Copper, BDNF and Its N-terminal Domain: Inorganic Features and Biological Perspectives

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Abstract: Brain-derived neurotrophic factor (BDNF) is a neurotrophin that influences development, maintenance, survival, and synaptic plasticity of central and peripheral nervous systems. Altered BDNF signaling is involved in several neurodegenerative disorders including Alzheimer’s disease. Metal ions may influence the BDNF activity and it is well known that the alteration of Cu²⁺ homeostasis is a prominent factor in the development of neurological pathologies. The N-terminal domain of BDNF represents the recognition site of its specific receptor TrkB, and metal ions interaction with this protein domain may influence the protein/receptor interaction. In spite of this, no data inherent the interaction of BDNF with Cu²⁺ ions has been reported up to now. Cu²⁺ complexes of the peptide fragment BDNF(1–12) encompassing the sequence 1–12 of N-terminal domain of human BDNF protein were characterized by means of potentiometry, spectroscopic methods (UV/Vis, CD, EPR), parallel tempering simulations and DFT-geometry optimizations. Coordination features of the acetylated form, Ac-BDNF(1–12), were also characterized to understand the involvement of the terminal amino group. Whereas, an analogous peptide, BDNF(1–12)D3N, in which the aspartate residue was substituted by an asparagine, was synthesized to provide evidence on the possible role of carboxylate group in Cu²⁺ coordination. The results demonstrated that the amino group is involved in metal binding and the metal coordination environment of the predominant complex species at physiological pH consisted of one amino group, two amide nitrogen atoms, and one carboxylate group. Noteworthy, a strong decrease of the proliferative activity of both BDNF(1–12) and the whole protein on a SHSY5Y neuroblastoma cell line was found after treatment in the presence of Cu²⁺. The effect of metal addition is opposite to that observed for the analogous fragment of nerve growth factor (NGF) protein, highlighting the role of specific domains, and suggesting that Cu²⁺ may drive different pathways for the BDNF and NGF in physiological as well as pathological conditions.

Introduction

Since the 1950s when the first nervous system growth factor (nerve growth factor, (NGF)), was discovered,[1] approximately 50 nervous system growth factors have since been identified.[2] Distinct families based on similarities in their structure include the “classic” neurotrophin family consisting of NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophine 4 (NT4). BDNF is one of the most abundant growth factors in the brain and was first identified in 1982.[3] Pro-BDNF is packaged into neuronal secretory vesicles[4] and released through either constitutive secretion or activity-dependent release.[5] After its release, the pro-domain of pro-BDNF is proteolytically cleaved by plasmin or metalloproteinases, to generate mature BDNF.[6–7] This is the predominant form and of great relevance in the adult CNS physiology,[8,9] being widely distributed throughout the brain in almost all cortical areas, as well as several subcortical and spinal cord regions. BDNF as a dimer binds two receptors of distinct classes: the type B tyrosine kinase receptor of the tropomyosin-related kinase family (TrkB)[10] and the p75 receptor, which is a member of the tumor necrosis factor (TNF) receptor family.[11] TrkB is the key receptor for BDNF in the adult brain, owing to its wide pattern of expression and a higher binding affinity for BDNF than p75. BDNF has a role in modulating dendritic branching and dendritic spine morphology.[12,13] as well as synaptic plasticity and long-term potentiation (LTP).[14,15] BDNF is therefore involved in learning and memory processes.[16–18] Altered BDNF signaling has been shown to be involved in several neurodegenerative disorders including AD.[19] BDNF has indeed shown an important role in supporting neuronal survival, which has encouraged studies of its therapeutic potential.[20] Recent studies have shown that decreased levels of BDNF correlate with the severity of AD-related cognitive impairment,[21] suggesting that reduced BDNF availability may be an early cofactor involved in AD development. Interestingly, Zn²⁺ and Cu²⁺ have been shown to be important modulators of this pathway because these metal ions facilitate the maturation of BDNF from pro-BDNF through the activation of metal-dependent matrix metalloproteinases.[22–24] The relevant decrease of BDNF levels observed in 3xTg-AD mice can be counteract-
ed by zinc(II) ion supplementation that induces a strong potentiation of MMP-2 and MMP-9 and leads to a four-fold increase of BDNF levels. In addition, ZnT3-KO mice that lack synaptic Zn$^{2+}$ show signs of cognitive impairment and have decreased BDNF and TrkB levels. Further involvement of metallostasis in promoting a neurotrophic response has been demonstrated: metal ionophore treatment favors spine survival by elevating the levels of the components of the BDNF pathway, that is, BDNF, pro-BDNF, and TrkB. The direct interaction of d-block metal ions with NGF and related neurotrophins has been investigated. Zn$^{2+}$, Cu$^{2+}$, and Pd$^{2+}$ have been reported to alter the conformation of NGF, whereas Co$^{2+}$, Ni$^{2+}$, and Cd$^{2+}$ have been shown to play no role based on the different chemical cross-linking of NGF N-terminus between the two groups of transition-metal ions. The selective effects of both Zn$^{2+}$ and Cu$^{2+}$ have been attributed to a distorted square-pyramidal geometry involving two imidazole nitrogen atoms (His-4 and His-8 residues) from one NGF monomer, and an imidazole (His-84), and a carboxylate group (Asp-105) from the other NGF unit as determined by means of theoretical calculations. The structural modification induced by metal binding to the NGF dimer has been thought to prevent the trophic factor from binding TrkA receptors, thus inactivating signal transduction pathways and biological activity. Zn$^{2+}$ and Cu$^{2+}$ may indeed inhibit the crosslinking of NGF promoters by bis(sulfosuccinimidyl) suberate. As BDNF and NT-3 share Asp-105 with NGF, and taking into account that their N-termini contain residues able to chelate metal ions, BDNF and NT-3 have been tested in assay systems sensitive to neurotrophin conformation and both proteins displayed similar changes in the presence of the zinc(II) ion with respect to conformation, binding to p75 and neurite outgrowth of cultured neurons.

Previous studies have shown that the N-terminal region of neurotrophins is critical for the binding selectivity and activation of their cognate Trk receptors. The crystal structure of NT-4/5 in complex with the fifth subdomain of TrkB (TrkB-d5) shows that the N-terminal region of the neurotrophin interacts with the “specificity patch” of the receptor.

Since no structural data are available for the BDNF/TrkB complex, a 3D model has been derived from the existing high-resolution X-ray structures of the NT-4/5/TrkB complex and the BDNF/NT-3 dimer. A binding pocket located in TrkB-d5 is thought to drive the selectivity of the interaction with BDNF. The N-terminal end of neurotrophins has been shown to affect Trk receptor binding and activation capacities and to fit into a binding pocket formed by a patch of charged amino acids in the fifth subdomain of Trk receptors; this results in it being highly variable in terms of amino acid composition. It has been reported that copper not only activated extracellular signal-regulated kinase 1/2 and Src tyrosine kinase, signaling molecules activated downstream of TrkB, but also decreased levels of pro-BDNF in cells and increased levels of pro- and mature BDNF in the medium. Nevertheless, experimental data for the coordination features of metal ions with BDNF have not yet been reported.

In this study, we report the synthesis of a BDNF N-terminus peptide fragment encompassing the residues 1–12 blocked at the C-terminus, namely, HSDPARRGELSV-NH$_2$ (BDNF(1–12), Scheme 1) to ascertain: 1) the coordination features of Cu$^{2+}$ complexes with the BDNF N-terminal domain, 2) the affinity of this neurotrophin for this metal ion, 3) the metal-driven conformation change, and 4) the effects on neuroblastoma cell culture proliferation.

The Cu$^{2+}$ complexes with BDNF(1–12) were studied by means of potentiometry, spectroscopy (UV/Vis, CD and EPR), parallel tempering simulations and DFT-based geometry optimizations. The coordination features of the acetylated form Ac-HSDPARRGELSV-NH$_2$ (Ac-BDNF(1–12)) were also characterized to prove (or disprove) the involvement of the terminal amino group (see Scheme 1). Both of these peptides contain His and Asp residues and are a satisfactory model of the BDNF N-terminus domain. In addi-
tion, the role of the Asp residue was assessed by the synthesis of a single mutated peptide HSNPARGELSV-NH₂ (BDNF(1–12)D3N). Moreover, the functional interaction of Cu²⁺ with BDNF, whole protein or peptides, was tested by measuring the effects on the proliferation rate of SHSY5Y neuroblastoma cell line culture.

Results and Discussion

Protonation constants and conformational features of BDNF(1–12), BDNF(1–12)D3N and Ac-BDNF(1–12): Protonation constants of the studied peptides were determined by potentiometric titrations and are reported in Table 1; the ligands show four (BDNF(1–12)) and three (Ac-BDNF(1–12), BDNF(1–12)D3N) proton-accepting centers, as expected.

Table 1. Protonation constants ($\log f_{\text{pH}}$) and $pK_a$ values for BDNF(1–12), BDNF(1–12)D3N and Ac-BDNF(1–12) ($T = 298 \, K$, $I = 0.1 \text{mol dm}^{-3}$ KNO₃).[a]

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<th>Ac-BDNF(1–12)</th>
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[a] Standard deviations (3σ values) are given in parentheses. Charges are omitted for clarity.

The amino group is the most basic center of BDNF(1–12) and BDNF(1–12)D3N. The two protonation equilibria of the aspartic and glutamic residues in BDNF(1–12) and Ac-BDNF(1–12) partly overlap; the comparison with the $pK_a$ value of glutamate residue of BDNF(1–12)D3N suggests that the lowest $pK_a$ values of the wild-type peptides belong to the aspartic residue. This assessment is in agreement with the general finding indicating that aspartic β-carboxylic group is more acidic than glutamic γ-carboxylic one. The $pK_a$ value obtained agrees with that found for other peptides containing aspartic and glutamic residues.[41–43] Taking into account the small differences in the $pK_a$ values of BDNF(1–12) and the mutated BDNF(1–12)D3N, an electrostatic interaction between the aspartic carboxyl group and the amino group of the histidine residue can be ruled out. The imidazole basicity of the histidine residue in BDNF(1–12) and BDNF(1–12)D3N is lower than that displayed by the heterocyclic residue in the acetylated peptide Ac-BDNF(1–12). This difference in the $pK_a$ values originates from electrostatic interaction with the more basic amino group, an effect observed in other peptides encompassing the histidine in the first position of the sequence.[44]

Parallel tempering simulations confirm the potentiometric data. The conformations of BDNF(1–12), Ac-BDNF(1–12) and BDNF(1–12)D3N span extended and bent states, shown in Figure 1, which are featured by intra-chain hydrogen bonds. The N-terminal amine group is prone to form hydrogen bonds involving Nε nitrogen of His-1 in those peptides which are not N-terminally blocked, as BDNF(1–12) and BDNF(1–12)D3N (Figure 1) justifying the different $pK_a$ values of imidazole in comparison with the acetylated peptide. Moreover, the carboxyl groups of Asp-3 belonging to BDNF(1–12) and Ac-BDNF(1–12) form hydrogen bonds with Arg-6 or Arg-7 (Figure 1b and 1c of Ac-BDNF(1–12)). The former weak interactions are lost in Ac-BDNF(1–12) (concerning the amine group) and in BDNF(1–12)D3N (concerning the Asp-3 residue; Figure 1).

On the whole, the investigated peptides do not display a definite secondary structure region and the far-UV CD spectra confirm that all three peptides adopt predominantly a random coil conformation over the pH range investigated (4–11) (data not shown). On the other hand, the intra-chain hydrogen bond between the N-terminal group and the histidine residue involves only the first amino acid residue and the electrostatic interaction between the aspartate carboxylate group and the arginine guanidine group is present at all the pH ranges investigated (pH 4–11). Therefore, the absence of variations, at increasing pH values, is justified.

The addition of Cu²⁺ does not induce significant changes on the secondary structure region of BDNF(1–12) and BDNF(1–12)D3N peptides, in contrast to that observed for the acetylated fragment Ac-BDNF(1–12). In the Cu-BDNF(1–12) system a maximum at 215 nm appears at increasing pH values and the apo-subtracted difference spectra show a slight increase of a turn structure determined by the binding of copper(II) amide nitrogen atoms (Figure 2a and inset).

This effect is less evident in the analogous system with the mutated peptide BDNF(1–12)D3N (Figure 2b). The copper(II) addition to the Ac-BDNF(1–12) peptide induces a gradual loss of intensity of the negative signal at 199 nm and the appearance of a positive band at 210 nm (Figure 2c). The CD spectra differences indicate a clear increase of the turn conformation of the peptide, resulting from the involvement of backbone amide nitrogen atoms in Cu²⁺ binding.[45]

The comparison of CD data indicate that the involvement of the amino group in copper(II) binding in BDNF(1–12) and BDNF(1–12)D3N (see next section) induces less conformational change in the peptide backbone in comparison to the acetylated peptide fragment.

Speciation, stability constants, and coordination modes of Cu²⁺ complexes with BDNF(1–12), BDNF(1–12)D3N, and Ac-BDNF(1–12): The overall stability constant values of the metal complexes with the peptides are listed in Table 2, whereas the distribution diagrams are reported in Figure 3.

The first species formed by BDNF(1–12) is [CuH₂L]⁺. The stepwise stability constant value calculated for this complex species ($\log K = \log K_{121} = \log K_{231} = 3.43$) suggests the involvement of one nitrogen atom without the assistance of a carboxyl group.[46,47]
Taking into account the strong acidic pH range of this species formation, it is possible that the glutamate carboxylate group is still protonated, with one between amino and imidazole nitrogen atom deprotonated.\footnote{48} Due to its low percentage of formation, it is not possible to determine its spectral parameters.

The stability constant value calculated for the next complex species $[\text{CuHL}]^+$ ($\log K = \log b_{111} - \log b_{011} = 5.74$) indicates the coordination of two nitrogen atoms to the metal ion.\footnote{47} The log$K$ value calculated for the analogous species formed with single mutated peptide BDNF(1–12)D3N is similar ($\log K = \log b_{111} - \log b_{011} = 5.31$) and in agreement with the hypothesis of a histamine-like coordination of copper(II) ion, with the glutamate group still protonated. The slightly higher value for BDNF(1–12) is due to a weak interaction of aspartic carboxylate group in copper(II) binding. However, the simultaneous presence of other complex species does not permit the determination of spectroscopic parameters.

![Figure 1](image_url). The three main clusters of BDNF(1–12), Ac-BDNF(1–12), and BDNF(1–12)D3N obtained from Parallel Tempering molecular dynamics simulations. The hydrogen bonds involving the N-terminal amine and the N$\gamma$ nitrogen of His-1 can occur only in BDNF(1–12) (a and c) and BDNF(1–12)D3N (a–c). The hydrogen bonds involving Asp-3 and Arg-6 or Arg-7 can occur only in BDNF(1–12) (b and c) and Ac-BDNF(1–12) (b and c). Carbon atoms are shown in black, nitrogen atoms in blue, and oxygen atoms in red spheres.
Increasing the pH, the deprotonation of the glutamate γ-carboxylate group occurs and this is not bound to the metal ion in the [CuL] \( (\log K = \log K_{11} - \log K_{10} = 4.25; pK_{COOH} = 4.22) \). The stability constant value for the analogous species formed by BDNF(1–12)D3N is similar and also in this case the logK deprotonation step corresponds to the pK value of glutamic carboxylate group (Table 1).

However, the UV/Vis parameters \( (\lambda_{max} = 648 \text{ nm}; \epsilon = 55 \text{ mol}^{-1} \text{ dm}^{3} \text{ cm}^{-1}; \) see Table 3) indicate a stronger ligand field around copper(II) ion for Cu-BDNF(1–12) system, which is in comparison with the analogous species formed by BDNF(1–12)D3N, indicating the formation of a CuN\textsubscript{O} chromophore with the metal ion bound to two nitrogen atoms and one carboxylate (\( N_{\text{Nef}}, N_{\text{im}}, COO^{-}_{\text{Asp}} 2O_{\text{water}} \); Figure 4).

The UV/Vis parameters of [CuL]\(^+\) formed by BDNF(1–12)D3N are similar to those reported for the analogous species formed by histamine and His–Gly in which the metal ion shows a \( N_{\text{Nef}}, N_{\text{im}} \) coordination mode (Figure 4).\(^{[48-50]}\) Moreover, for this last species it was possible to determine the EPR parameters at pH 5 \( (g_{\perp} = 2.283 \text{ and } A_{\perp} = 179 \times 10^{-4} \text{ cm}^{-1}) \), which confirm a histamine-like coordination.\(^{[49]}\) Noteworthy, in the case of Cu-BDNF(1–12) system, it was not possible to determine EPR parameters for the poor resolution of the spectra up to pH 6. The reason for a similar \( \log \beta \) value for these two copper(II) complex species, in which the metal ion display a different coordination environment, lies in the loss of different intramolecular weak interactions between the two peptides as determined by parallel tempering simulations (see previous section). Therefore, the increase of the stability constant determined by the coordination of the amino group to metal ion is counterbalanced by the break of electrostatic bonding between the Asp-4 carboxylate and guanidine group of Arg-6 and/or Arg-7.

The CD parameters, determined by combined pH-metric and spectroscopic titrations (see the Experimental Section) for [CuL] species (Table 3) are similar to those reported for other copper(II) complexes, in which the metal ion is bound to donor atoms in a macrochelate ring, without the involvement of the peptide backbone.\(^{[51]}\) The copper(II) complex with BDNF(1–12) shows a band at 270 nm due to the presence of the amino group in the metal ion coordination environment.\(^{[52]}\) Further support for the amino group binding is provided by the absence of this band in the analogous [CuL] species formed by the acetylated peptide Ac-BDNF(1–12).

[CuL] is the first complex species formed by Ac-BDNF(1–12), with a \( (N_{\text{im}}, COO^{-}_{\text{Asp}} 2O_{\text{water}} \) coordination mode. This copper complex species prevails up to pH 6.5. The stability constant value is 4.03, a value higher than that generally reported for the coordination of only one imidazole nitrogen, which indicates that metal binding of aspartate β-carboxyl group occurs besides water oxygen atoms (Figure 4). It is interesting to note that the \( \log \beta \) value determined is lower to that reported for analogous system in which copper ions display a similar coordination environment and a similar membered macrochelate ring\(^{[53,54]}\) but also in this case the \( \log \beta \) value is influenced by the break of the weak electrostatic interaction between the carboxylate and the guanidine group present in the apo-ligand. The EPR parameters reported in Table 3 \( (g_{\perp} = 2.323 \text{ and } A_{\perp} = 160 \times 10^{-4} \text{ cm}^{-1}) \), confirm the involvement of both the carboxylate group and the imidazole nitrogen atom in metal binding.\(^{[53-55]}\) The parallel hyperfine coupling constant value \( (A_{\perp}) \) is higher than those reported for other CuNO\textsubscript{3} chromophores, in which the metal is coordinated by an imidazole nitrogen and a carbonyl oxygen atom, being indicative of a

Figure 2: Far-UV CD spectra at pH 5 (---), 7.4 (---), and 10 (-----) of copper(II) complexes with a) BDNF(1–12), b) BDNF(1–12)D3N, and c) Ac-BDNF(1–12). Inset: The apo-subtracted difference spectra.

stronger equatorial field due to the negative charge of the carboxylate oxygen.\[^{[39]}\]

The involvement of the glutamic carboxylate group in the metal ion coordination can be ruled out for all studied peptides, taking into account the primary sequence (the aspartic is in third position, whereas Glu is the ninth amino residue). However, in general the glutamic γ-carboxylic group has a lower influence in comparison with the shorter β-carboxylic side chain group of the aspartic residue.\[^{[40]}\]

The formation of \([\text{CuH}_2\text{L}]\) involves the deprotonation of an amide nitrogen atom. The pK value (Table 2) for the Cu-BDNF(1–12) system indicates that this deprotonation is favored by the coordination of the carboxylate group. The pK value of the analogous species formed with the mutated BDNF(1–12)D3N is one logarithmic unit higher (Table 2). The UV/Vis parameters calculated by means of titration experiments (\(\lambda_{\text{max}} = 555 \text{ nm} \); \(\varepsilon = 102 \text{ mol}^{-1} \text{ dm}^{3} \text{ cm}^{-1} \)) show a strong blueshift in comparison with the \([\text{CuL}]\) species. The amino and imidazole nitrogens, one deprotonated amide nitrogen, and the carboxylate group bind Cu\(^{2+}\). This coordination mode results in a slightly distorted geometry: the three nitrogen donors cannot contemporary coordinate in the equatorial plane without give rise to a significant distortion of metal coordination geometry.\[^{[49]}\] The EPR parameters determined at pH 6.5, in which the \([\text{CuH}_2\text{L}]^-\) is the predominant species, are indicative of a planar disposition of at least three nitrogen atoms. Indeed, the UV/Vis spectra obtained for this species display a wide band caused by the presence of two isomers in which the metal has a different coordination environment (Figure 5).

An analogous system in which the copper(II) ion is bound to \(\text{N}_{\text{Asp}}, \text{N}_{\text{am}}, \text{N}^+\), and \(\text{COO}^-\) shows a \(\lambda_{\text{max}}\) value of around 600 nm.\[^{[39]}\] The comparison with spectroscopic data UV/Vis and CD assigned to the \([\text{CuH}_2\text{L}]^-\) species indicate that one isomer may be \([\text{CuH}_2\text{L}(\text{H})]^-\) with two deprotonated amides and the imidazole protonated. The deprotonation and coordination of the second amide nitrogen results in the formation of a very stable \([\text{CuH}_2\text{L}]^-\) species, with a \([5,5,5]\) chelation ring, due to the presence in the third position of an aspartic residue (Figure 6).\[^{[57,58]}\] The EPR parameters are unchanged in the pH range 6.5–10 and strongly support the absence of the ligand in an axial position. The UV/Vis parameters (Table 3) are in agreement with the presence of \(\text{N}_{\text{His}}, \text{N}^+\), COO\(^{-}\) donor atoms in the metal equatorial plane, and the CD spectrum shows an increase of the \(\mathbf{N}^-\rightarrow\mathbf{Cu}^{2+}\) charge transfer band at 355 nm, whereas the band centered at 270 nm is unaffected.

To evaluate the metal coordination geometry in \([\text{CuH}_2\text{L}]^-\) and \([\text{CuH}_2\text{L}]^{2-}\), DFT-based geometry optimizations were carried out. The coordination polyhedron parameters of the \([\text{CuH}_2\text{L}]^-\) and \([\text{CuH}_2\text{L}]^{2-}\) species are reported in Table S1 (the Supporting Information). The minimum energy structures of the former complexes predicted through DFT are reported in Figure 7. \([\text{CuH}_2\text{L}]^-\) adopts a type-II tetragonal coordination geometry in which Cu\(^{2+}\) is bound to \(\text{N}_{\text{His}}, \text{N}^+\), COO\(^{-}\) and the metal ion lies in the ligand square plane, in agreement with experimental data. On the other hand, in the \([\text{CuH}_2\text{L}]^-\) species, the copper(II) coordination environment with lower energy involves the N-terminal amine group and the backbone nitrogen of His-1, the carbonyl oxygen of Ser-2, and carboxylate side-chain of Asp-3, adopting a type-II distorted tetragonal coordination geometry.

The highest deviation from the planarity in the \([\text{CuH}_2\text{L}]^-\) is observed in the carbonyl oxygen of Ser-2 and in the carboxylate of Asp-3 (Figure 7a and Table S1, the Supporting Information). The former ligands are involved in a macrochelate ring upon the coordination to Cu\(^{2+}\). In particular, at variance with the planar \([\text{CuH}_2\text{L}]^{2-}\) polyhedron, the \([\text{CuH}_2\text{L}]^-\) complex is identified by a larger \(\text{NH}_2\text{Cu-O}_{\text{Asp}}\) bonding angle (111.3 versus 95.5\(^\circ\) in \([\text{CuH}_2\text{L}]^{2-}\)). Moreover, the improper torsion of the \([\text{CuH}_2\text{L}]^-\) coordination plane deviates 28.4\(^\circ\) from the planarity, whereas only 5.1\(^\circ\) is observed in the \([\text{CuH}_2\text{L}]^{2-}\) species.

The \([\text{CuH}_2\text{L}]^-\) is only a minor species for BDNF(1–12)D3N and its spectroscopic parameters cannot be determined. Also the next deprotonation step is higher in comparison with the analogous species of BDNF(1–12). The spectroscopic parameters suggest the involvement in the binding of \(\text{N}_{\text{His}}, \text{N}^+\), and carbonyl group, ruling out the presence of imidazole nitrogen atom coordinated in axial position (Table 3) \[^{[49]}\]

The characterization of the copper(II) complex species formed by Ac-BDNF(1–12) highlights the main role played by the amino group in the metal binding to BDNF(1–12). The stability constant values of Cu-Ac-BDNF(1–12) reveal that the macrochelate formation in this case delays the first amide nitrogen deprotonation step and the metal ion coordination of amide group in the \([\text{CuH}_2\text{L}]^-\). This is due to the different size of chelate rings \(7,5\), which occurs when the imidazole is the anchoring site and the deprotonation takes place towards the C-terminus.\[^{[48–49]}\] The pK of the second amide deprotonation, owing to the metal complex formation, is very similar to the first, whereas generally it is at

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Table 2. Stability constants (log_\(pK_{\text{app}}\)) and pK values of Cu\(^{2+}\) complexes with BDNF(1–12), BDNF(1–12), and Ac-BDNF(1–12) (\(T = 298 \text{ K}, I = 0.1 \text{ mol dm}^{-3} \text{ KNO}_3\)).\[^{[39]}\]

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</tr>
<tr>
<td>(pK(-2/-3))</td>
<td>6.93</td>
<td>7.37</td>
<td>6.84</td>
</tr>
<tr>
<td>(pK(-3/-4))</td>
<td>8.31</td>
<td>8.72</td>
<td>9.48</td>
</tr>
</tbody>
</table>

[a] Standard deviations (30 values) are given in parentheses. Charges are omitted for clarity; \(pK(n/m)\) values reflect the pK value of copper(II) complexes.
least one logarithmic unit higher.\cite{58-60} The metal ion coordination mode is characterized by the involvement of the N_{Im}, 2N\textsubscript{1}/C\textsubscript{0}, COO\textsubscript{1}/C\textsubscript{0} donor atoms (Figure 6). The UV/Vis parameters are very different in comparison with those reported for analoguous species formed by BDNF\textsubscript{A C H T U N G T R E N N U N G} (1–12) (Table 3). This is clearly due to the formation of a sequence of [7,5,6]-membered chelate rings in which the more basic amino group is substituted by imidazole nitrogen. These parameters are similar to those reported for analogous species formed with other acetylated peptides and consistent with a CuN\textsubscript{3}O\textsubscript{1} coordination mode involving one imidazole and two amide deprotonated nitrogen atoms.\cite{53,61-63} The relatively high $\lambda_{\text{max}}$ and $\varepsilon$ value, indirectly suggests an involvement of the carboxylate group in binding to Cu\textsuperscript{2+}. The EPR parameters (Table 3) indicate an apical interaction in the copper(II) complexes. In fact, differently to that observed for the Cu-BDNF system, the different chelate ring size induces a lower rigidity of the equatorial plane formed by donor atoms. The relatively high value of hyperfine coupling con-
Table 3. Spectroscopic parameters of copper(II) complexes with BDNF(1–12), BDNF(1–12)D3N, Ac-BDNF(1–12) [4].

<table>
<thead>
<tr>
<th>L Species</th>
<th>UV/Vis CD</th>
<th>EPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHL</td>
<td>λ [nm]</td>
<td>δ_1 [10^{-4} cm^{-1}]</td>
</tr>
<tr>
<td>BDNF (1–12)</td>
<td>648 (44)</td>
<td>304 (–0.156); 686 (0.255); 502 (0.138); 686 (0.181)</td>
</tr>
<tr>
<td>1–11</td>
<td>555 (102)</td>
<td>312 (0.564); 502 (0.204)</td>
</tr>
<tr>
<td>1–21</td>
<td>540 (128)</td>
<td>310 (0.568); 500 (0.298)</td>
</tr>
<tr>
<td>1–31</td>
<td>540 (130)</td>
<td>310 (0.568); 500 (0.298)</td>
</tr>
<tr>
<td>BDNF (1–12)</td>
<td>670 (40)</td>
<td>334 (–0.113); 701 (0.129)</td>
</tr>
<tr>
<td>D3N</td>
<td>11–1</td>
<td>300 (–0.495); 639 (0.72)</td>
</tr>
<tr>
<td>1–21</td>
<td>550 (140)</td>
<td>313 (0.903); 490 (0.222); 585 (0.225)</td>
</tr>
<tr>
<td>1–31</td>
<td>525 (130)</td>
<td>310 (0.696); 491 (0.495); 565 (0.452)</td>
</tr>
<tr>
<td>1–41</td>
<td>525 (130)</td>
<td>308 (0.610); 490 (0.492); 565 (0.614)</td>
</tr>
<tr>
<td>Ac-BDNF (1–12)</td>
<td>708 (51)</td>
<td>305 (0.319); 361 (–0.100)</td>
</tr>
<tr>
<td>1–11</td>
<td>–</td>
<td>305 (–1.051); 357 (0.886); 511 (0.100); 581 (–0.104); 649 (0.319)</td>
</tr>
<tr>
<td>1–21</td>
<td>620 (110)</td>
<td>305 (–1.402); 357 (1.211); 491 (0.104); 567 (–0.252); 653 (0.369)</td>
</tr>
<tr>
<td>1–31</td>
<td>581 (114)</td>
<td>301 (–0.506); 352 (0.601); 521 (–0.626); 663 (0.357)</td>
</tr>
</tbody>
</table>

[a] [L] = 1 × 10^{-4} mol dm^{-3}. Errors in λ = (+2 nm) and ε = 5%. Charges are omitted for clarity.

...ant and theoretical studies carried out on similar system, suggest that the β-carboxylate group is coordinated in equatorial position in a tetrahedrally distorted square planar arrangement. [64]

By increasing the pH, other deprotonated species are formed. These are not relevant for the biological activity, taking into account that their maximum percentage formation is over pH 9. The deprotonation step in the [CuH_2L]~ formation should involve: 1) the amide nitrogen atom of the fifth residue, alanine, 2) water hydrolysis, or 3) the guanidine group of arginine. In the Cu-BDNF(1–12) system such a species displays the same spectroscopic parameters of the previous [CuH_2L]~*, indicating the same coordination environment for the metal ion, so the deprotonation may involve a group not involved in metal binding. On the contrary, the [CuH_2L]^+ species formed by mutated BDNF(1–12)D3N shows a blueshift in the λ_{max} and a decrease of the δ_1 value, indicating the formation of a 4N chromophore with the deprotonation of the alanine amide or arginine guanidine group. [65,66]

The presence of an aspartyl carboxylate side chain in the third position explains the difference observed between BDNF(1–12) and BDNF(1–12)D3N; the carboxylate coordination to the copper(II) ion facilitates the cooperative deprotonation of the preceding two amide functions, but precludes the deprotonation of the subsequent ones. The [CuH_2L]~ formed by Ac-BDNF(1–12) shows a blueshift and a decrease of ε. The spectroscopic parameters indicate the presence of a 4N chromophore so involving the deprotonation of the acetyl nitrogen atom. The hyperfine coupling constant is lower than that reported for the analogous complex species with a similar coordination mode due to the binding of imidazole nitrogen in axial position, as confirmed by the presence also for this species of the N_{im}→Cu^2+ charge transfer band at 355 nm in the CD spectrum.

Proliferative effects of BDNF protein and its N-terminus peptide fragments BDNF(1–12) on SHSY5Y cells: Neurotrophins and their receptors are known to be involved in the proliferation and survival of various cell types either neuronal or non-neuronal, in physiological as well as pathologic conditions. [60] The proliferative effect of BDNF(1–12), BDNF(1–12)D3N, Ac-BDNF(1–12) was tested on neuronal undifferentiated cell cultures (neuroblastoma SHSYSY) at 60–70% confluence, and compared to that obtained by the whole protein BDNF both in the presence and in the absence of copper(II) ions. After 72 h of treatment with BDNF full protein or peptides, a general increase of the cell number was observed (Figure 8).

The results indicate that these peptides can mimic the activity of the BDNF protein under these conditions, although the proliferative effect is achieved when the peptide concentration is 100-fold higher than the whole protein, as reported for the peptide fragment encompassing the N-terminal domain of the NGF protein. [69] It is interesting to note that Ac-BDNF(1–12) and BDNF(1–12)D3N show a higher proliferative effect than that of the wild-type BDNF(1–12). This finding can be rationalized in a different interaction with the TrkB receptor due to different intramolecular/intermolecular interactions.

Notably, a computational modeling approach revealed that BDNF(1–12), BDNF(1–12)D3N and Ac-BDNF(1–12) peptides when docked to domain 5 of TrkB receptor (TrkB-D5) show similar binding poses to those of the N-terminal sections of neurotrophin 4/5 (pdb code 1HCF). [70] All the peptides tend to maintain their intramolecular interactions, such as those involving Asp-3 and the N-terminal amine...
(Figure 9). Binding poses involving Arg-6 or Arg-7 interacting with Asp-298 of TrkB-D5 are also observed (Figure 9).

In particular, when Asp-3 is mutated with Asn, this last residue tends to avoid a direct facing with TrkB-D5 (Figure 9), but, at the same time, the Arg residue can easily interact with Asp-298, explaining its higher activity in comparison with wild-type BDNF(1–12). On the other hand, the higher activity of acetylated peptide may be related to the absence of protonated amino group and its consequent higher hydrophobicity.

To evaluate the contribution of the copper(II) ions to BDNF proliferative activity, neuroblastoma cell cultures were treated with a mixture of metal/BDNF and metal/BDNF peptides (Figure 8). The Cu$^{2+}$ addition inhibited the proliferation induced by BDNF(1–12) by about 50%, reproducing similar effect on whole protein. This effect is opposite to that reported for NGF protein and the peptide NGF(1–14) encompassing the N-terminal domain, in which a synergic proliferative activity is observed.\[69\]

A different behavior is observed for the acetylated and single point mutated peptides: copper addition to Ac-BDNF(1–12) does not significantly affect its proliferative activity, whereas it significantly enhances that of mutated BDNF(1–12)D3N. The distribution diagram for Cu-Ac-BDNF(1–12), carried out at 10 mM (1:1 molar ratio, Figure 1S, the Supporting Information), shows that at physiological pH, the free ligand (4.3/C14810/C0 6 moldm$^{-3}$) and Cu$^{2+}$ (4.7/C14810/C0 6 moldm$^{-3}$) are present. Therefore, the proliferative activity of the Cu-Ac-BDNF(1–12) system is practically determined by the sum of free Ac-BDNF(1–12) and Cu$^{2+}$ activity. The distribution diagram of Cu-BDNF(1–12)D3N, as well as Cu-BDNF(1–12), at 1/C14810/C0 5 moldm$^{-3}$ (Figure 1S, the Supporting Information) show that the metal ion is bound to the ligand, at physiological pH, and the [CuH$_2$L] and [CuH$_2$L] complex species are predominant. Thus, the great difference in the proliferative activity of Cu-BDNF(1–12)D3N with that of Cu-BDNF(1–12) system could be due to the different metal-ion coordination environment.

We can speculate that copper(II) binding to BDNF(1–12)D3N peptide induces the Arg residue to face more towards the receptor binding site. Computational and experimental works are in progress to for a full understanding of the proliferative activity of copper(II) complex species of investigated peptides. However, it is evident that a prominent
role is played by the amino terminus and aspartic carboxylate groups.

**Conclusion**

The neurotrophin (NT) family comprises the nerve growth factor (NGF), the brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). These are small, structurally related proteins essential for neuronal survival and axonal guidance that play key roles in cognition and memory formation. The activity of each NT is overlapped to that of the others but every neurotrophin has its own specialization and localization in the brain. The homeostasis of the NT is altered during the pathogenesis of different neurodegenerative disorders, in particular Alzheimer’s disease, as well as that of metal ions. Metal ions can directly modulate the activity of the neurotrophins, likely through conformational changes, and/or indirectly by activating their downstream signaling in a neurotrophin-independent mode. In particular the N-terminal domain of the protein represents the main metal-ion binding region. Such domain encompasses recognition site of Trk receptor that is specific for each NT. In previous work, we have shown how the metal-ion coordination environment can drive the proliferation activity of a peptide fragment, owing to the N-terminal domain of NGF, as well as that of the whole NGF protein.[69]

Here we report the characterization of the Cu2⁺ complexes with the peptide fragment BDNF(1–12) encompassing the N-terminal sequence 1–12 of BDNF, by means of potentiometry, EPR, CD, UV/Vis spectroscopic techniques, and DFT-geometry optimizations. We demonstrate that the predominant Cu2⁺ complex species, at physiological pH, is the [CuH₂L₂]²⁻ in which the metal ion is bound to an amino, two amide nitrogen atoms and a carboxylate group (NH₂, 2N, COO⁻₉₄₈₈) in a planar environment. The comparison with the Cu2⁺ complex species formed by the acetylated peptide Ac-BDNF(1–12) and the single point mutated BDNF(1–12)D₃N, in which the aspartate has been substituted by an asparagine residue, were helpful to put into light the essential role of the amino and carboxylate group in metal binding. The peptide BDNF(1–12) displays proliferative activity at μM concentrations and as the whole protein at nM concentration. Interestingly, Ac-BDNF(1–12) and BDNF(1–12)D₃N peptides display higher proliferative activity than wild-type BDNF(1–12). Parallel tempering and docking simulations show that Ac-BDNF and BDNF(1–12)D₃N may interact better with the receptor recognition site due to the absence of a determined intramolecular interaction involving the N-terminal amino group and/or the aspartate carboxylate side chain. The addition of Cu²⁺ induces a decrease in the proliferative activity of BDNF(1–12) as well as of that of the whole protein. This effect is opposite to that observed for the analogous peptide NGF(1–14) and the NGF protein.[69] Although the biological tests presented here are to be regarded as preliminary results, undoubtedly they indicate that Cu²⁺ may differently drive the activity of the two neurotrophins NGF and BDNF.
Experimental Section

Materials: All NovaSyn-TGR resin, N-fluoromethylthoxycarbonyl (Fmoc)-protected amino-acids, and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), were obtained from Novabiochem (Switzerland); N,N-Diisopropyl-ethylamine (DIEA), N,N-di-methylformamide (DMF, peptide synthesis grade) and 20% piperidine-DMF solution were from Applied Biosystems; N-hydroxybenzotriazole (HOBt), triisopropylsilane (TIS), trifluoroacetic acid (TFA), 3-morpholino-propan-1-sulfonic acid (MOPS) were purchased from Sigma/Aldrich. All the other chemicals were of the highest available grade and were used without further purification.

Peptide synthesis and purification: The peptides HSDPARRGELSV-NH₂, Ac-HSDPARRGELSV-NH₂, Ac-BDNF(1–12), and HisNPARRGELSV-NH₂:BDNF(1–12)D3N were assembled using the solid phase peptide synthesis strategy on a Pioneer Peptide Synthesizer. All amino acid residues were added according to the TBTU/HOBT/DIEA activation method for Fmoc chemistry on a NovaSyn-TGR resin (loading 0.18 mmol g⁻¹, 0.33 mmol scale synthesis). Other experimental details have already been described.⁴⁵ The peptides were purified by means of a preparative reversed-phase high-performance liquid chromatography (RP-HPLC). Purification was performed on a Varian PrepStar 200 model SD-1 chromatography system equipped with a ProStar photo diode array detector with detection at 222 nm. They were eluted with solvent A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) on a Vydac C₁₈ 250 x 22 mm (300 Å pore size, 10–15 μm particle size) column, at a flow rate of 0.2 mL min⁻¹. Analytical RP-HPLC analyses were performed using an Agilent 1200 series instrument, equipped with a DAD detector. Samples were analyzed using gradient elution with solvent A and B on a Vydac C₁₈ 250 x 4.6 mm (300 A Å pore size, 5 μm particle size) column, at a flow rate of 1 mL min⁻¹. The peptides were eluted according to the following protocol: from 0 to 5 min isocratic gradient in 0% B, then linear gradient from 0 to 15% B over 25 min, finally isocratic gradient in 15% B from 25 to 40 min. The peptides were characterized by means of electron spray ionization mass spectrometry (ESI-MS).


Potentiometric titrations: Potentiometric titrations were performed with two home-assembled fully automated apparatus sets (Metrohm 6564 pH-meter, combined micro pH glass electrode, Orion 9103SC, Hamilton digital dispenser, Model 665) controlled by the appropriate software set up in our laboratory. The titration cell (2.5 mL) was thermostatted at (298.0±0.2) K, and all solutions were kept under an atmosphere of argon, which was bubbled through a solution having the same ionic strength and temperature as the measuring cell. KOH solutions (0.1 mol dm⁻³) were added through a Hamilton burette equipped with 1 cm² syringes. The ionic strength of all solutions was adjusted to 0.10 moldm⁻³ (KNO₃). To determine the stability constants, solutions of the ligands (protonation constants) or the ligands with Cu²⁺ (copper(II) complex constants) were titrated by using 0.1 mol dm⁻³ potassium hydroxide. The peptide concentration ranged from 1.8 to 3.0 x 10⁻⁵ and from 3.0 to 4.0 x 10⁻⁵ mol dm⁻³ for the protonation and complexation experiments, respectively. A minimum of three independent runs were performed to determine the protonation constants, whereas four independent experiments were run for the copper(II) complexation constants. The initial pH was always adjusted to 2.4. To avoid systematic errors and verify reproducibility, the EMF values of each experiment were taken at different time intervals. Other details were as previously reported.⁴⁵ To obtain protonation and complexation constants, the potentiometric data were refined using the HYPERQUAD program,⁴⁶ which minimizes the error square sum of the measured electrode potentials through a nonlinear iterative refinement of the sum of the squared residuals, U, and also allows for the simultaneous refinement of data from different titrations: 

\[ U = \sum(E_{\text{exp}} - E_{\text{calc}})^2 \]

in which \( E_{\text{exp}} \) and \( E_{\text{calc}} \) are the experimental and calculated electrode potentials, respectively. Errors in stability constant values are reported as three times standard deviations. The formation reaction equilibrium of ligands with protons and copper(II) ions is given in Equation (1):

\[ pCu + qH + rL = CuH_qL_r \]

in which L are the peptides under study. The stability constant \( \beta_{qpr} \) is defined in Equation (2):

\[ \beta_{qpr} = [CuH_qL_r]/[Cu^{q+}]:[H]^r:[L]^r \]

The species distribution as a function of the pH was obtained by using the computer program Hysys.⁴⁷

Spectroscopic studies: UV/Vis measurements: UV/Vis spectra were recorded at 25°C, by using an Agilent 8453 or a Varian Cary 500 spectrophotometer. The concentrations of the peptides and copper(II) used to record absorption spectra were the same as those for the potentiometric titrations. Combined spectroscopic and potentiometric metal-complex titrations were performed into a 3 mL quartz cuvette with a 1 cm path length to get the spectrum in the visible region at each pH value simultaneously. These experiments were replicated at least three times for each copper-peptide system. Spectroscopic data were processed by using the HYPERQUAD program.⁴⁷

CD measurements: CD spectra were obtained at 25°C under a constant flow of nitrogen on a Jasco model 810 spectropolarimeter at a scan rate of 50 mm min⁻¹ and a resolution of 0.1 nm. The path lengths were 1 or 0.1 cm, in the 190-800 nm range. The spectra were recorded as an average of 10 or 20 scans. Calibration of the instrument was performed with a 0.06% solution of ammonium camphorsulfonate in water. The CD spectra of the copper(II) complexes on varying the solution pH were obtained in both the 240–400 and 300–800 nm wavelength regions. All the solutions were freshly prepared by using double distilled water. The copper(II) ion and peptide concentrations used for the acquisition of the CD spectra in the visible region were identical to those used in the potentiometric titrations. CD spectra in the region 240–400 were acquired by using copper(II) ion and peptide concentrations of 5.0 x 10⁻⁵ mol dm⁻³. The results are reported as e (molar absorption coefficient) and 𝛿 (molar dichroic coefficient) in mol⁻¹ dm³ cm⁻¹.

EPR measurements: A Bruker Elexys E500 CW-EPR spectrometer driven by a PC running the XEpr program under Linux and equipped with a Super-X microwave bridge, operating at 9.3–9.5 GHz, and a SHIF cavity was used throughout this work. All EPR spectra of frozen solutions of copper(II) complexes were recorded at 150 K by means of a ER4131VT variable temperature apparatus. EPR magnetic parameters were obtained directly from the experimental EPR spectra, always calculating them from the 2nd and the 3rd line to get rid of second order effects. Instrumental settings of EPR spectra recordings of the copper(II)-peptide complexes were as follow: number of scans 1–5; microwave frequency 9.344–9.376 GHz; modulation frequency 100 kHz; modulation amplitude 0.2–0.6 mT; time constant 164–327 ms; sweep time 2.8 min; microwave power 20–40 mW; receiver gain 1 x 10⁻⁶–2 x 10⁻⁶. Copper(II) complexes were prepared by addition of the appropriate amount of isotopically pure copper, taken from a 15Cu(NO₃)₂ 0.05 mol dm⁻³ solution, to the peptide solution. Copper(II) complexes solutions were prepared in 10% methanol-water mixture.

Computational methods: Parallel tempering simulations: BDNF(1–12), Ac-BDNF(1–12) and BDNF(1–12)D3N underwent 10 ns of parallel tempering (PT) simulations in explicit solvent with a total volume of 60 x 40 x 40 Å³, after have been equilibrated through 2 ns of MD in explicit solvent. GROMACS 4.5.5 package was used.⁴⁷ For all the systems the
N-terminal amine group was considered protonated, histidine was deprotonated at Nε, and aspartic acid was considered in the dissociated form. The overall charge of the system was neutralized by adding 1 chloride ion for BDNF(1–12), whereas 2 chloride ions were added for BDNF(1–12)D3N. Periodic boundary conditions were applied. The AMBER99SB force field was used for the biomolecules and counter ions, and the TIP3P force field was used for water molecules. Electrostatic interactions were calculated using the Particle Mesh Ewald method. A cutoff (0.9 nm) was used for the Lennard-Jones interactions. The time-step was set to 2 fs. All bond lengths were constrained to their equilibrium values using the SHAKE algorithm for water and the LINCS algorithm for the peptide. We simulated 64 replicas distributed in the temperature range 300–500 K following a geometric progression. All replicas were simulated in NVT ensemble using a stochastic thermostat with a coupling time of 0.1 ps. A thermostat that yields the correct energy fluctuations of the canonical ensemble is crucial in parallel tempering simulations of such systems. The resulting D.02 interface.

Docking simulations: The starting coordinates of domain-5 of TrkB (TrkB-D5) were taken from the X-ray structure of TrkB-D5 bound to neurotrophin 4/5 (pdb code 1HCF). The former complex was used as template for the alignment of the main PT simulation clusters of BDNF(1–12), Ac-BDNF(1–12) and BDNF(1–12)D3N prior to the docking to TrkB-D5. Docking simulations have been performed using HADDOCK interface. All residues of BDNF(1–12), Ac-BDNF(1–12) and BDNF(1–12)D3N were included as ‘active residues’ for the Haddock docking, as well as T280 to P311 and K326 to D349 belonging to TrkB-D5. Structures underwent rigid body energy minimization, semirigid simulated annealing in torsion angle space, with a final clustering of the results.

Density functional theory calculations: The starting coordinates were considered from the PT simulation clusters of BDNF(1–12), Ac-BDNF(1–12) and BDNF(1–12)D3N. We selected the ‘HSdp’ section, adding copper(II) ion and thereby deprotonating one amide of His-1 for the [CuH -L] and two amide of His-1 and Ser-2 for the [CuH2L] complexes. All DFT calculations were performed using the Gaussian 03 program (revision D02). The optimized geometries have been calculated using the PBE pseudopotential[10] and TZVP basis sets[11]. For both complexes frequency calculations were performed to ensure that the geometries identify local energy minima. Solvent effects were included through the polarized continuum model (PCM).[12]

Biological assays: Biological assays were performed on SHSY5Y neuroblastoma cell line cultures. The cells were maintained in Dulbecco’s modified eagle’s medium (DMEM; purchased from Lonza), supplemented with 10% fetal bovine serum (FBS; Lonza) and streptomycin in a humidified atmosphere of 95% air 5% CO2 at 37°C in an incubator (Hera Cell 150). SHSY5Y cells were treated with BDNF(1–12), (10–4 mol dm–3), and BDNF protein (10–5 mol dm–3) also in the presence of CuSO4 (10–6 mol dm–3). Treatments of SHSY5Y cells were performed in the same culture medium at 60–70% confluence. Proliferation was qualitatively estimated after 72 h through microscopy observation (LEICA DMI 4000 B) and quantitated through cell counting, after 72 h. This was achieved by Hoechst staining of nuclei and analyses of the captured images using the tool provided by Image-J, image analyses software (NIH).

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