Benzodifurans for biomedical applications: BZ4, a selective anti-proliferative and anti-amyloid lead compound

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Aim: Our goal is to evaluate benzodifuran-based scaffolds for biomedical applications. Methodology: We here explored the anticancer and anti-amyloid activities of a novel compound (BZ4) in comparison with other known benzodifuran analogs, previously studied in our group, and we have explored its ability to interact with different DNA model systems. Results: BZ4 shows antiproliferative activity on different cancer cells; does not affect noncancerous control cells and alters the aggregation properties of β-amyloid, as ascertained by circular dichroism, fluorescence spectroscopy and scanning electron microscopy analysis. An overall, qualitative picture on the mechanistic aspects related to the biological activities is discussed in light of the dynamic light scattering, UV, circular dichroism and fluorescence data, as well as of the metal ion-binding properties of BZ4.

Graphical abstract:
Benzofuran derivatives are heterocyclic compounds found in natural sources, as well as synthetic materials, that have been extensively studied as therapeutic agents for several cancer forms, showing a marked therapeutic activity associated to their binding to receptors like estrogen receptor-α (ERα) [1] and to their potent aromatase inhibitory activity [2,3]. Due to the increasing need to develop new effective anticancer drugs and taking into account the great potential provided in this field by benzofuran compounds, we aimed at exploring the anticancer potential of their tricyclic analogs, benzodifurans. These compounds are mostly investigated in the field of new materials, especially as organic dyes in the development of solar cells [4], and only few studies have been addressed thus far at the investigation of their biological activity [5–7]. Recently, the synthesis and structural characterization of various substituted benzodifuran derivatives, as well as their biological activities have been reported in the literature. These compounds showed interesting antiproliferative effects on different cancer cell lines, also with cell-selective activity [8]. They formed nanoaggregates stable at physiological temperature, probably responsible for their bioactivity, as evidenced for other self-assembling biologically active molecules [9]. Among the three novel benzodifurans previously described, only BZ1 (Figure 1) was able to bind Cu²⁺ ions, suggesting that copper ion depriving ability is not a mechanism of action common to all these compounds and that probably each benzodifuran derivative exerts cytotoxic effects by virtue of specific mechanisms or different combinations of them [8]. Overall, these benzodifuran derivatives proved to be valuable lead compounds in anticancer therapies, but their poor solubility (in some cases limited also in dimethyl sulfoxide) impaired further investigations.

Thus, with the aim of improving the solubility properties of BZ1, that is the most promising compound among the previously investigated benzodifuran derivatives, we here designed a new analog with longer oligoethylene glycol chains, named BZ4 (Figure 1), which could confer better solubility properties and in general a good hydrophilic–lipophilic balance for the studied scaffold, as previously demonstrated in the case of other small molecules [10–12].

We here report the synthesis and characterization, by spectroscopic and spectrometric techniques, of BZ4, as well as the evaluation of its biological properties. In particular, in analogy with previous works and in continuation with studies on the search for effective antineoplastic small molecules [13,14], we explored the antiproliferative activity of BZ4 on different human cancer and normal control cells, comparing the observed effects with those previously associated to BZ1, BZ2 and BZ3 derivatives (Figure 1) [8]. Furthermore, aiming at shedding light on the potential biological targets and mechanisms at the origin of the biological activity of BZ4, we evaluated the ability of BZ4 to bind various DNA model systems (in single strand, duplex and G-quadruplex forms) and/or biologically relevant Cu²⁺ ions, as well as its aggregation properties.

Moreover, we evaluated also the interaction of BZ4 with the β-amyloid (Aβ) peptide, a peptide related to a number of pathologies, using different techniques. Aggregation and deposition of Aβ peptide leads to pathogenic plaques observed in the brain of Alzheimer’s disease (AD) patients, the most common form of neurodegenerative diseases [15]. Aβ40 and Aβ42 are the two prevailing isoforms of Aβ peptide in the human brain, of which the longer Aβ42 isoform more readily self-assembles into aggregates [15,16]. Aggregation of Aβ42 peptides is an early event in the disease progression of AD. Thus, any strategy interfering with the Aβ self-assembly is potentially of high therapeutic interest in order to retard or prevent AD onset [16]. Several compounds of different nature were identified in the past as effective inhibitors of amyloid formation [17,18], and among these, a pivotal role is played by aromatic or heteroaromatic compounds such as curcumin or quinone derivatives [19,20]. Furthermore, amyloid-induced toxicity on neuronal cells was prevented by microRNAs, sulfonates and liposomes [21–23], while gold nanoparticles were successfully employed to visualize Aβ42 aggregation, thus providing new tools in diagnostic and prognostic strategies for neurodegenerative disease treatment [24]. Interestingly, compounds containing benzofuran moieties have been described to bind amyloid plaques, as well as neurofibrillary tangles [25] and can play an important role in decreasing Aβ-induced toxicity by interfering with Aβ binding to plasma membrane [26], as well as inhibiting Aβ aggregation [27,28]. The latter effect on Aβ produced by benzofuran derivatives is closely related with their ability to directly bind the Aβ and is associated with their intrinsic self-aggregation tendency [29]. Additionally, a specific recognition site for benzofurans on Aβ, different from those of nonbenzofuran Aβ disaggregators, is involved during their interference process on amyloid fibril formation [29].
Benzofuran-based derivatives were previously shown to interact with *in vitro*-generated Aβ40 aggregates with subnanomolar binding affinity [30]. However, these compounds also showed slow wash out and long retention in the brains of control mice suggesting nonspecific interaction with brain constituents other than Aβ aggregates. Redesign of benzofuran-based derivatives aimed at retaining their high binding affinity but improving their selectivity is thus beneficial.

In this context, the introduction of a third fused furan cycle is a chemical modification of the starting benzofuran scaffold never tested before, whose effect on amyloid aggregation and toxicity on primary mouse cortical neurons is also the object of the present study.

Thus, aiming at the development of specific Aβ42-targeting neurodrugs, the anti-amyloid activity of benzodifurans, unprecedentedly explored, was here studied. The Aβ42 anti-aggregation potential of BZ4 as well as of the previously described benzodifurans [8] were evaluated by means of fluorescence assays (thioflavin T, ThT). The benzodifuran-Aβ42 interaction and consequent amyloid anti-aggregation effect were confirmed by circular dichroism (CD) and scanning electron microscopy (SEM) microscopy studies.

**Experimental section**

**Abbreviations**

Alzheimer’s disease (AD); circular dichroism (CD); dimethyl sulfoxide (DMSO); Dulbecco’s Modified Eagle’s Medium (DMEM); dynamic light scattering (DLS); fetal bovine serum (FBS); human cervix adenocarcinoma cells (HeLa); liver hepatocellular carcinoma (Hep-G2); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); human dermal fibroblast (HDF); liquid chromatography-electrospray ionization-mass spectrometry (LC–ESI/MS); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); nuclear magnetic resonance (NMR); oligodeoxyribonucleotide (ODN); phosphate-buffered saline (PBS); scanning electron microscopy (SEM); thioflavin T (ThT); melting temperature (Tm); total ion current (TIC); time of flight (TOF) and malignant melanoma (WM266).

**Materials & methods**

All chemicals and solvents were purchased from Sigma-Aldrich (Amsterdam, The Netherlands).

Human cervix adenocarcinoma cells (HeLa), liver hepatocellular carcinoma (Hep-G2) and malignant melanoma (WM266) cells were obtained from ATCC (Manassas, VA, USA). Human dermal fibroblasts (HDFs) were a kind gift from Dr Annalisa Tito (Arterra Biosciences, NA, Italy).
TLC analyses of the benzodifuran derivatives were performed on SiO2 aluminium-supported plates from Macherey–Nagel (60, F254 – from Delchimica S.G. Srl, NA Italy) using the mixture CH2Cl2/CH3OH 95:5 (v/v) as eluent.

All the oligonucleotide sequences listed below were prepared by using standard phosphoramidite solid phase synthesis (ABI Expedite 8909 synthesizer) on the scale of 1 μmol:

- d(5′ CCTCTGGTCTCC3′) (A);
- d[5′ GGAGACCAGAGG3′] (B);
- d[5′ TCACACACACACACACACACTT3′] (C);
- d[5′ (TTAGGG)4TT3′] (tel26).

Deprotection and detachment of the oligodeoxyribonucleotides (ODNs) from the resin were obtained by treatment with ammonia (aq.) at 55°C for 12 h, after which the oligomers were purified by C18 cartridges (GlenPack of GlenRes, purchased from Primm Srl, MI Italy), desalted by dialysis versus H2O and lyophilized.

1H and 13C NMR spectra were recorded on a Bruker WM-400 spectrometer using DMSO-d6 as solvent. All chemical shifts (δ) are expressed in parts per million (ppm) with respect to the residual solvent signal both for 1H NMR [DMSO-d6 = 2.50 ppm] and 13C NMR (DMSO-d6 = 39.5 ppm). All the coupling constants (J) are quoted in Hertz (Hz).

Abbreviations used for the multiplicities are: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; b = broad.

LC–MS ESI–TOF analysis on the final purified benzodifuran BZ4 was carried out on an Agilent 6230B TOF LC–MS system, composed of a HPLC analytical system (1260 Infinity) with binary pump and a TOF–MS as detector with an ESI source. Reverse phase HPLC was performed on a C18 column (Agilent ZORBAX from Agilent Technologies, MI Italy, 1.8 μm, 50 × 4.6 mm). The benzodifuran samples for analysis were prepared by withdrawing 1 μl of a 2 mM solution of the compound in DMSO and diluting it to 500 μl H2O; 20 μl injections of these solutions were analyzed with a flow rate of 0.4 ml/min. Column elution was conducted by using H2O (A) and CH3CN (B) as eluents. Gradient: from 5% B in A to 95% B over 5 min and 95% B in A for 3 min. The MS analysis was operated in the positive ion mode (100–1000 m/z range).

The UV-vis experiments were carried out on a UV-vis spectrophotometer JASCO (Easton, MD USA) V-550 coupled to a Peltier Thermostat JASCO ETC-505T by using a quartz cuvette with a path length of 1.0 cm (internal volume = 1.0 ml). The spectra, subtracted of the appropriate baseline, were recorded with the following parameters: medium response, 100 nm/min scanning speed, 2.0 nm bandwidth. A scan rate of 1°C/min and a fixed absorbance value was used for the temperature-scanning UV measurements. The concentrations of the oligonucleotides were obtained by measuring the absorbance values at 260 nm and 85°C of solutions obtained by dissolving the oligomers in a known bidistilled H2O volume. The melting temperature value of the duplex was calculated by the first derivative method.

CD experiments were carried out in analogy with previous studies.[31,32] CD spectra were obtained on a Jasco J-715 spectropolarimeter coupled to a PTC-348WI temperature control system.

SEM: few drops of the benzodifuran samples eluted in tetrahydrofuran were dried on Al stubs and coated with Au and Pd for the microscopic observations, using a Scanning Electron Microscope (Nova NanoSem 450, FEI-Thermo Fisher – Thermo Fisher Scientific, MA USA).

The dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano ZSP (Malvern Instruments, Worcestershire, UK) equipped with a temperature controller. Solutions of the benzodifuranas at 10 μM concentration were prepared by diluting 2 mM stock solutions of the compounds dissolved in DMSO to 1 ml PBS buffer (140 mM NaCl, 10 mM phosphate buffer and 3 mM KCl) previously filtered on a 0.2 μm filter. The data were collected at a scattering angle of 173° at a given temperature until stabilization of the signal and carried out in triplicate.

Emission fluorescence spectra (5 nm slit widths), from 370 to 570 nm, of BZ4 at 7 μM concentration were recorded in PBS at 20°C, by using a quartz cuvette with a path length of 1 cm and a 0.5 ml volume capacity. The excitation wavelength was 314 nm.

**Synthesis of 2,6-diamino-benzo[1,2-b:4,5-b’]difuran-3,7-dicarboxylic acid bis-[2-(2-ethoxy-ethoxy)-ethyl] ester (BZ4)**

For the synthesis of BZ4, 2-(2-ethoxyethoxy)ethyl 2-cyanoacetate (2) was first prepared as depicted in Supplementary Scheme 1. Then, 4.5 ml of the crude ester 2 were added to a solution of 1,4-benzoquinone (0.5 g, 4.6 mmol)
in absolute ethanol (4.0 ml) and the resulting mixture left under stirring at room temperature. The addition of 30% aqueous ammonia solution (3.0 ml) caused a fast exothermic reaction, with temperature rising up to 40°C. After 2 h, ethanol and ammonia were removed under reduced pressure and 15 ml of water were added. A dark grey solid precipitate was recovered by filtration under vacuum and purified by crystallization using ethanol/acetone: 0.55 g (23% yield) of a product, identified as BZ4 by NMR and LC–MS analyses, were obtained. The synthetic procedure is outlined in Figure 2.

1H NMR (400 MHz, DMSO-d6): δ 7.65 (s, 4H), 7.36 (s, 2H), 4.32 (t, J 4.8 Hz, 4H), 3.76 (t, J 4.8 Hz, 4H), 3.60 (t, J 4.6 Hz, 4H), 3.51 (t, J 4.8 Hz, 4H), 3.43 (m, 4H), 1.06 (t, J 7.0 Hz, 6H).

13C NMR (100 MHz, DMSO-d6): δ 165.1, 164.2, 145.7, 121.1, 99.6, 81.8, 69.8, 69.2, 68.7, 65.6, 61.9, 15.1.

LC–ESI/MS: m/z 509.2 (M+H)+, 1017.4 (2M+H)+, 1039.3 (2M+Na)+, tR = 5.02 min.

TLC (CH2Cl2/CH3OH, 95:5 v/v) Rf = 0.70.

MTT assays on HeLa, Hep-G2, HDF, WM266 cells
HeLa, Hep-G2 and HDF cells were maintained in DMEM and WM266 in RPMI with 10% FBS, 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin, in a humidified incubator at 5% CO2 at 37°C [33].

BZ4 (10 mg) was further purified on a prepacked C18 minicolumn and 2 mg of the collected compound were dissolved in DMSO, so to have a 2 mM stock solution. Cisplatin was dissolved in 0.9% NaCl aq. solution.

A total of 2500 HDF cells and 3000 for all the other cancer cell lines were plated in 96-well tissue culture microplates and incubated at 37°C for 24 h. The benzodifuran or cisplatin solution was added to the cells at the proper concentration. As control, an equal amount of DMSO or 0.9% NaCl, respectively, was used. MTT assay was performed after 24 h of treatment [34]. In detail, for each well, 100 μl of a MTT solution at a 0.5 mg/ml final concentration, diluted in medium without red phenol, was added and incubated for 4 h. Then, the solution was discarded and formazan crystals were dissolved in isopropanol containing TRITON-X100 10% and HCl (0.04–0.1 N). Finally, the absorbance was determined at 570 nm by an automated spectrophotometric plate reader. The experiment was performed at least in triplicate.

Primary cortical neuron culture preparation
These cultures were prepared from E14–E16 mouse embryonic brains. Cells were seeded in a 96-well plate at 60,000 cells/well on polyethyleneimine (PEI) precoated plates. To prepare the culture medium, neurobasal medium (Gibco, Landsmeer, The Netherlands) was supplemented with the 2% (v/v) of B27-supplement, glutamine at a concentration of 0.5 mM) and 0.2% Penicillin-Streptomycin [P/S] – ThermoFisher Scientific, Landsmeer, The Netherlands). Cells were first cultured in this medium for 48 h after which cytosine arabinoside (Sigma) was added at a concentration of 10 μM for 24 h in order to inhibit the growth of nonneuronal cells. Afterward, the medium was substituted with fresh neurobasal medium and after a week the neuronal cells (<1% contaminating glial cells) were treated for the MTT assay performed as described before.
Circular dichroism experiments

The CD spectra were registered in a quartz cell with a path length of 1 cm, at 20°C or 37°C (for Aβ42 binding experiments) with a response of 1 s, a scanning speed of 100 nm/min and a 2.0 nm bandwidth. All the spectra were averaged over three scans.

For the experiments with DNA, a 2 μM solution of the oligonucleotide – dissolved in PBS containing NaH2PO4 (10 mM) and KCl (100 mM), pH = 7.2 – and a benzodifuran 1.5 mM stock solution in DMSO were used. The CD titration curves were recorded after the addition of increasing aliquots of the ligand (0.5 equiv. at a time, 0.65 μl) up to 5 equiv. (10 μM concentration) to the various DNA solutions. Each sample addition was followed by a suitable equilibration time after which the spectra were recorded. In all cases, no DMSO contribution to the oligonucleotide CD spectra was detected. We obtained the melting curves recording the CD at 290 nm (in case of G4) or 276 nm (for duplex DNA) upon temperature increase with a scan rate of 1°C/min. Tm values were obtained by the first derivative method.

Experiments with Aβ42 were carried out using a 5 μM concentration of Aβ42 in PBS (overall volume = 2 ml, pH 7.2) and a twofold concentration of BZ4. All samples were incubated at 37°C for 0, 24 and 48 h.

Copper(II) binding experiments

The binding experiments were monitored by UV/Vis spectroscopy as follows: BZ4 was used at 25 μM in PBS at 25°C and pH 7.2, in order to have a stable UV signal over the experiment period; then, the addition of two equivalents of a Cu²⁺ solution (withdrawn from a 10 mM stock solution of copper(II) chloride) was carried out. Thereafter UV/Vis spectra were registered for an overall period of 3 h subsequent the addition of the metal salt. Each experiment was performed in duplicate.

Aβ peptide solubilization

Solutions of recombinant Aβ42 peptide (rPeptide, GA, USA) were prepared according to a previously published procedure [35]. In short, Aβ was sequentially dissolved in hexafluoroisopropanol (HFIP) and DMSO. The DMSO was removed from the Aβ solution by using a HiTrap™ desalting column (GE Healthcare, Zwijndrecht, The Netherlands) and elution with PBS at pH 7.4. We measured the Aβ42 concentration by the Coomassie (Bradford, UK) Protein Assay Kit (ThermoFisher, Landsmeer, The Netherlands) and, afterward, the final concentration required for the subsequent experiments was achieved after dilution. Aβ peptide aggregation, in the presence or absence of BZ4, was evaluated at 37°C under quiescent conditions.

Thioflavin-T assay

Amyloid fibril formation was measured by a ThT fluorescence assay. We adjusted the Aβ42 concentration to 25 μM diluting the peptide solution with PBS buffer (pH 7.4), while a final ThT concentration of 12 μM was realized in a 96-well plate (Greiner flat bottom transparent black, Sigma – cat. M9685). The emitted fluorescence intensity of ThT was measured upon excitation at a wavelength of 450 nm by detection in the range 480–600 nm. The fluorescence intensity from ThT at its maximum (485 nm) was reported in a graph in dependence of the different concentrations of BZ4. We performed measurements in triplicate, averaged the values recorded and subtracted background measurements that corresponded to buffer containing 12 μM ThT and the tested benzodifuran compound.

Molecular models

Energy-minimized 3D structure models (random low energy conformers) were obtained by DataWarrior (http://openmolecules.org/) and MOLVIEW (http://molview.org) softwares. Molecular volume of BZ4 was estimated by Molinspiration (http://www.molinspiration.com).

SEM analysis

The samples for microscopic observations were derived from CD experiment solutions. The solvent was left to slowly evaporate, and then, pure BZ4, Aβ42, and mixture of BZ4 and Aβ42, were coated on a conductive layer of Au–Pd. Then, the prepared samples were analyzed on a Nova NanoSem 450 FEI SEM instrument using a kilovolt ranging from 2.00 to 5.00 in high vacuum.
Results & discussion

Design, synthesis & characterization of the new benzodifuran derivative BZ4

In order to improve the solubility in aqueous solutions and the general hydrophilic–lipophilic balance of BZ1 (Figure 1), in other words, the most promising among the benzodifuran derivatives we have previously investigated[8], we here designed BZ4. With respect to the original compound, this new derivative carries longer oligoethylene glycol chains, attached through stable ester bonds to the central benzodifuran scaffold.

In our design, intramolecular H-bonds formed between the ethylene glycol chains with NH2 residues should be somehow prevented by their tendency to point in different directions (Supplementary Figure 1). Thus, potential intermolecular associations can be driven by both chains and benzodifuran moiety, the latter being able to in principle interact via π–π stacking with other aromatic molecules (e.g., nucleic acid bases or other BZ4 units) leading to potentially relevant complexes or self-assemblies.

For the synthesis of the target compound containing two free amino groups and two ethylene glycol chains, respectively, in 2 (and 2’) and 3 (3’) positions of the benzodifuran core, a recent protocol based on the Craven reaction, in analogy to a previous work (8), was employed. Starting materials were the commercially available 1,4-benzoquinone (1; Figure 2) and the ad hoc synthesized 2-(2-ethoxyethoxy)ethyl 2-cyanoacetate ester (2; Supplementary Scheme 1) mixed in 1:2 ratio in the presence of ammonia (30% aq.) and ethanol. This one-pot reaction, depicted in Figure 2, allowed obtaining the target BZ4, which was then purified after a simple work-up, consisting of precipitation from water and crystallization from ethanol/acetone and isolated as a pure compound in a 23% yield.

Compound BZ4 was analyzed and characterized by LC–ESI/MS (Figure 3 & Supplementary Figure 2A) and 1H/13C NMR (Supplementary Figures 2B & 3) techniques, which confirmed the identity and purity of the desired product. The higher polarity of BZ4 compared with its analog BZ1 was evidenced by its lower Rf on a TLC silica plate (eluent = CH2Cl2/CH3OH, 95:5 v/v; BZ4 Rf = 0.70; BZ1 Rf = 0.77).

Then the spectroscopic properties of the benzodifuran derivative, dissolved in an aqueous saline buffer, were analyzed by UV and fluorescence spectroscopies. The UV spectrum of a 7 μM solution of BZ4 in PBS buffer at room temperature showed two absorption bands at 314 and 269 nm (Figure 4A) which were approximately stable over 4 h monitoring (Supplementary Figure 4) after dilution of the compound from a concentrated stock solution in DMSO. The fluorescence spectrum of BZ4 showed an emission band of low intensity at 426 nm upon excitation at 314 nm (Figure 4B).
Aggregation properties of BZ4 in solution

In order to investigate the self-assembling behavior of BZ4 in solution, the absorption or scattering properties of the molecule were monitored over time and at various temperatures.

UV kinetic properties were explored by solubilizing BZ4 at 10 and 50 μM concentrations in a pseudo-physiological solution (PBS, pH 7.2) and acquiring the UV/Vis spectra of the obtained solutions at different times. Monitoring the UV spectrum of a 10 μM solution of BZ4 in PBS over time showed that the absorption band at 314 nm was quite stable during the first 4 h after dissolution and the overall signal intensity decreased by 7.3% after 24 h (Supplementary Figure 5). These results could indicate the formation of some small aggregates that were not detectable by visual inspection. Aggregation of the compound was further confirmed by a more rapid and marked decrease of the absorption intensity over time when BZ4 was solubilized in PBS at higher concentration (50 μM): the absorbance value at 314 nm decreased by 45% with respect to the original intensity after 5 h and by 76% after 24 h (Supplementary Figure 6A). After stabilization of the UV absorption at 48 h, UV spectra were collected upon increasing the temperature to 85°C (Supplementary Figure 6B). Interestingly, by maintaining the temperature at 85°C for 140 min, the UV signal recovered the initial intensity (Supplementary Figure 6C) and the UV spectrum at 85°C (Supplementary Figure 6D) was almost superimposable to the one at 25°C immediately after dissolution of the benzodifuran (t = 0; Supplementary Figure 6A), data indicative of the dissociation of the aggregates at high temperature.

This last datum, together with the consistency of the mass spectra of BZ4 before and after the kinetic (0 to 48 h) and thermal (2.5 h at 85°C) experiments, allowed us to exclude decomposing processes of the compound, instead evidencing its good stability over time and temperature.

The formation of aggregates in a pseudo-physiological solution was further supported by the observed deviation from linearity of the 314 nm absorbance values of BZ4 dissolved in PBS at different concentrations (Figure 5): the signal intensity increased linearly with the concentration up to about 20 μM, whereas at concentrations higher than 25 μM divergences from linearity were observed. With respect to the behavior of the previously reported benzodifuran derivatives [8], BZ4 showed a higher solubility in aqueous media, as expected: indeed, the deviation from linearity of the UV absorbance versus concentration for BZ1, the more polar compound of the previous series, occurred already above 10 μM, and the stability of its UV signal with time denoted a faster reduction of the absorption band than in the case of BZ4 [8]. Furthermore, from visual inspection of a set of freshly prepared solutions of the benzodifurans in PBS, we observed the presence of white suspensions for BZ4 starting from 35 μM samples and for BZ1 already at 20 μM: these data, in accordance with the UV results, provide evidence, with good approximation, that the aqueous solubility is ca. 3–4 × 10⁻⁵ M for BZ4 and 1–2 × 10⁻⁵ M for BZ1, according to the values found for other polycyclic aromatic compounds [36].

In addition to UV spectroscopy, DLS provided insight into the ability of BZ4 to self-aggregate, as evidenced for other benzodifurans [8]. Upon incubation at 25°C for 20 min at 10 μM concentration, DLS analysis showed aggregates with a size distribution composed of one prevailing population (93.3%) with average hydrodynamic
Figure 5. UV absorbance of BZ4 monitoring at 314 nm at different concentrations (5–60 μM) in phosphate-buffered saline (pH 7.2) at 25°C. The measurements were performed on freshly prepared solutions obtained by diluting BZ4 in phosphate-buffered saline from concentrated stock solutions in DMSO (from 0.75 to a maximum of 9 mM), maintaining a constant DMSO concentration (0.67%), necessary to dissolve the samples.

Figure 6. Representative dynamic light scattering spectra of BZ4 (at 10 μM concentration in phosphate-buffered saline solution) at different temperatures (25°C and 37°C). The time required for signal stabilization at each temperature investigated was also reported.

diameter of 224 nm and a small population (6.7%) of 42 nm (Figure 6). Upon incubation of these samples for 10 min at 37°C, a general reduction in the particle size was observed, with confluence of the system into a single narrow-shaped population having a size-distribution centered around 168 nm.
Figure 7. Cytotoxic effects of BZ4 on different cell lines in comparison with cisplatin. Cells were incubated with the tested compounds at 10 μM concentration at 37°C for 24 h. Proliferating cells are reported as percentage respect to the control (vehicle-treated cells) and are expressed as means ± SE. All the cell experiments on the antiproliferative activity of BZ4 were carried out at least in triplicate. Statistical significance was analyzed using Student’s t-test, unpaired and two-sided. *p < 0.05.

HDF: Human dermal fibroblast; HeLa: Human cervix adenocarcinoma cells; HepG2: Liver hepatocellular cells; WM266: Malignant melanoma.

The aggregation ability found for BZ4 could be due to its potential to form intermolecular interactions, as hypothesized on the basis of models (Supplementary Figure 1). Considering the molecular volume of BZ4 estimated using the Molinspiration software (450.75 Å³, Supplementary Table 1), leading to a molecular radius of 4.75 Å (r = [3V/4π]1/3), and combining this result with the experimentally determined hydrodynamic radius of clusters formed by multiple BZ4 units (at 10 μM concentration of the benzodifuran at 25°C), which is 112 nm (1120 Å) as deduced from DLS experiments, several hundreds of monomers form the supramolecular assembly of BZ4.

Antiproliferative activity of BZ4
The novel benzodifuran derivative BZ4 was then tested for its antiproliferative activity using the MTT assay (Figure 7). Three human cancer cell lines (HeLa, Hep-G2 and WM266) and normal HDF were incubated with BZ4 at 10 μM concentration for 24 h, using cisplatin at the same concentration as a positive control. While BZ4 induced moderate cytotoxic activity on the three cancer cell lines, no effect was observed on HDF cells. In analogous experiments, cisplatin reduced the viability of normal HDF by 33%. This apparent selectivity, similar to what previously found for the other benzodifuran derivatives [8], makes BZ4 an interesting starting compound to develop new potential anticancer drugs.

In order to elucidate the possible bioactivity mechanisms of BZ4, its ability to bind various DNA model systems and biologically relevant metal ions, as well as its aggregation properties, were also evaluated.

Studies on the interaction with DNA
Oligonucleotide binders play a key role in the development of new anticancer drugs. For this reason, great interest has been recently devoted to the synthesis of molecules able to selectively bind with nucleic acids strands [37-40]. The interaction of BZ4 with DNA in solution was here analyzed using the following model systems: three random coil single stranded ODNs (A, B and C); a bimolecular 12-mer duplex (from the association of complementary ODNs A and B) and a unimolecular G-quadruplex (G4) obtained by folding of a tract of the human telomere sequence (the 26-mer tel26), an important target in anticancer strategies. In particular, ODNs A and B contain consecutive purines in their sequence, while ODN C does not contain consecutive purines. All the interaction studies were carried out in a 100 mM KCl-phosphate buffer solution (pH 7.2), analogous to recent works [8,41,42].
As the benzodifuran did not show a CD signal at the chosen concentrations (data not shown), CD titration experiments were carried out by adding increasing equivalents of BZ4 to each selected DNA system (2 μM concentration). Thus, we monitored the difference in the CD spectrum of the ODNs after each addition. In Figure 8 and Supplementary Figure 7, the overlapped CD spectra of the ODN systems alone (black lines) and in the presence of five equivalents of BZ4 (final point of the titration experiments, red curves) were reported.

Minor changes in the CD spectra of the single strands containing consecutive purines in the sequence (A and B; Figure 8A & Supplementary Figure 7A) were observed in the titrations, whereas no effect in the case of C (the ODN without consecutive purines; Supplementary Figure 7B) was found.

Regarding the structured systems, the titrations with BZ4 produced little variations in the CD spectrum of the G-quadruplex structure (Figure 8B), whereas no effect on the duplex A/B was observed (Supplementary Figure 7C), demonstrating that in both cases no significant conformational change occurred.

However, CD melting experiments on the mixtures duplex-BZ4 and tel26-BZ4 (obtained after the titration experiments) revealed a slight destabilization of the ODN secondary structures, reflected in Tm values 4 and 2°C lower than those of the untreated duplex and G-quadruplex, respectively (data not shown). These data demonstrated that BZ4 is able to interact with the analyzed duplex and G-quadruplex systems, even if this occurs without significantly affecting their overall conformation and thermal stability. Thus it seems plausible to exclude a DNA-binding mechanism as the main mechanism of action for the observed antiproliferative activity of BZ4.

Ability of BZ4 to bind copper II ions
Considering the crucial role that copper plays in tumor progression [43] and the observed effect of copper chelators as antancer drugs by blocking tumor angiogenesis and growth in animal models [44], we also investigated the capability of BZ4 to bind copper(II) ions. The UV/Vis-monitored binding experiments were performed by incubating BZ4 with two equivalents of a Cu²⁺ solution and then the spectra were recorded over a 3-h period.

As shown in Figure 9A, the incubation of BZ4 with CuCl₂ for 20 min resulted in the appearance of a new band at 550 nm, indicative of copper (II) recognition in agreement with literature reports [45,46].

It is interesting to notice that also the homologous benzodifuran BZ1 showed a similar behavior with copper II ions, whereas BZ2 and BZ3 were not able to bind Cu²⁺, as we previously reported [8]. Analyzing the structures of all the benzodifurans (Figure 1) and their copper-binding ability, we concluded that the Cu²⁺ coordination involves the amino groups on furan rings, as well as the ether oxygen atoms of the ester chains.

Thus, in analogy to BZ1 [8], we can hypothesize a hexacoordinated copper(II) dimeric complex (2 BZ4 molecules per Cu²⁺, Figure 9B), as repeating unit of a multimeric supramolecular network which could be arisen due to the symmetric nature of BZ4.
Ability of BZ4 to interact with the amyloid peptide Aβ42

Aβ peptide – the main constituent of plaques associated with AD – has been suggested to play an important role in AD. Here, we tested the ability of BZ4 to interact with Aβ42 peptide and interfere with its aggregation process. The aggregation of Aβ42, incubated in PBS at 37°C, was monitored by CD spectroscopy at different times (0, 24 and 48 h). The spectra obtained at the different incubation times showed changes similar to those reported in literature [47]. In parallel, Aβ42 was incubated at 37°C in the presence of BZ4 (which did not show any contribution to the CD signal) and CD spectra at different incubation times were recorded. When comparing the obtained CD spectra after 24 and 48 h of incubation, it became apparent that the presence of BZ4 induced differences in the structural organization of Aβ42. (Figure 10). The spectral changes, impacting both intensity and shape of the negative valley at 225 nm already described by Guo et al. [48], suggested that BZ4 partly suppresses the β-sheet structure of Aβ42 leading to new structural elements.
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Figure 11. BZ4 exerts a dose-dependent inhibiting effect on the amyloid fibril formation of Aβ42. Solutions containing Aβ42 at a concentration of 25 μM were incubated in the presence and absence of BZ4 at 37 °C for 1.5, 5 and 24 h. Amyloid fibril formation was detected using ThT fluorescence intensity measurements at a detection wavelength of 485 nm. The reported values represent the results obtained from three independent experiments. The statistical significance of the replicates was assessed by p-values using paired two-tailed t-tests (GraphPad Software). *p < 0.05; **p < 0.005.

Ability of BZ4 to interfere with Aβ aggregation propensity

The potential interaction between BZ4 and Aβ42 suggests the possibility that BZ4 may also impact on the aggregation of this peptide. Aggregation of Aβ42 was monitored at different times (1.5, 5 and 24 h) upon incubation at 37°C in the presence or absence of BZ4 using ThT fluorescence. ThT is a dye commonly used to measure the aggregation propensity of Aβ42, as it generates a characteristic fluorescence emission upon binding to amyloid β-sheet structures [47,49]. The incubation of the peptide alone generated an increase in the fluorescence over time, indicating that the peptide self-assembled into ThT-binding fibrils. On the other hand, when Aβ42 was incubated with BZ4 at various concentrations, fibril formation was significantly inhibited in a dose-dependent manner, as shown by the lower ThT fluorescence intensities (Figure 11). In particular, BZ4 was active at concentrations exceeding 25 μM and after 5 h incubation.

Anti-aggregation activity of BZ4 was compared with those of the previously synthesized benzodifuran derivatives of Figure 1 [8]. Interestingly, compounds BZ1, BZ2 and BZ3 showed no significant inhibitory activity, demonstrating that the specific structural features of BZ4 are essential for this effect (Supplementary Figures 8–10).

Having found this activity only for BZ4, in view of further exploring its potential as a therapeutic agent in neurodegenerative diseases, we tested its cytotoxicity on primary mouse cortical neurons using the MTT assay. We found that BZ4 was nontoxic up to a concentration of 20 μM, compared with cytotoxic staurosporine (Figure 12).

Interestingly, in analogy to BZ4, another polycyclic aromatic compound, imidazoquinoline dactolisib (NVP-BEZ235), showed antiproliferative activity [50], as well as significant neuroprotective effects as evidenced in studies carried out on the Aβ [51]. The dual activity of dactolisib relies on the PI3K/mTOR pathway involved in protection against neurodegeneration in hippocampal neuronal cultures and mice while inducing an antiproliferative effect [51].

SEM analysis of aggregated Aβ morphology in absence & presence of BZ4

The effect of BZ4 on the morphology of Aβ42 aggregates was investigated after 48 h incubation at 37°C by SEM. The SEM micrograph depicted in Figure 13A showed the surface morphology of pure BZ4 crystals: only a regular roughness of about 10 nm appeared. Aβ42 incubated for 48 h at 37°C (in PBS solution at pH 7.2) in the absence of compound BZ4 showed both a granular morphology (Figure 13B), and filaments of considerable length (Figure 13C) and with thickness in the range 20–30 nm (Figure 13D), visible on the surface, in line with previous
reports [52]. After incubation of Aβ42 with BZ4 under the same experimental conditions, SEM micrographs of the mixture evidenced a different granular morphology with visual absence of filamentous materials (Figure 13E). Thus, SEM analysis demonstrated an altered morphology of Aβ42 in samples where BZ4 was present with respect to those only containing the pure Aβ peptide, with disappearance of the typical filaments.

**Conclusion**

In this paper, we report the synthesis and characterization of a new benzodifuran derivative, named BZ4, and the evaluation of its biological properties, including anticancer and anti-amyloid activities, as well as its ability to interact with several DNA model systems. BZ4 was designed starting from a previously reported benzodifuran derivative (BZ1), showing promising biological activities, by elongating the two oligoethylene glycol chains, with the aim of improving its solubility in aqueous solutions and, in general, the hydrophilic–lipophilic balance of the molecule. The newly synthesized compound showed significant antiproliferative activity on a panel of human cancer cells, but not on normal HDF or primary neuronal cells. Analogously to the previously tested benzodifuran derivatives [8], the antiproliferative activity of BZ4 could be connected to the formation of nanoaggregates, evidenced by UV-vis spectroscopy and DLS analysis, as reported in other literature examples for several anticancer drugs [53].

Another interesting property of this compound is its ability to act as a copper (II) ion chelator, as the rameic cation binding was clearly evidenced by UV. In addition, thioflavin T fluorescence assays showed that BZ4 inhibits, in a dose-dependent manner, aggregation of the Aβ peptide, one of the most relevant targets in current therapeutic strategies to treat AD. Instead, fibril formation was not significantly affected by BZ1 and BZ2, and was potentiated by incubation of Aβ peptide with the most apolar derivative of the benzodifuran series (BZ3). The interaction of BZ4 with Aβ was also confirmed by CD spectroscopy and SEM microscopy. These aspects, along with the absence of toxicity on neuronal primary cells, provide an interesting picture of the properties of this compound. This novel species could be a valuable core scaffold to generate libraries of optimized analogs in the search for new anticancer and anti-Alzheimer drugs investigating the final benzodifuran effective extra- and/or intracellular targets and its potential mechanism of action.
Future perspective

Overall, these findings indicate BZ4 as a valuable scaffold to evolve potential candidate drugs for anticancer and Aβ-associated neuropathies and strongly encourage further efforts in the search for benzodifuran derivatives with pharmacological activity.

Besides the therapeutic applications that could derive from the present work, in future also the diagnostic potential of benzodifurans in disease imaging could be investigated, in analogy to previous reports on aromatic molecule applications in this area [54–58].

Supplementary data

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Ethical conduct
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

Summary points
• In the context of the biological evaluation of benzodifuran compounds, this study reports on the synthesis, characterization and bioactivity of a new derivative, named **BZ4**.
• This compound showed antiproliferative activity on a panel of human cancer cells, but no significant cytotoxicity on normal human dermal fibroblast or primary neuronal cells.
• The antiproliferative activity of **BZ4** could be connected to the formation of nanoaggregates, evidenced by UV-Vis spectroscopy and dynamic light scattering analysis, and/or with its ability to act as a copper (II) ion chelator, demonstrated by UV studies.
• Thioflavin T fluorescence assays, as well as circular dichroism and scanning electron microscopy analyses, showed that **BZ4** interacts with the β-amyloid (Aβ) peptide altering its structure and morphology, and interfering with its aggregation properties.

References
Papers of special note have been highlighted as: ● of interest; •• of considerable interest


•• This is a key work on the relationships between bioactivity and chemistry of benzodifuran compounds.


