.5 μM doses, reaching a plateau with the higher

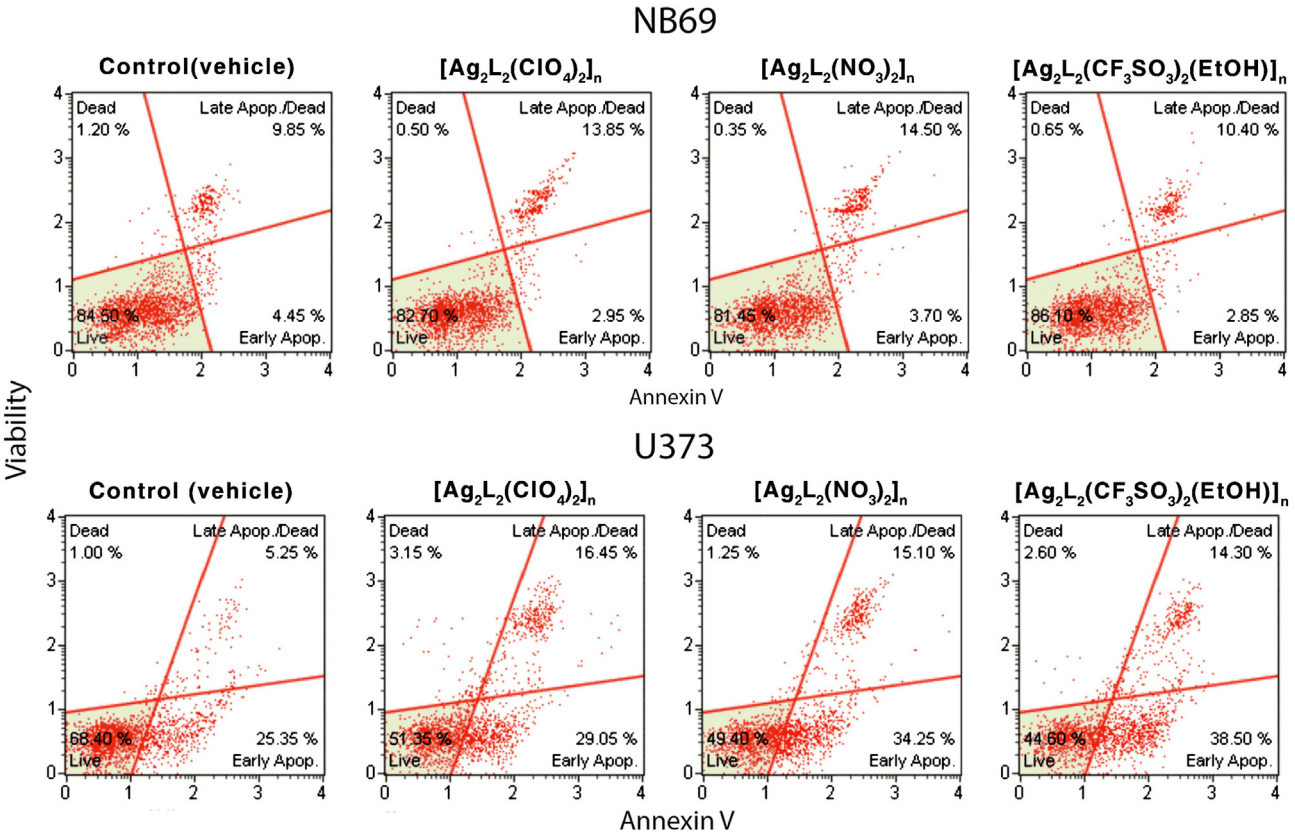

Fig. 5. Examples of dot plots for the events in each quadrant showing annexin V *vs.* viability in human NB69 neuroblastoma and U373-MG glioma cells after the treatment with the silver complexes.

dose (5 μM). However, in NB69 cells, significant changes are only found for late apoptotic/dead cells, indicating a necrotic more than pro-apoptotic effect of these compounds in this cell type (Figs. 4 and 5).

On the contrary, the three silver complexes show an inhibitory effect on APA activity in a dose-dependent manner (Fig. 6), although U373-MG cells are more sensitive to this inhibitory effect than NB69 cells. In fact, APA activity is not detected in U373-MG cells after treatment with either $Ag_2L_2(ClO_4)_2$ 3–5 μM or $Ag_2L_2(NO_3)_2$ 5 μM. In the same way, none of the silver compounds tested showed effects on APN activity in NB69 neuroblastoma cells, whereas all of them showed a dose-dependent stimulatory effect on APN activity in U373-MG glioma cells with a similar potency (Fig. 6). It indicates that these new bioactive molecules act through the specific mechanisms of cell growth in which the RAS is involved, and may also act through the angiogenic and invasive processes mediated by angiotensin peptides. Furthermore, no effects on other acid-, basic- and omega-type related proteolytic regulatory enzymes tested have been found, which also indicates that the silver complexes are effective in targeting specific proteases.

The *in vitro* antiproliferative efficacy and the differential effects of the three silver complexes on RAS-regulating APA and APN specific activities, in the human neuroblastoma and glioma cell lines NB69 and U373-MG have been studied; the results allow to propose them as compounds with specific antitumor activity against brain tumor cells, mainly glioma, acting through the paracrine regulating system mediated by tissue RAS. It supposes a first step to examine the efficacy and toxicity of these compounds as therapeutic agents. The importance lies in that we have previously described *in vivo* changes in RAS-regulating aminopeptidase activities in tumor tissue and in relationship to tumor growth in a rat model of glioma, in which no changes were observed in the circulating RAS. Thus, we found a time-dependent significant decrease in the specific activity of soluble and membrane-bound APN, whereas the specific activity of soluble (but not membrane-bound) APA increased concomitantly with tumor growth [11]. In RAS cascade, APA degrades AngII to form not only AngIII, but also angiotensinogen to form des-Asp-AngI, which in turn is converted to AngIII by ACE. APN is the enzyme responsible for the degradation of AngIII to form AngIV [23]. These two main bioactive peptides of the RAS, AngII and its direct metabolite AngIII, exert their actions through their receptors AT_1 and AT_2, whereas AngIV interact with the AT_4 receptor [24,25]. It is well known that the peptides of the RAS are expressed, independently of the circulating RAS peptides, in normal nonvascular tissues, including the nervous system [26,27], where they play a role in regulating cardiovascular functions [28] and other physiological processes such as cell growth, cell differentiation and apoptosis [29]. However, it has been shown that some neurons or glial cells and glioma cells express several of the components of the RAS and their receptors, although the participative mechanisms are unclear [5,30–32]. Thus, it has been described that APA was up-regulated and enzymatically active in blood vessels of human tumors, but was not detected in normal blood vessels [33]. In addition, APA expression was found on dysplastic cells and was increased in precancerous lesions and invasive cervical cancer [34]. These data and others about expression of APA on prostate cancer cells [35], suggest that APA may play a regulatory role in neoplastic transformation and disease progression in various types of cancer. On the other hand, others authors have reported that several kinds of carcinomas including those of colon, kidney, breast and lung, exhibited little expression of APN [36,37]. Although APN has been considered a proteolytic enzyme with the ability to facilitate tumor cell invasion through the extracellular matrix degradation [38], lower expression of APN implies that APN may enzymatically function in ways other than extracellular matrix degradation. Our data in experimental glioma *in vivo* suggests a predominant action of AngIII *vs.* AngII, whereas the silver complexes tested here potentiate APN and decrease APA activities,

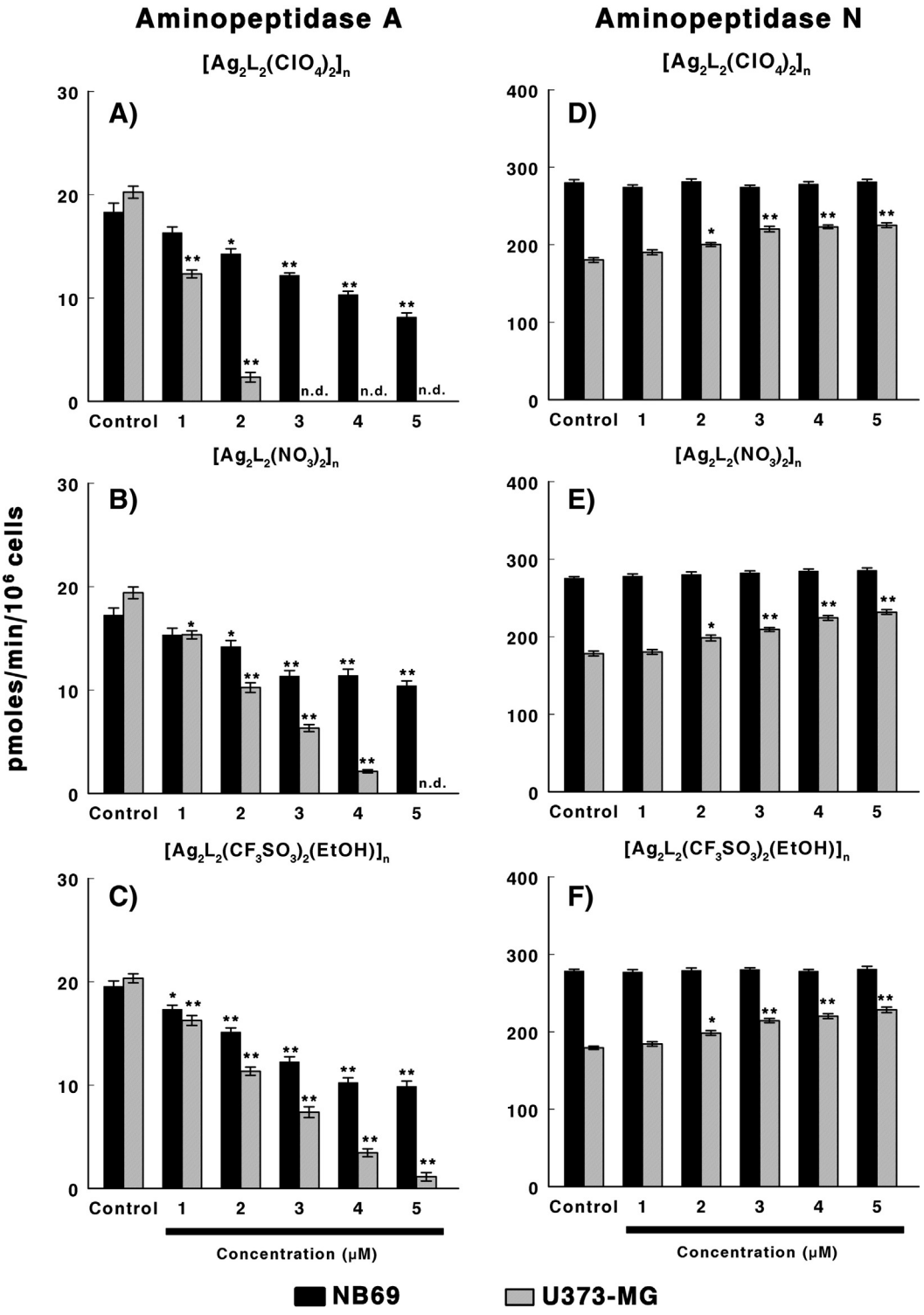

Fig. 6. Aminopeptidase A (APA) and aminopeptidase N (APN) specific activities in human NB69 neuroblastoma and U373-MG glioma cell lines after the treatment with the silver complexes **1** (A,D), **2** (B,E) and **3** (C, F). Results are expressed in pmol/min/10^6 cells (mean ± SEM; *P < 0.05; **P < 0.01; n.d.: not detected; n = 4).

supporting a predominant action of AngII and increase the clearance of AngIII, as the support of their antiproliferative actions, mainly on human glioma U373-MG cells. In NB60 human neuroblastoma cells, only a decrease on APA activity was found with the silver compounds, which support only the predominant action of AngII. These changes in the bioavailability of the angiotensins are very important; although the amount of each angiotensin is also important, the ratio between them, which stimulate cell growth and angiogenesis, is more important [35]. In fact, it is known that gliomas are accompanied by extensive angiogenesis, essential for tumor growth and invasiveness [3]. Thus, some reports indicate that AngII up-regulates several growth factors production, induces neovascularization [6,7,39] and promotes the expression of proto-oncogenes [40], with these effects being blocked by AT_1 but not AT_2 antagonists [39,41], which also supports the importance of the AngII/AngIII relationship, and that selective blockage of one receptor could increase the effect of the other [31,42]. Also, it seems that the selective blockage of AT_1 may lead to disequilibrium of the AT_1/AT_2 relationship because it may also increase the bioavailability

of AngII, producing up-regulation and overstimulation of the AT_2 receptor, which in turn, could produce some beneficial effects, including vasodilatation and antiproliferative/apoptotic responses [30,42,43]. In fact, we demonstrate here the pro-apoptotic effects of the three silver complexes tested on U373-MG cells but not in NB69 cells.

The increase found in APN also potentiates the formation of AngIV. AngIV exerts its action through the AT_4 receptor. To date, no information is available about the role of AT_4 in brain tumors, but it is known that AngIV seems to show protective effects against AngII effects mediated by AT_1 receptors acting through tyrosine-kinase-mediated signaling [44], also suggesting the importance of the counterbalance of the signals of several angiotensins to promote cell proliferation *versus* cell death.

Furthermore, on the structure–activity relationships, we have no data which allow us to distinguish if the effects found here are due to a direct interaction of the complexes with the proteolytic regulatory enzymes of the RAS or if they are due to interactions with any step of the multiple intracellular signaling cascades/routes which could modify gene expression and/or influence post-translational modifications of the proteolytic regulatory enzymes. Therefore, at present we cannot honestly suggest any points of discussion to clarify this particular. Further research is necessary to shed light on this subject.

4. Conclusion

The *in vitro* analysis of the antiproliferative efficacy, the apoptotic properties and the effects on RAS-regulating APA and APN specific activities of the three new disilver-6-hydroxyiminolumazine complexes on the human neuroblastoma and glioma cell lines NB69 and U373-MG allows us to propose them as compounds with specific antitumor activity against brain tumor cells, mainly glioma, acting through the paracrine regulating system mediated by tissue RAS. This report supposes a first step to examine the efficacy and toxicity of these compounds as therapeutic agents; further studies will allow to calculate more reliable Dm values as well as to analyze their bioavailability and efficacy *in vivo*.

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References

[1] L.M. de Angelis, N. Engl. J. Med. 2 (2001) 114–123.
[2] O. Arrieta, P. Guevara, S. Reyes, A. Ortiz, D. Rembao, J. Sotelo, Eur. J. Cancer 34 (1998) 2101–2106.
[3] D. Wang, H.J. Huang, A. Kazlauskas, W.K. Cavenee, Cancer Res. 59 (1999) 1464–1472.
[4] B.R. Zetter, Annu. Rev. Med. 49 (1998) 407–424.
[5] L. Juillerat-Jeanneret, S. Lohm, M.F. Hamou, F. Pinet, Lab. Invest. 80 (2000) 973–980.
[6] L.A. Fernandez, J. Twickler, A. Mead, J. Lab. Clin. Med. 105 (1985) 141–145.
[7] S.P. Andrade, C.C. Cardoso, R.D. Machado, W.T. Beraldo, Int. J. Microcirc. Clin. Exp. 16 (1996) 302–307.
[8] D. Chansel, R. Ardaillou, Nephrologie 19 (1998) 427–432.
[9] M.J. Ramírez-Expósito, J.M. Martínez, I. Prieto, F. Alba, M. Ramírez, Horm. Metab. Res. 32 (2000) 161–163.
[10] A.J. Barrett, N.D. Rawlings, J.F. Woessner, Handbook of Proteolytic Enzymes, Elsevier Academic Press, Amsterdam, Boston, MA, 2004.
[11] M.D. Mayas, M.J. Ramírez-Expósito, M.P. Carrera, M. Cobo, J.M. Martínez-Martos, Anticancer Res 32 (2012) 3675–3682.
[12] M. Munakata, L.P. Wu, G.L. Ning, Coord. Chem. Rev. 198 (2000) 171–203.
[13] A.N. Khlobystov, A.J. Blake, N.R. Champness, D.A. Lemenovskii, A.G. Majouga, N.V. Zyk, M. Schröeder, Coord. Chem. Rev. 222 (2001) 155–192.
[14] S.-L. Zheng, M.-L. Tong, X.-M. Chen, Coord. Chem. Rev. 246 (2003) 185–202.
[15] C.-L. Chen, B.-S. Kang, C.-Y. Su, Aust. J. Chem. 59 (2006) 3–18.
[16] G.M. de Oliveira, L.R. Soccol-Gris, L. de Lourenço-Marques, E. Schulz-Lang, Z. Anorg. Allg. Chem. 633 (2007) 610–614.
[17] S. Abuskhuna, J. Briody, M. McCann, M. Devereux, K. Kavanagh, J.B. Fontecha, V. McKee, Polyhedron 23 (2004) 1249–1255.
[18] V.T. Yilmaz, E. Gocmen, C. Icsel, M. Cengiz, S.Y. Susluer, O. Buyukgungor, J. Biol. Inorg. Chem. 19 (2014) 29–44.
[19] S.B. Jiménez-Pulido, F. Hueso-Ureña, M.P. Fernández-Liencres, M. Fernández-Gómez, M.N. Moreno-Carretero, Dalton Trans. 42 (2013) 530–541.
[20] I. Schmidt, W.J. Krall, C.H. Uitenbogaart, J. Braun, J.V. Giorgi, Cytometry 13 (1992) 204–208.
[21] T.C. Chou, P. Talalay, Adv. Enzyme Regul. 22 (1984) 27–55.
[22] Calcusyn 2.1 for Windows: Software for Dose–Effects Analysis (Biosoft (c) 1996–2007).
[23] R. Beynon, J. Bond, Proteolytic Enzymes, Oxford University Press, New York, 2001.
[24] Y. Kambayashi, S. Bardhan, K. Takahashi, S. Tsuzuki, H. Inui, T. Hamakubo, T. Inagami, J. Biol. Chem. 268 (1993) 24543–24546.
[25] M. Mukoyama, M. Nakajima, M. Horiuchi, H. Sasamura, R.E. Pratt, V.J. Dzau, J. Biol. Chem. 268 (1993) 24539–24542.
[26] A. Milsted, B.P. Barna, R.M. Ransohoff, K.B. Brosnihan, C.M. Ferrario, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 5720–5723.
[27] J.W. Wright, J.W. Harding, Pflugers Arch. 465 (2012) 133–135.
[28] S. Morimoto, M.D. Cassell, C.D. Sigmund, J. Biol. Chem. 277 (2002) 33235–33241.
[29] E. Escobar, T.S. Rodríguez-Reyna, O. Arrieta, J. Sotelo, Curr. Vasc. Pharmacol. 2 (2004) 385–399.
[30] L. Juillerat-Jeanneret, J. Celerier, C. Chapuis Bernasconi, G. Nguyen, W. Wostl, H.P. Maerki, R.C. Janzer, P. Corvol, J.M. Gasc, Br. J. Cancer 90 (2004) 1059–1068.
[31] E. Rivera, O. Arrieta, P. Guevara, A. Duarte-Rojo, J. Sotelo, Br. J. Cancer 85 (2001) 1396–1399.
[32] O. Arrieta, B. Pineda-Olvera, P. Guevara-Salazar, N. Hernández-Pedro, D. Morales-Espinosa, T.L. Cerón-Lizarraga, C.H. González-De la Rosa, D. Rembao, B. Segura-Pacheco, J. Sotelo, Br. J. Cancer 99 (2008) 160–166.
[33] S. Marchio, J. Lahdenranta, R.O. Schlingemann, D. Valdembri, P. Wesseling, M.A. Arap, A. Hajitou, M.G. Ozawa, M. Trepel, R.J. Giordano, D.M. Nanus, H.B. Dijkman, E. Oosterwijk, R.L. Sidman, M.D. Cooper, F. Bussolino, R. Pasqualini, W. Arap, Cancer Cell 5 (2004) 151–162.
[34] H. Fujimura, K. Ino, T. Nagasaka, N. Nakashima, H. Nakazato, F. Kikkawa, S. Mizutani, Oncology 58 (2000) 342–352.
[35] J. Teranishi, H. Ishiguro, K. Hoshino, K. Noguchi, Y. Kubota, H. Uemura, Prostate 68 (2008) 1666–1673.
[36] J. Dixon, L. Kaklamanis, H. Turley, I.D. Hickson, R.D. Leek, A.L. Harris, K.C. Gatter, J. Clin. Pathol. 47 (1994) 43–47.
[37] A. Varona, L. Blanco, J.I. López, J. Gil, E. Agirregoitia, J. Irazusta, G. Larrinaga, Am. J. Physiol. 292 (2007) F780–F788.
[38] I. Saiki, H. Fujii, J. Yoneda, F. Abe, M. Nakajima, T. Tsuruo, I. Azuma, Int. J. Cancer 54 (1993) 137–143.
[39] R. Tamarat, J.S. Silvestre, M. Durie, B.I. Levy, Lab. Invest. 82 (2002) 747–756.
[40] J.L. Cook, J.F. Giardina, Z. Zhang, R.N. Re, J. Mol. Cell. Cardiol. 34 (2002) 1525–1537.
[41] S. Fujiyama, H. Matsubara, Y. Nozawa, K. Maruyama, Y. Mori, Y. Tsutsumi, H. Masaki, Y. Uchiyama, Y. Koyama, A. Nose, O. Iba, E. Tateishi, N. Ogata, N. Jyo, S. Higashiyama, T. Iwasaka, Circ. Res. 88 (2001) 22–29.
[42] O. Arrieta, P. Guevara, E. Escobar, R. García-Navarrete, B. Pineda, J. Sotelo, Br. J. Cancer 92 (2005) 1247–1252.
[43] H. Matsubara, Circ. Res. 83 (1998) 1182–1191.
[44] H. Yang, X.J. Zeng, H.X. Wang, L.K. Zhang, X.L. Dong, S. Guo, J. Du, H.H. Li, C.S. Tang, Peptides 32 (2011) 2108–2115.