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Antibiofilm activity of cationic carbosilane BD132 dendron and its synergistic effect with amphotericin B against *Candida* spp.

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The BD132 is a cationic carbosilane dendron containing peripheral ammonium groups, which impairs antimicrobial properties, and the chaperone 4-phenylbutyrate, with anti-inflammatory and antibacterial properties. Its in vitro activity was evaluated against biofilm formation and against established biofilms in *Candida albicans* and *Nakaseomyces glabratus* (former named *Candida glabrata*). In addition, the BD132 dendron was used in combination with amphotericin B against both species. Its cytotoxicity was evaluated using HeLa cells. Our data showed that BD132 dendron was an excellent compound that prevented biofilm formation. The Minimum Biofilm Inhibitory Concentration was 16 mg/L for *N. glabratus* and 32 mg/L for *C. albicans*, both concentrations were not cytotoxic. Furthermore, in the studies against established biofilms, the dendron was effective eradicating them. Experiments showed that the combination of the dendron with amphotericin B improved the activity of both by reducing the effective concentration of both compounds required to obtain the same results as when used individually. Therefore, the BD132 dendron is a very interesting compound that exhibits low cytotoxicity at active concentrations that prevent the formation of biofilms of these microorganisms. In addition, these results suggest that the BD132 dendron may have great benefits in the development of antifungal agents.

Keywords Amphotericin, Biofilm, Biocide synergy, Candida albicans, Nakaseomyces glabratus, Dendritic systems

Antimicrobial resistance is a worldwide health problem, and the future projection is not optimistic. Among these resistant pathogens, there are species of relevant clinical significance like *Candida* spp., which are difficult to eliminate using clinical drugs, especially in the biofilm state[1]. This biofilm-associated resistance to antimicrobials is due to the increase of cell density and the exclusion of the antimicrobial compounds[2,3]. These biofilms are problematic because they can grow on implant, catheters or other medical materials and may cause significant harm to patients, especially those immunocompromised or with other underlying pathologies[4–6]. Therefore, there is a growing need for new antimicrobials agents to fight drug resistance and treat fungal diseases.

In this regard, there are some new molecules that are able to eradicate *Candida* spp. biofilms; however, these drugs usually are cytotoxic against eukaryotic cells such as those of mammals. *Candida* is also eukaryotic, making it difficult to find compatible compounds for clinical use that target only the pathogen[7]. In addition, another problem is the development of multi-drug resistant *Candida* strains and the emergence of intrinsically resistant pathogens. Since the 2000s, there has not been a new family of antifungal compounds for clinical use. The most recent was the approval of an azole, isavuconazole, in 2015[8].

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To overcome this problematic, cationic dendritic systems have raised as a promising tool to treat different microbial infections. The controlled synthesis of these compounds makes them of great interest due to the wide range of possibilities and applications that it provides[9]. Within this group of systems, the family of carbosilane (CBS) dendritic macromolecules presents a common structure based on carbon-carbon and carbon-silicon bonds. This framework is highly hydrophobic, but adequate modifications give them water solubility and favors their biomedical applications[10]. In the literature, it can be found information about recent in vitro studies that shown activity against prokaryote and eukaryote, in planktonic cells[11–15] and biofilm state[16–21] of these systems. In vivo studies with dendritic systems have also been reported[22].

Dendritic systems can be developed in different topologies as spherical or wedges (dendrons)[23]. The last contains two different active positions, the periphery and the focal point (Fig. 1). This makes these systems very attractive for heterofunctionalization keeping a well-designed structure. Hence, it is possible to generate cationic dendrons, with antibacterial activity, including at the focal point active moieties as antibiotics[24] or anti-inflammatory[25] drugs.

The aim of this article was to synthesise a new dendritic compound, based on previous studies[18,26], to generate an improved molecule capable of preventing the formation of *Candida* biofilms, as well as eradicating an already established biofilm of different microorganisms, *C. albicans and N. glabratus*. On the one hand, herein we have combined a microbicidal CBS dendron with the chaperone 4-phenylbutyrate (PBA). This molecule has been widely studied in various biological systems, including the evaluation of antibacterial[27] and anti-inflammatory activity[28]. On the other hand, the cationic moieties were modified with hydroxyl units that hinders the positive charge of the ammonium groups providing a higher solubility and better biocompatibility[29]. Studies have also been carried out on the effect of combining this molecule with a clinical antimicrobial compound such as amphotericin, with the purpose of reducing the effective concentration of this clinical compound. Finally, the cytotoxicity of the new dendritic compound has been evaluated on human cell lines.

Materials and methods

Synthesis of PBAG2(S-NMe$_2$-OH-Br)$_4$ (BD132)

The reactions were carried out under inert conditions using dry solvents. The NMR spectra were recorded on Bruker Advance Neo 400 at ambient temperature (400.13 MHz for ^{1}H), 100.60 MHz for ^{13}C). Chemical shifts (δ) are given in ppm. ^{1}H and ^{13}C resonances were measured relative to solvent peaks considering TMS = 0 ppm. When it was necessary, the assignment of resonances was done from HSQC, HMBC, DOSY and TOCSY NMR experiments. The elemental analyses were carried out on a INTEC EA31OO instrument. Precursor PBAG2(S-NMe2) was prepared following the methodology previously described[30]. The synthesis of the BD132 dendron is described below.

A solution of PBAG2(S-NMe$_2$) (0.125 g, 0.13 mmol) in dry THF (10 ml) was introduced in a Teflon-valved ampoule under inert atmosphere. Br(CH$_2$)$_2$OH was added (0.072 g, 0.57 mmol) and the mixture was heated at 60 °C for 3 days. Afterward, the volatiles were removed under vacuum and the yellowish wax was washed with ether and finally with hexane, yielding BD132 as yellow solid (0.161 g, 79%).

Characterization of BD132 (Fig. 1 and Figs. S1–S5)

^{1}H-NMR (CD$_3$OD): 0.01 (s, 3 H, SiMe(e)), 0.13 (s, 6 H, SiMe(i)), 0.60 (m, 2 H, SiCH$_2$(d)), 0.66 (m, 4 H, SiCH$_2$), 0.73 (m, 4 H, SiCH$_2$), 1.01 (m, 8 H, SiCH$_2$(j)), 1.43 (m, 6 H, CH$_2$(g) and CH$_2$(c)), 1.70 (m, 2 H, CH$_2$(b)), 1.95 (m, 2 H, CH$_2$(c')), 2.36 (m, 2 H, CH$_2$(b')), 2.68 (m, 2 H, CH$_2$(d')), 2.76 (m, 8 H, SCH$_2$(k)), 3.04 (m, 8 H, SCH$_2$(l)), 3.27 (s, 24 H, NMe$_2$(n)), 3.59 (m, 8 H, NCH$_2$(o)), 3.73 (m, 8 H, NCH$_2$(m)), 4.05 (m, 8 H, OCH$_2$(p)), 4.13 (m, 2 H, OCH$_2$(a)), 7.22 (m, 3 H, C$_6H_5$), 7.29 (m, 2 H, C$_6H_5$).

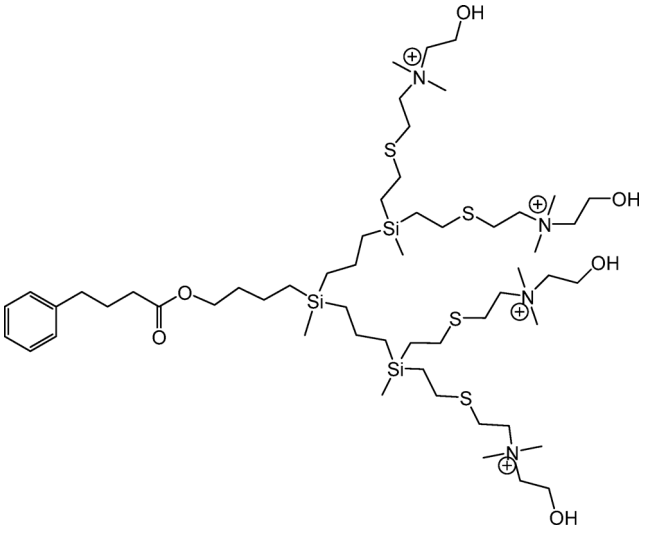

Fig. 1. Drawing of the cationic BD132 dendron (bromide anions have been omitted for clarity).

^{13}C-NMR (CD_3OD): -6.1 (SiMe(i)), -5.8 (SiMe(e)), 13.5 ($SiCH_2$(d)), 14.6 ($SiCH_2$(j)), 17.7 (CH_2(c)),18.0 ($SiCH_2$), 18.8 ($SiCH_2$), 20.2 (CH_2(g)), 26.3 (CH_2(c')), 24.1 (SCH_2(l)), 26.8 (SCH_2(k)), 32.3 (CH_2(b)), 32.9 (CH_2(b')), 34.8 (CH_2(d')), 51.1 (NMe_2(n)), 55.7 (OCH_2(p)), 64.1 (OCH_2(a)), 64.7 (NCH_2(m)), 64.9 (NCH_2(o)), 125.7, (C_6H_5), 128.0 (C_6H_5), 128.1 (C_6H_5), 141.2 (C_6H_5), 173.4 (CO).

Elemental analyses for $C_{55}H_{116}Br_4N_4O_6S_4Si_3$ (1461.67 g/mol): Calc. C, 45.20; H, 8.00; N, 3.83; S, 8.77; Obt. C, 45.91; H, 8.29; N, 3.63; S, 8.47.

Microorganisms: strains and culture conditions

The microorganisms used in this study were obtained from Colección Española de Cultivos Tipo (CECT): *Nakaseomyces glabratus* CECT 1448 (ATCC 2001) and *Candida albicans* CECT 1002 (ATCC 18804). Both strains were stored at − 20 ∘C with 20% glycerol (Sigma-Aldrich, Saint Louis, MO, USA) until use. *C. albicans* and *N. glabratus* were grown on Sabouraud-Chloramphenicol agar (Scharlab, Barcelona, Spain). Then, colonies were transferred into YPD medium (Yeast Extract (1%), Peptone (2%) and Dextrose (2%), Scharlab, Barcelona, Spain) and incubated at 37 °C with slightly agitation (150 rpm) for 24 h.

In vitro antibiofilm susceptibility test in *C. albicans* and *N. glabratus*

The BD132 dendron, soluble in water, was tested to study its ability in vitro to prevent biofilm formation and eliminate *C. albicans* and *N. glabratus* established biofilms. In 96-well microtiter plates, the dendron was tested using a series of two-fold dilutions (1 to 1024 mg/L).

Treatment antibiofilm formation of Candida with the BD132 dendron

Using the YPD culture, each *Candida* strain inoculum was adjusted to 0.5 McFarland and added to 96-well microtiter plates (50 µL/well) with medium alone (control) orBD132 concentration gradient (50 µL/well). Plates were incubated in the dark at 37 °C for 48 h. Then, plates were incubated with resazurin (see Sect. 2.5) and well content was transferred onto agar plate to perform the drop plate method (see Sect. 2.6)[26].

Treatment of established biofilms of Candida with the BD132 dendron

Using the YPD culture, each *Candida* strain inoculum was adjusted to 0.5 McFarland and added to 96-well microtiter plates. Plates were incubated in the dark at 37 °C for 48 h to produce a biofilm. Then, plates were washed with PBS and BD132 dendron was added with new medium RPMI. Plates were incubated in the dark at 37 °C for 48 h. Then, plates were incubated with resazurin (show Sect. 2.5) and well content was transferred onto Sabouraud agar plates to perform the drop plate method (show Sect. 2.6)[26].

Combination therapy of the BD132 dendron with amphotericin B in *C. albicans* and *N. glabratus*

The combination study was performed against biofilm formation and established biofilms. The interaction between the compounds was evaluated by the popularized checkerboard method. The procedures were as previously explained in Sect. 2.3.1 and 2.3.2. Amphotericin B was used in combination with the BD132 dendron. To test activity against biofilm formation, the concentration of the BD132 dendron used were from 0.5 mg/L to 16 mg/L in *N. glabratus* and from 1 mg/L to 32 mg/L in *C. albicans*, and the concentrations of amphotericin used were 0.003 mg/L to 0.125 mg/L and 0.006 mg/L to 0.25 mg/L, respectively. However, the concentrations of the BD132 dendron used against established biofilm were from 2 mg/L to 64 mg/L in *N. glabratus* and from 8 mg/L to 256 mg/L in *C. albicans*, and the concentration of amphotericin used were from 0.12 to 4 mg/L for both species. After treatments, resazurin assay and drop plate method were performed.

To determine synergy activity, the fractional inhibitory concentration index (FICI) was calculated:

$$\begin{aligned}\mathrm{FICI} = &[\mathrm{MBIC\ or\ MBEC\ (dendron + amphotericin)/MBIC\ or\ MBEC\ (dendron\ alone)}]\\ &+ [\mathrm{MBIC\ or\ MBEC\ (amphotericin + dendron)/MBIC\ or\ MBEC\ (amphotericin\ alone)}].\end{aligned}$$

The result was interpreted as synergistic when FICI ≤ 0.5, additive when 0.5 < FICI ≤ 1, indifferent when 1 < FICI < 4, and antagonistic when FICI ≥ 4[15].

Resazurin assay

To perform this viability assay, biofilms were washed with PBS after treatments. Then, 100 µL of PBS and 20 µL of 0.01% sterilized resazurin were added. Plates were incubated for 20 h and read at 570 and 600 nm in a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific™). The value of the absorbances allowed us to determine the Minimum Biofilm Inhibitory Concentration (MBIC) in biofilms in formation and the Minimum Biofilm Damage Concentration (MBDC) in established biofilms[31]. Resazurin assay was used for individual and combination treatments.

Drop plate method

To perform this assay, wells were scrapped, and content was homogenized. Then 5 µL of each well were transferred onto Sabouraud agar plates. After 24–48 h incubation, we obtained the minimum fungicide concentration in biofilm (MFCB) and the minimum biofilm eradicate concentration (MBEC) in established biofilm[31]. Drop plate method was used for individual and combination treatments. Using this method, we determined the exact concentrations that produce 99.9–100% cell death.

	Biofilm formation		Established biofilm	
	MBIC[a] (mg/L)	MFCB[b] (mg/L)	MBDC[a] (mg/L)	MBEC[b] (mg/L)
C. albicans	16–32	16–32	64	256–512
N. glabratus	8–16	8–16	32	128

Table 1. Antibiofilm activity of the BD132 dendron against *C. albicans* and *N. glabratus*. [a]Value obtained by resazurin method. [b]Value estimated by drop plate method.

Effect of BD132 Dendron preventing biofilm formation (MBIC)					
	Individual (mg/L)		In combination (mg/L)		
	BD132	Amphotercin B	BD132	Amphotericin B	FICI[a]
C. albicans	16–32	0.25	1	0.06	0.27
			4	0.03	0.24
N. glabratus	8–16	0.12	1	0.03	0.30
			2	0.01	0.24

Table 2. In vitro combination studies of the BD132 dendron and amphotericin B against *C. albicans* and *N. glabratus* in biofilm formation. [a]FICI ≤ 0.5: synergic activity.

Cytotoxicity evaluation assay

The BD132 dendron cytotoxicity was evaluated using HeLa cells (ATCC®CCL-2, American Type Culture Collection). Assays were performed in 24-well plates (NUNC™) in Dulbecco's Modified Eagle Medium supplemented with 10% foetal bovine serum (Sigma-Aldrich Ltd.) and 1% antibiotic mix (10.000 U penicillin, 10 mg streptomycin (Sigma-AldrichLtd.)). Cells were seeded at a density of 1×10^4 cells/well in 500 µL of fresh medium. Plates were incubated at 37 °C in a 5% CO_2 atmosphere for 4 days, until a confluent monolayer was formed. Then, medium was discarded and replaced by 400 µL of the serial concentrations of the BD132 dendron diluted in fresh medium. Control wells received 400 µL of fresh medium. After 48 h of incubation, the culture medium was discarded, wells were washed with PBS and 500 µL of medium were added to each well. To evaluate the cytotoxicity, each well received 50 µL of MTT (5 mg/mL) (Sigma-Aldrich Ltd.) and plates were incubated for 4 h at 37 °C. Subsequently, the well content was discarded and 500 µL of dimethyl sulfoxide were added to dissolve formazan crystals. Absorbance values were recorded in a microplate absorbance reader at 570 nm (BioTek Instruments Inc. Model: ELX 800). Experiments were performed in triplicate and repeated at least twice. Reduction in viability percentages < 10% were considered non-cytotoxic, values between 10 and 25% were considered low cytotoxicity, and values between 25 and 40% were considered moderate cytotoxicity levels. High cytotoxicity was considered when viability values reduced more than 40%[18,32].

Results and discussion

In vitro antibiofilm activity of the BD132 dendron in *C. albicans and N. glabratus*

The BD132 dendron is a CBS dendron with a 4-phenyl butyric (PBA) focal point and ammonium peripheral groups with pendant hydroxyl units. This dendron structure is similar to the previously reported CBS dendron that contains trimethylammonium cationic groups, known as BDTL056 (Fig. S6)[18,26]. The later compound showed interesting results eliminating different *Candida* biofilms[18,26]. However, with this minimal structural change, a high improvement in the antimicrobial activity was achieved against different *Candida* strains. Data showed that the MBIC values were 16–32 and 8–16 mg/L against *C. albicans* and *N. glabratus*, respectively (Table 1). The concentration required to eliminate all cells during the biofilm formation (MFCB) was 32 mg/L against *C. albicans* and 16 mg/L against *N. glabratus* (Table 2).

Regarding to studies against established biofilms, the BD132 dendron was able to drastically damage (MBDC) *C. albicans* biofilms at 64 mg/L (Table 1). In addition, this dendron eradicated (MBEC) an established biofilm of *C. albicans*, while BDTL056 dendron could not eradicated the biofilm completely[18]. Besides, against *N. glabratus* a lower MBEC value was determined using the BD132 dendron (128 mg/L) than using the BDTL056 dendron (256–512 mg/L)[26].

These differences that are related to the substitution of one methyl by one ethylalcohol group, can be justified by the ability of the hydroxyl group to establish additional interactions with cell membrane and biofilm structure. These interactions can be the formation of hydrogen bonds along with an increase in the polarity of the dendron periphery[33].

Synergistic study between the BD132 dendron and amphotericin against of *C. albicans* and *N. glabratus* biofilms

Preventing biofilm formation using combined the BD132 dendron and amphotericin against C. albicans and N. glabratus

The search for new antifungals with activity at low concentrations is problematic. This fact is important because treatments with low concentrations are essential, especially, with the aim to reduce the probability of the appearance of resistance and to reduce the cytotoxicity[34]. For this reason, combination studies are frequently

Effect of BD132 Dendron eradicanting biofilm formed (MBEC)					
	Individual (mg/L)		In combination (mg/L)		
	BD132	Amphotericin B	BD132	Amphotericin B	FICI[a]
C. albicans	256–512	NEB	64	2	< 0.5
			8	2	< 0.5
N. glabratus	128	NEB	64	0.12	< 0.5
			32	4	< 0.5

Table 3. In vitro combinate effect of the BD132 dendron and amphotericin B against *C. albicans* and *N. glabratus* in biofilm formed. *NEB* No Biofilm Eradication. [a]FICI ≤ 0.5: synergic activity. A biofilm eradication activity using amphotericin alone was not recorded (NEB). Therefore, the highest concentration tested of amphotericin (32 mg/L) was used to calculate the FICI values.

required. In this work, data showed that the combination of the BD132 dendron and amphotericin were synergistic against *C. albicans* and *N. glabratus* MBIC). Table 2 shows the best synergistic combinations with the lowest FICI. Both compounds, the BD132 dendron and amphotericin together, prevented biofilm formation against *C. albicans* at 4 mg/L (32 mg/L alone) and 0.03 mg/L (0.25 mg/L alone), respectively. Interestingly, we even registered a greater reduction of both drugs against *N. glabratus*, being the effective concentrations 2 mg/L (16 mg/L alone) of BD132 dendron and 0.01 mg/L (0.12 mg/L alone) of amphotericin (Table 2). In both cases, the FICI values calculated were below 0.5 that indicated a synergistic action when both compounds were used in combination.

Elimination of all active biofilm-forming cells by combined use of the BD132 dendron and amphotericin against C. albicans and N. glabratus

The prevention of biofilm formation is an important fact; however, the complete eradication of active cells forming an established biofilm is more relevant. The BD132 dendron eradicated all biofilm active cells when tested individually (Table 1). Nevertheless, our studies using amphotericin individually determined that cells were not eradicated at any of the evaluated concentrations (Table 3). The combination studies showed that the BD132 dendron and amphotericin had a synergistic activity against *Candida*. Table 3 shows the best synergistic combinations with the lowest FICI. The lower combined concentration of the BD132 dendron and amphotericin for eradication of biofilm active cells was reached using 8 mg/L of the BD132 dendron and 2 mg/L amphotericin against *C. albicans* and 32 mg/L of the BD132 dendron and 4 mg/L amphotericin against *N. glabratus* (Table 3). This synergistic activity was also observed in previous studies using the BDTL056 dendron in combination with amphotericin and with other compounds (EDTA and $AgNO_3$)[18,26]. Many drugs cannot be used due to their low solubility, stability or even their high cytotoxicity[35,36]. However, the use of the BD132 dendron, a hydrophilic and soluble compound, as a carrier for amphotericin could improve the clinical applications of this commercial antifungal.

Cytotoxic evaluation in HeLa cells of compound BD132

To verify cytotoxicity, the BD132 dendron was tested in HeLa cells. After treatments, cytotoxicity was not observed at 16 mg/L or lower concentrations, and low cytotoxicity was shown at 32 mg/L (Fig. 2). These concentrations were required to inhibit biofilm formation (MBIC) with the BD132 dendron in *C. albicans* (Table 1). In addition, the concentration required to damage (MBDC) an established biofilm (64 mg/L) (Table 1) produced a 49.4 ± 2.1%. reduction in HeLa cell viability. Previous studies showed that 16 mg/L of the BDTL056 dendron, predecessor molecule of the BD132 dendron, showed high cytotoxicity against Hela cells[18]. Hence, the presence of the pendant ehtylalcohol group favor biocompatibility to the cationic CBS dendron, since these groups shield the cationic charge, is bigger than a methyl, and the hydroxyl groups are widely distributed in biomacromolecules and improving hydratation of the cationic dendron[37]. Therefore, with the structure modifications included in the BD132 dendron based on our previous studies[18,26], a more effective and less cytotoxic compound has been developed with promising applications in future studies using in vivo models.

Conclusions

Nowadays, there is a crucial need to develop and find new active molecules against *Candida* cells, since there are currently only three classes of compounds approved for the treatment of systemic fungal infections and some of them do not have enough activity due to resistances or they show cytotoxicity at effective concentrations. In this work, we have made a significant progress in optimizing a dendritic compound, making it more powerful at a therapeutic level. This newly synthesized design has been shown to be a more active compound (BD132 dendron) than its predecessor (BDTL056 dendron). The dendron inhibited biofilm formation at non-cytotoxic concentrations and was able to eradicate established biofilms. The data obtained suggested that this new the BD132 dendron could be an interesting candidate, due to its antifungal properties, for future studies using in vivo infection models.

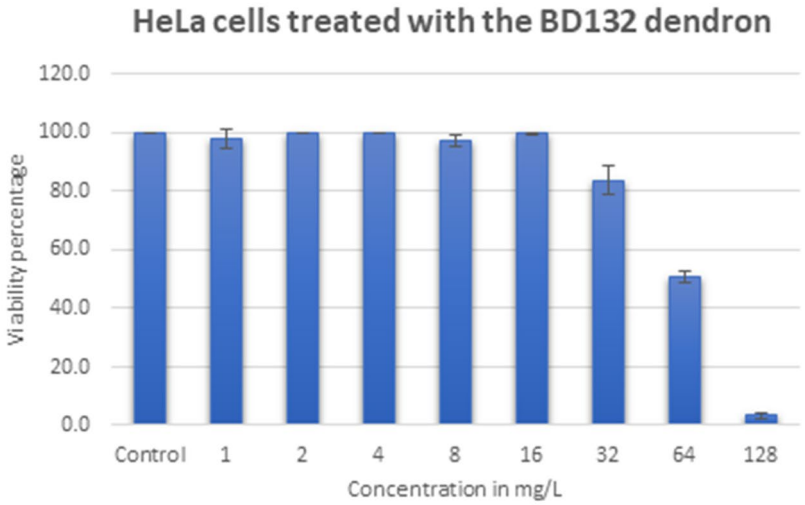

Fig. 2. Viability percentage of HeLa cells treated with the BD132 Dendron. Reduction in viability percentages < 10% were considered non-cytotoxic, values between 10–25% were considered low cytotoxicity, and values between 25–40% were considered moderate cytotoxicity levels. High cytotoxicity was considered when viability values reduced more than 40%.

Data availability

The data obtained and analyzed during the study are available from the corresponding author on reasonable request.

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Author contributions

N.G.-C. conceptualization, methodology, formal analysis and investigation, data curation, original draft preparation, and resources. J.L.C.-P. conceptualization, analysis and investigation, review and editing manuscript, resources, and supervision. R.G. funding acquisition and resources. J.S.-N. methodology, formal analysis and investigation, review and editing manuscript, and resources. I.H.-B. conceptualization, methodology, formal analysis and investigation, data curation, original draft preparation, funding acquisition, resources, and supervision. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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